

# Identification of a Novel Cryptochrome Differentiating Domain Required for Feedback Repression in Circadian Clock Function<sup>\*[S]</sup>

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**Background:** Mammalian CRY1 and CRY2 have distinct functions in circadian clock mechanisms.

**Results:** A core domain within the photolyase homology region of CRY1 differentiates CRY1 from CRY2 in clock function.

**Conclusion:** The CRY1/2 differentiating domain is required for strong transcriptional repression and rhythm generation, whereas the divergent tail domain fine tunes clock function.

**Significance:** This study provides novel insights into functional evolution of photolyase/cryptochrome flavoproteins.

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 sequence, domain structure, and biochemical activity, they play distinct roles in clock function. However, detailed biochemical studies have not been straightforward and *Cry* function 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, whereas *Cry2* cannot. Using this novel functional assay, we identify a cryptochrome differentiating  $\alpha$ -helical domain within the photolyase homology region (PHR) of CRY1, designated as CRY1-PHR(313–426), that is required for clock function and distinguishes CRY1 from CRY2. Contrary to speculation, the divergent carboxyl-terminal tail domain (CTD) is dispensable, but serves to modulate rhythm amplitude and period length. Finally, we identify the biochemical basis of their distinct function; CRY1 is a much more potent transcriptional repressor than CRY2, and the strength of repression by various forms of CRY proteins significantly correlates with rhythm amplitude. Taken together, our results demonstrate that 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.

In mammals, many aspects of behavior and physiology, most notably the sleep-wake cycle, are regulated by endogenous circadian clocks and are subject to daily oscillations (1, 2). The mammalian circadian time-keeping system is a hierarchical, multioscillator network with the central clock in the suprachiasmatic nucleus (SCN)<sup>2</sup> synchronizing and coordinating peripheral oscillators elsewhere in the body (3). Although virtually all cells in the body have circadian clocks (4–6), the SCN clocks are qualitatively more robust because of functional intercellular coupling mechanisms that are present in the SCN, but absent in most, if not all, peripheral oscillators (7, 8). As a result, peripheral tissues or cells, when cultured *in vitro*, display cell-autonomous circadian rhythms.

The various clock cells in different tissues share a remarkably similar biochemical mechanism, the autoregulatory negative feedback loop, consisting of negative and positive molecular components (1, 9, 10). The positive components include the two basic helix-loop-helix/PAS domain-containing transcription factors, BMAL1 and CLOCK, that form a heterodimeric transcriptional complex to activate target gene expression via E/E'-box enhancer elements. Periods (*Per1*, -2, and -3) and cryptochromes (*Cry1* and -2) constitute the negative components of the loop. The PERs and CRYs repress transcription of target genes, by directly interacting with and inhibiting BMAL1-CLOCK complex activity. In particular, the *Per* and *Cry* genes themselves are targets of the BMAL1-CLOCK and in turn repress their own transcription, thereby forming the autoregulatory negative feedback loop (11–13). Genetic studies established that CRYs are essential clock components (8, 14, 15). This observation, together with the finding that CRYs are much more potent repressors than PERs for BMAL1-CLOCK complex activity (16, 17), placed the CRYs at the heart of the core clock mechanism.

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<sup>[S]</sup> This article contains supplemental Table S1 and Figs. S1–S5.

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<sup>2</sup> The abbreviations used are: SCN, suprachiasmatic nucleus; PHR, photolyase homology region; CTD, C-terminal tail domain; RRE, ROR/REV-ERB-binding element; CC2, coiled-coil 2.

## Differential Functions of Cryptochromes 1 and 2

The CRYs belong to the photolyase/cryptochrome (PHL/CRY) superfamily of flavoproteins. All CRYs from different species share a highly conserved core domain at the N terminus, the photolyase homology region (PHR), whereas the C-terminal tail domain (CTD), on the other hand, has diverged during evolution (18, 19). Although photolyases lack this tail region, CRYs from plants to animals contain an extended CTD, but of variable length and amino acid composition. Despite similarity in sequence and domain structures, these flavoproteins play diverse biological roles. Bacterial photolyases, upon activation by light, are DNA repair enzymes that revert UV-induced photoproducts to normal bases to maintain genetic integrity (20). In eukaryotes, however, the CRYs do not exhibit photolyase activity, and the CRYs in plants and *Drosophila* are photoreceptors. Although CRYs in plants function to mediate phototropism, growth, and development (21, 22), *Drosophila* dCry is directly involved in the light input pathway for circadian clock entrainment (23, 24). In contrast, the mammalian CRYs are neither photolyases nor photoreceptors; rather, they function as light-independent transcriptional repressors (16, 17). Although less well characterized, *Drosophila* CRY was shown to exhibit repressor function in certain peripheral tissues (25, 26). Functional evolution of this superfamily of flavoproteins remains one of the most intriguing questions in circadian biology. However, detailed biochemical studies have not been straightforward and Cry function has not been examined in real clock cells using kinetic measurements.

Experimental data suggest that *Cry1* and *Cry2* have overlapping but differential functions in the clock mechanism. Although they are both repressors, *Cry1* and *Cry2* play opposite roles in regulating animal behavior: *Cry1*<sup>-/-</sup> and *Cry2*<sup>-/-</sup> mice display shorter and longer free-running period lengths of locomotor activity rhythms, respectively, compared with wild type mice (14, 15). Similarly, SCN explants from *Cry1*<sup>-/-</sup> mice exhibit shorter period length than wild type, whereas *Cry2*<sup>-/-</sup> SCN explants exhibit longer periods (8). Interestingly, *Cry1* and *Cry2* play distinct roles in generating and maintaining cell-autonomous circadian rhythms. For example, dissociated individual SCN neurons derived from *Cry1*<sup>-/-</sup> mice are arrhythmic or only transiently rhythmic, whereas neurons from *Cry2*<sup>-/-</sup> SCN show persistent rhythms of higher amplitude with longer period lengths than in wild type (8). Similarly, peripheral tissue explants and cells from *Cry1*<sup>-/-</sup> mice are arrhythmic (8, 27). The more essential role of *Cry1* is also supported by behavioral phenotypes of compound knockouts; *Cry1*<sup>+/-</sup>:*Cry2*<sup>-/-</sup> mice show more persistent rhythms than *Cry1*<sup>-/-</sup>:*Cry2*<sup>+/-</sup> mice, and whereas *Per2*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> mice are rhythmic, *Per2*<sup>-/-</sup>:*Cry1*<sup>-/-</sup> mice are arrhythmic (14, 28). Taken together, these studies indicate that *Cry1* is required for cellular rhythmicity and plays a more prominent role than *Cry2* in the clock mechanism.

However, molecular details underlying the functional distinction between the two are not well understood. It is known that *Cry1* is regulated by a combinatorial transcription mechanism and strongly rhythmic in most tissues including the SCN, whereas *Cry2* has only weak rhythms (16, 29–34). Their differential expression patterns may partially explain the differential roles in clock function *in vivo*. Alternatively, the CRY1 protein

level may be higher than CRY2, or CRY1 may be a stronger repressor than CRY2. In this study, we 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 (see below).

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, whereas the *Bmal1* gene is essential, its rhythmic expression is dispensable for core clock function (35). In contrast, rhythmic expression of *Cry1* is required for cell-autonomous circadian oscillation (29). In addition to the E/E'-box (responsible for morning-time phase of gene expression, e.g. *Rev-erba*) at the core of the clock mechanism, at least two other circadian cis-elements are involved: the DBP/E4BP4 binding element (D-box; daytime phase, e.g. *Per3*) and the ROR/REV-ERB-binding element (RRE; nighttime 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. Furthermore, through genetic complementation, we showed that this distinctive delayed phase of *Cry1* expression is required to restore circadian rhythmicity in arrhythmic *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts (29).

In the present study, we took advantage of the *Cry1* rescue assay to dissect the differential functions of *Cry1* and *Cry2*. First, we confirmed that *Cry1* is required for cell-autonomous circadian rhythms, whereas *Cry2* is dispensable. Through systematic analyses of protein domain structure-function relationships, we identified a highly conserved  $\alpha$ -helical domain within the PHR that distinguishes CRY1 from CRY2. Contrary to previous speculation, the least conserved CTD is dispensable for circadian oscillation, but serves to modulate rhythm amplitude and period length. Finally, we demonstrated that CRY1 is a much stronger repressor than CRY2, and that repression strength positively correlates with rhythm amplitude. Thus, our data demonstrate that CRY1-specific repression is necessary for normal clock function.

## MATERIALS AND METHODS

**Plasmid Construction**—The *Cry1* expression vector, pMU2-P(*Cry1*)-intron-*Cry1*, was made in a previous study (29). 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'-TCTAGATGGCAAACAGCTATTATGGGTATTATGGGTGCGGCGGCTGCTGTGGTG-3'; underline, XbaI restriction site) and reverse primer (5'-GTCGACTGCCATTTTCATTACCTCTTTCTCCGCACCCGACATAGATTGAGGAGTCCTTGCT-3'; underline, SalI). The PCR product was cloned into pCR2.1-TOPO vector (Invitrogen) and the digested XbaI/SalI fragment was then subcloned into pMU2 vector (36) in place of the *Cry1* gene.

Domain swap constructs were generated by overlapping PCR. The primers (supplemental Table S1) were designed so that swap junctions reside in highly conserved or identical sequences, so as to minimize major structural changes and protein folding problems. Site-directed mutagenesis using overlapping PCR was performed to generate single mutations within

the CRY1-PHR(313–426). Similarly, the PCR products were cloned into pCR2.1-TOPO and subsequently into the pMU2 vector, as described above. For construction of pMU2-P(CMV)-*Cry2*, the full-length coding sequence of *Cry2* was digested from pMU2-P(SV40)-*Cry2* (29) with *PI*-PspI and *PI*-SceI, and the *Cry2* fragment was cloned into the *PI*-PspI-*PI*-SceI sites immediately downstream of the CMV promoter.

Each *Cry* construct (1  $\mu$ g) was co-transfected with either empty vector (0.4  $\mu$ g) or *Bmal1/Clock* (0.2  $\mu$ g each) in 293T cells or in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts in a 12-well plate. Forty-eight hours after transfection, cells were lysed in RIPA buffer containing protease inhibitors. The lysates were cleared by centrifugation and supernatants were used for Western blot analysis with guinea pig polyclonal antibodies against CRY1 or CRY2 as described previously (16, 30, 31, 37) or against FLAG tag according to the manufacturer's protocol (Sigma).

**Kinetic Bioluminescence Recording and Data Analysis**—Real-time circadian reporter assays were performed using a Lumicycle luminometer (Actimetrics, Inc.) as previously described (8, 29). Briefly, *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> mouse embryonic fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin and 100  $\mu$ g/ml of streptomycin). One day prior to transfection,  $4 \times 10^5$  cells were plated onto 35-mm culture dishes. Cells were cotransfected using FuGENE 6 (29) with 3.95  $\mu$ g of pGL3-P(*Per2*)-dLuc reporter plasmid (38) and 0.075  $\mu$ g of a *Cry* expression plasmid. For the *Cry1* dose-response experiment, the amount of plasmid was adjusted to 5.45  $\mu$ g with empty vector. Three days post-transfection, the medium was replaced with HEPES-buffered recording medium supplemented with B-27 and containing 0.1 mM luciferin and 10  $\mu$ M forskolin as previously described (29). Bioluminescence from each dish was continuously recorded with a photomultiplier tube for ~70 s 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.

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 (29). 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.

**Transcription Repression Assay**—*Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts were grown and transfected as described above with the following modifications. In transfection, 1  $\mu$ g of reporter plasmid, pGL3-P(*Per2*)-dLuc (38), pGL3-3x $E'$ -box-P(SV40)-dLuc, pGL3-3x $E$ -box-P(SV40)-dLuc, or pGL3-P(SV40)-dLuc (39) was used together with 2  $\mu$ g of a *Cry* expression plasmid. In some assays as presented in supplemental Fig. S5, 0.5  $\mu$ g each of

*Bmal1* and *Clock* plasmid DNA (40) was also included. Empty vector was used to make up the total amount of DNA to 4.1  $\mu$ g/well. As an internal control, 50 ng of a phRL-SV40 plasmid expressing *Renilla* luciferase (*RLuc*) (Promega) was added in each transfection. Forty-eight hours after transfection, cells were harvested and assayed with the Dual Luciferase Reporter Assay System (Promega). Luciferase activity was normalized by *RLuc* 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.

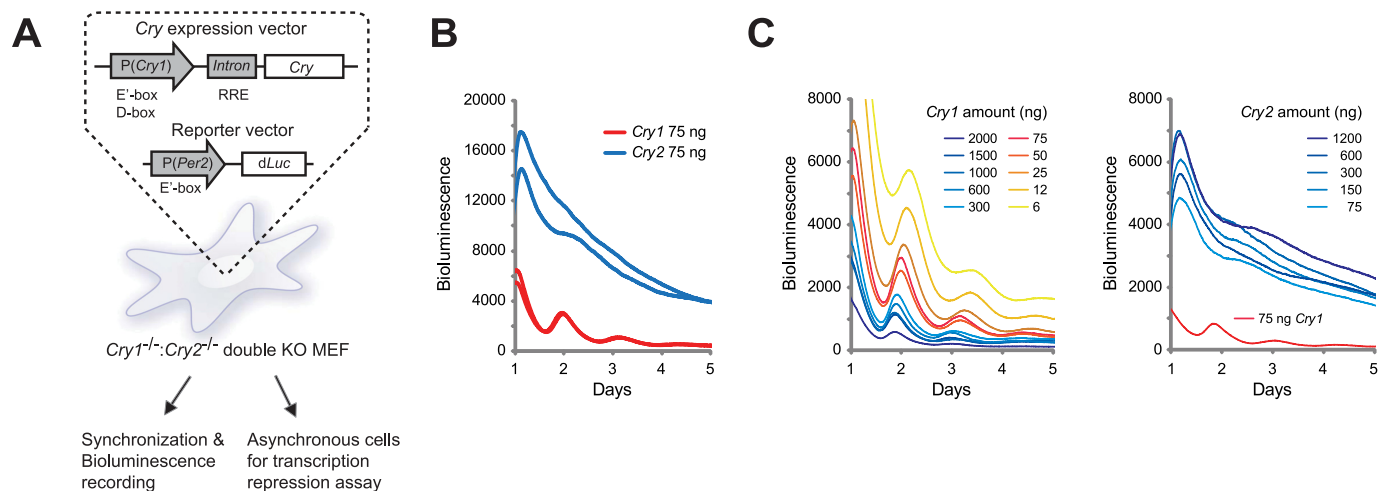
**Protein Structure Homology Modeling**—Homology models for full-length mCRY1 and mCRY2 were generated using the I-TASSER protein structure prediction server (41–43). This server first threads fragments of the target sequence to representative PBD structure templates with matched sequence identity greater than 70%. The fragments are then assembled into a full-length model, whereas 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 PBD structures. The quality of predicted structure was assessed with a scoring method, and five atomistic models with the highest scores were obtained for each input protein sequence. Images of predicted structures were created using PyMOL software, version 1.2r3pre (Schrödinger, LLC).

## RESULTS

***Cry1*, But Not *Cry2*, Can Restore Circadian Clock Function in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> 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*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> 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. 1A).

As expected, *Cry1* was able to restore rhythms in these cells (Fig. 1B), consistent with previous results (29), and the rescued cells showed longer period lengths than wild type, characteristic of *Cry2*<sup>-/-</sup> cells (8). In contrast, however, *Cry2* was unable to restore circadian oscillation to *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts, confirming results found for cells from *Cry1*<sup>-/-</sup> mice (8) (Fig. 1B). As the *Cry* expression level in these fibroblasts was below the detection limit, the ability of P(*Cry1*)-*Intron-Cry* constructs to express CRY proteins was tested by Western blot in transfected 293T cells (supplemental Fig. S1A). Additionally, to compare their relative expression in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts, we determined that 3xFlag-*Cry1* and 3xFlag-*Cry2* (functionally comparable with *Cry1* and *Cry2*, respectively, in the rescue assay; supplemental Fig. S1B, left panel) are expressed to similar levels in these cells (supplemental Fig. S1B, right panel). Interestingly, rescue of rhythmicity is largely independent of the dose of *Cry1*, ranging from nanograms to micrograms of DNA used in the transfection (Fig. 1C, left panel). On the other hand, *Cry2* of any amount failed to rescue circadian rhythmicity





**FIGURE 1. *Cry1*, but not *Cry2*, restores circadian rhythmicity in arrhythmic *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> 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* (dLuc) gene driven by the *Per2* promoter. Transfected *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts are either harvested for a transcription repression assay, or synchronized for kinetic bioluminescence recording. B, representative bioluminescence records from *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts expressing *Cry1* or *Cry2*. Genetic complementation of *Cry1* (red), but not *Cry2* (blue), restored circadian rhythms in these cells. Each expression construct was cotransfected with the P(*Per2*)-dLuc into the cells. Three days post-transfection, 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*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts. Experiments were done as in B.

in these cells (Fig. 1C, right panel). Thus, our data establish that *Cry1* and -2 play differential roles at the level of core clock function: whereas *Cry1* is essential for generation of cell-autonomous circadian clock function, *Cry2* is dispensable.

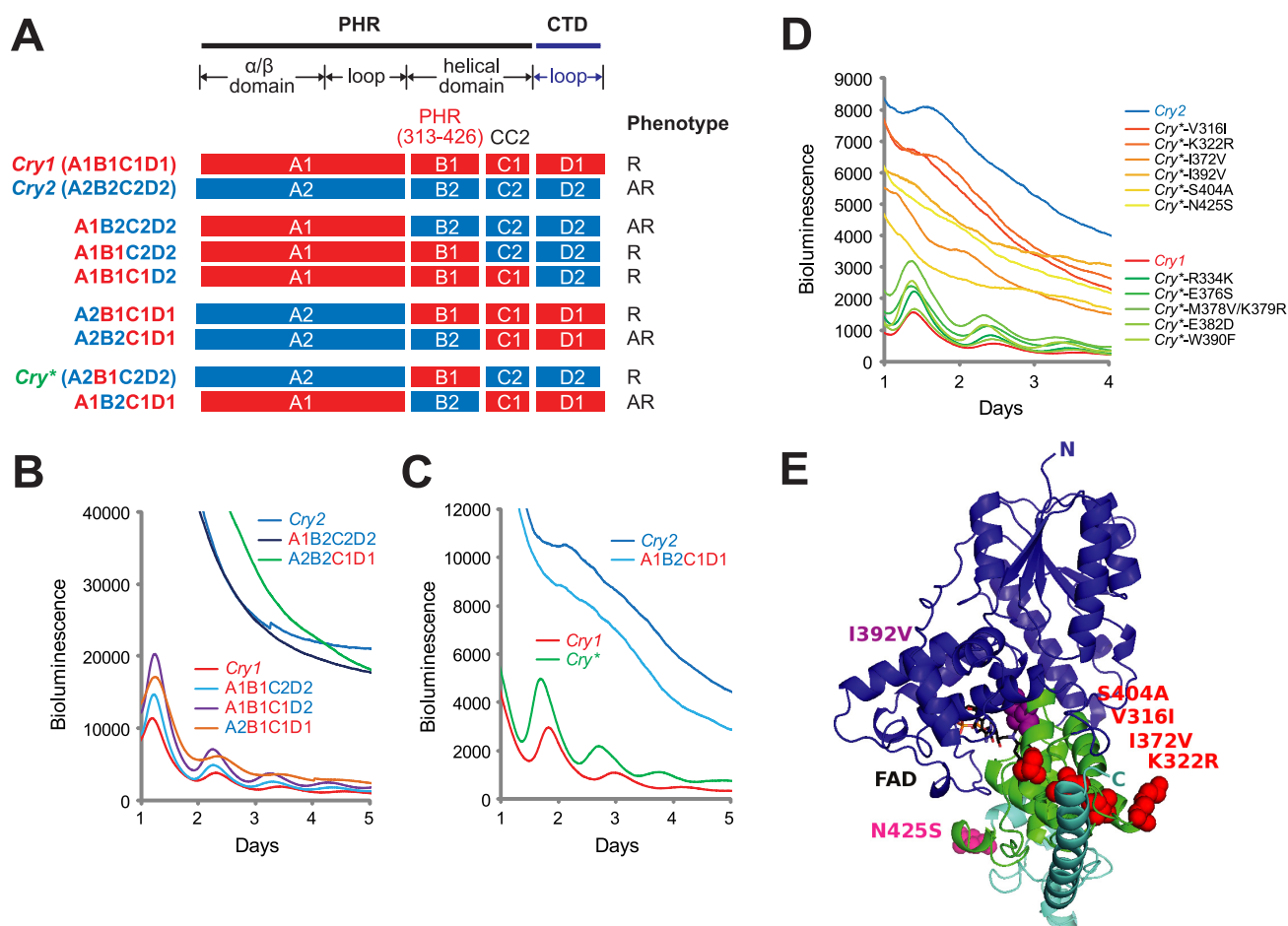
Unlike the high-amplitude rhythmic expression of *Cry1* in various tissues and cells, *Cry2* expression is either not rhythmic or rhythmic at very low amplitude (16, 30, 31, 37). It is thus possible that this differential rhythmic expression contributes to functional differences *in vivo*. In our *in 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*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts suggest that CRY1 and -2 possess different intrinsic biochemical properties at the protein level that call for further investigation.

**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 functions of PHL/CRY proteins (18, 19, 44), we divided CRY1 and -2 proteins into four regions, namely A, B, C, and D (Fig. 2A; supplemental Fig. S2). 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. 2A). To minimize major structural changes and protein folding problems, we selected highly conserved or identical sequences at swap junctions (supplemental Fig. S2). The ability of these chimeras and the mutant *Cry* constructs to express CRY proteins was tested by Western blot (supplemental Fig. S1). These chimeras were then tested for their ability to restore circadian rhythms in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> 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. 2B). However, when the B region in *Cry1* (*Cry1*-B) is replaced with the corresponding *Cry2*-B, the A1B2C1D1 chimera failed to restore rhythms, suggesting that *Cry1*-B is required for clock function (Fig. 2B).

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. 2C). In fact, all chimeras that harbor *Cry1*-B were able to sustain circadian oscillation, whereas those containing *Cry2*-B failed to do so (Fig. 2A). Interestingly, a previous mutagenesis study also hinted that this region likely differentiates CRY1 and CRY2 (45). Thus, we have identified a critical region within the highly conserved  $\alpha$ -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)**—Because 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), 12 are divergent between CRY1 and -2 (supplemental Fig. S2). Each of the 12 amino acids in *Cry\** was mutated to the corresponding residue in *Cry2*, one or two at a time. Because 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*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts. Among 12 mutants, six restored circadian rhythms in these cells, similar to *Cry1* and *Cry\**, whereas the other 6 failed to do so: *Cry\**-V316I, K322R, I372V, I392V, S404A, and N425S (Fig. 2D), indicating that these six residues within the CRY1-PHR(313–426) are critical for CRY function in the clock mechanism.

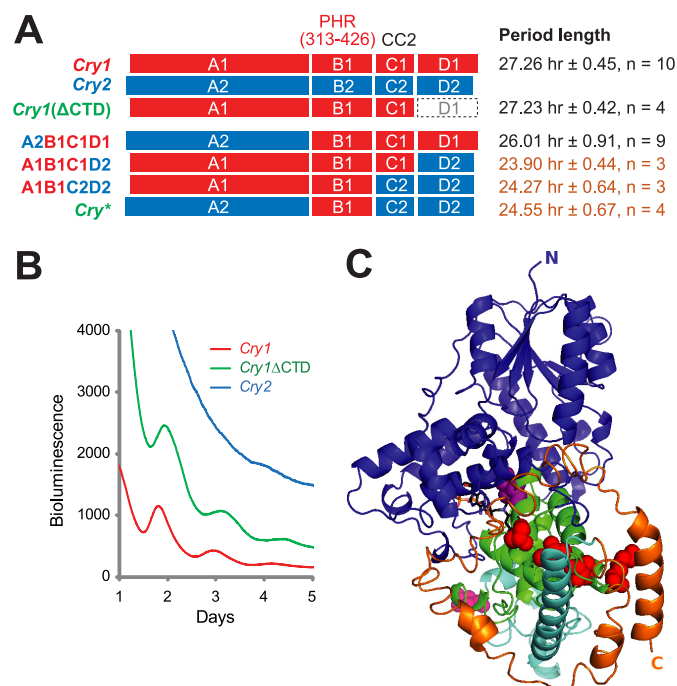


**FIGURE 2. CRY1-PHR(313–426) is critical for Cry1 function.** *A*, Cry expression constructs. Cry1 (red) and Cry2 (blue) were divided into four regions: 1) region A includes the N-terminal  $\alpha/\beta$  domain and the inter-loop domain of the PHR; 2) region B is the CRY1-PHR(313–426) and includes the core  $\alpha$ -helical domain of the PHR (from  $\alpha 13$  to  $\alpha 18$ ); 3) region C contains the rest of the  $\alpha$ -helical domain, including sequences immediately after  $\alpha 18$  and before the CTD where CC2 resides; and 4) region D is the CTD. For sequence details, see supplemental Fig. S2. 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 bioluminescence records from *Cry1*<sup>−/−</sup>:*Cry2*<sup>−/−</sup> fibroblasts expressing different Cry chimeras. Domain-swapped chimeras (in *B* and *C*) were tested for their ability to rescue rhythms in *Cry1*<sup>−/−</sup>:*Cry2*<sup>−/−</sup> fibroblasts as described in the legend to Fig. 1. 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. *D*, representative bioluminescence records from *Cry1*<sup>−/−</sup>:*Cry2*<sup>−/−</sup> 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. *E*, 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).

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–C, with a root mean square deviation 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 Ile-392 (Fig. 2*E*), which is located near the FAD-binding cavity. Asn-425 is localized within a loop motif between helix  $\alpha 18$  in region B and  $\alpha 19$  in region C, and is potentially involved in protein-protein interactions. The other four residues (*i.e.* Val-316, Lys-322, Ile-372, and Ser-

404) are readily available for potential interaction with the CTD (see below), CC2, or other clock factors. Ser-404 is localized within a recognition loop between  $\alpha 17$  and  $\alpha 18$ , which is recently implicated in interaction with the CTD of *Drosophila* CRY (46).

**The CTD Is Dispensable for Clock Function, but Modulates Rhythm Amplitude and Period Length**—The CTD represents the least conserved region among the CRYs. It is generally accepted that the CTD is critical for CRY function (47). To test the functional importance of the CTD, we generated a *Cry1* CTD-deletion construct, *Cry1*( $\Delta$ CTD) (Fig. 3*A*). To our surprise, *Cry1*( $\Delta$ CTD) was able to rescue circadian rhythms in *Cry1*<sup>−/−</sup>:*Cry2*<sup>−/−</sup> fibroblasts. Thus, contrary to expectation, our data suggest that the CTD is not absolutely essential for CRY1 function (Fig. 3*B*). This result is consistent with a previ-



**FIGURE 3. CTD is dispensable for CRY function, but modulates period length.** A, schematic diagram of various *Cry* constructs, including the truncation construct *Cry1*( $\Delta$ CTD) in which the CTD is deleted. Period length corresponding to each construct is shown on the right. Mean  $\pm$  S.D. (error bar) of two independent experiments are shown. Raw data are presented in Figs. 2, B and C, 3B, and supplemental Fig. S3. B, representative bioluminescence records from *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts expressing different *Cry* constructs. Deletion of CTD did not render *Cry1* unable to generate circadian rhythms, suggesting that the CTD is dispensable for CRY1 function. *Cry1*( $\Delta$ CTD), green. 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).

ous study, which found that CTD is not absolutely required for repression (45). 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 (47). Here we show that a larger C-terminal deletion, *Cry1*( $\Delta$ CC2-CTD), which lacks both CC2 and CTD, eliminated the ability of *Cry1* to maintain rhythmicity (supplemental Fig. S3), indicating an important role for CC2 in clock function.

Although the CTD of CRY1 is dispensable, rhythms rescued by *Cry1*( $\Delta$ CTD) showed decreased rhythm amplitude compared with 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 ( $\sim$ 27 h), characteristic of *Cry2*<sup>-/-</sup> cells, when *Cry1*-CTD is replaced by *Cry2*-CTD (chimera A1B1C1D2), the rescued cells displayed shorter period lengths that are comparable with wild type cells ( $\sim$ 24 h) (Fig. 3A and supplemental Fig. S3B). In fact, among all *Cry* chimeras containing the B region of CRY1 (and therefore conferring circadian rhythmicity), those that contain *Cry2*-CTD showed a period of  $\sim$ 24 h, whereas those that contain *Cry1*-CTD showed a longer period ( $\sim$ 27 h). Taken together, our data suggest that although the CTD is dis-

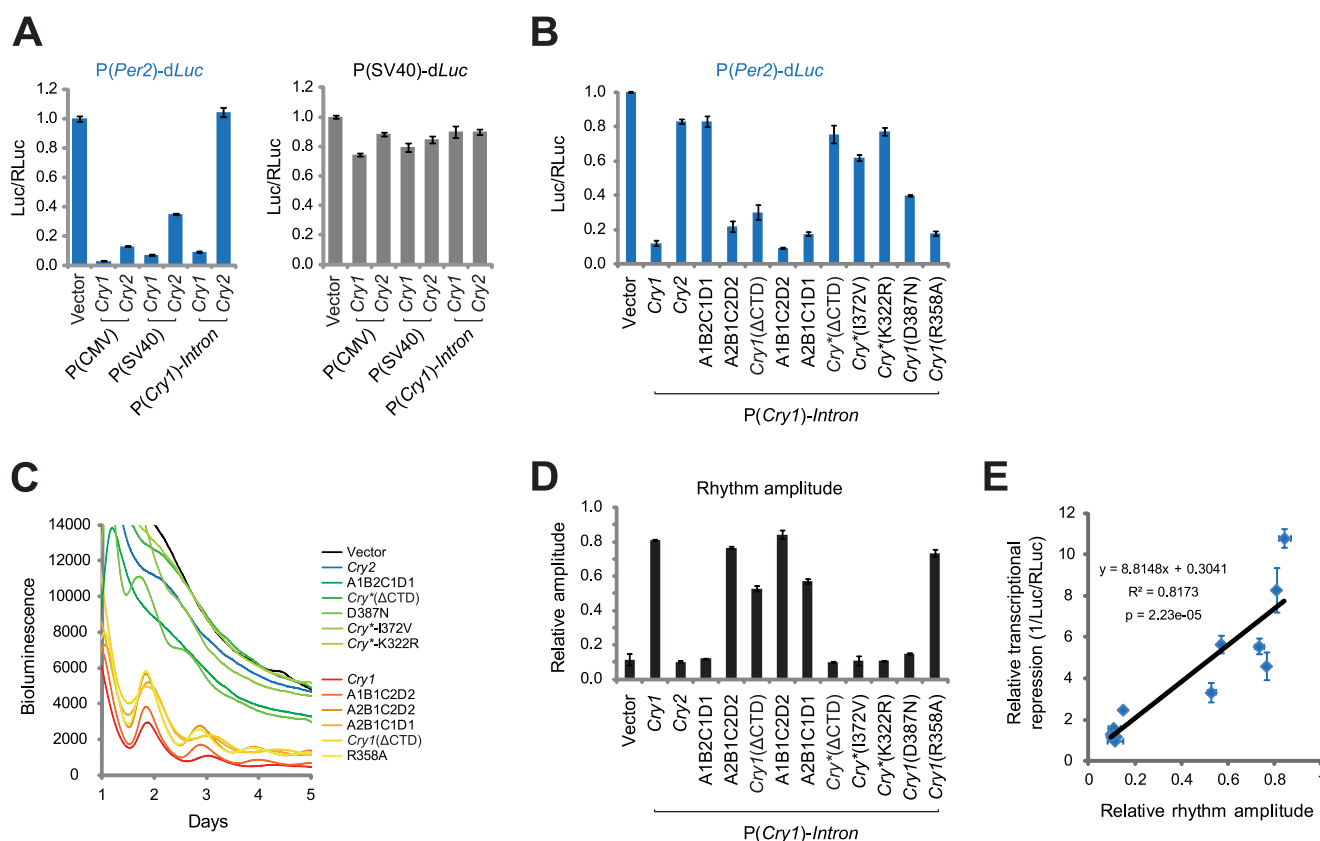
pensable for *Cry1* function, it plays important roles in modulating rhythm amplitude and period length.

Our homology models for full-length CRY1 and CRY2 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 (48). Among possible arrangements of the CTD, those involving interactions with CRY1-PHR(313–426) are energetically favored, especially interactions with the side chains of Val-316, Lys-322, Ile-372, and Ser-404 (Figs. 2E and 3C), each shown to be critical for CRY function. The observation that these residues reside in critical regions (e.g. Ile-392 and Ser-404) and/or at an interface (e.g. Val-316, Lys-322, Ile-372, and Ser-404) available for potential protein-protein interaction explains why mutating them impairs normal clock function.

**CRY1 and CRY2 Display Differential Transcription Repression Activity**—In kinetic rhythm assay experiments, we noticed low expression levels of the P(*Per2*)-dLuc reporter in rhythmic cells and high levels in arrhythmic cells, suggesting that rhythm amplitude may be related to potency of repression of BMAL1-CLOCK transcriptional activity. To examine this correlation more quantitatively, we measured P(*Per2*)-dLuc expression in the presence of *Cry1* or *Cry2* in transiently transfected, nonsynchronized cells. When assayed under nonrhythmic 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. 4A and supplemental Fig. S4), consistent with previous studies (16, 17, 38, 45). To test for differences in repression activity of CRY1 and CRY2, we measured *Cry* repression under our conditions of genetic complementation in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts, in which *Cry* is regulated by the *Cry1*-phase promoter. Under these conditions, CRY1 still displayed strong repression on the P(*Per2*)-dLuc reporter. CRY2, however, did not repress transcription to the same extent as CRY1, showing a repression activity 10 times weaker than CRY1 (Fig. 4A). This difference in repression by CRY1 and CRY2 was independent of the reporter used in the assay, as similar results were obtained with 3x $\Delta$ -box-P(SV40)-dLuc or 3x $\Delta$ -box-P(SV40)-dLuc (supplemental Fig. S4). Similar differential repression was also observed when *Bmal1* and *Clock* were co-transfected in these cells (supplemental Fig. S4). Therefore, we conclude that CRY1 is a much more potent transcriptional repressor than CRY2 when expressed under control of a *Cry1*-phase promoter.

**CRY Transcriptional Repression Positively Correlates with Rhythm Amplitude**—These 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. 4B). Importantly, we observed that all the constructs that were able to rescue the rhythms exhibited stronger repression activities, similar to *Cry1*, whereas those that failed to rescue have much weaker repression, similar to *Cry2* (Fig. 4C). For example, *Cry1* (A1B1C1D1) and chimera A2B1C1D1 exhibited low but similar P(*Per2*)-dLuc expression,





**FIGURE 4. Transcriptional repression positively correlates with rhythm amplitude.** A, dual luciferase reporter assay in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> 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  $\pm$  S.D. (error bars) of two independent experiments are shown ( $n = 3$  for each experiment). B, repression activities of various *Cry* chimeras and mutants. Dual luciferase reporter assay was done as in A. 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  $\pm$  S.D. (error bars) of two independent experiments are shown ( $n = 3$ ). C, representative bioluminescence records from *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts expressing various *Cry* chimeras and mutants. The *Cry* rescue assay was performed as described in the legend to Fig. 1B. D, relative amplitudes of rescued rhythms in C. Mean  $\pm$  S.D. (error bar) of two independent experiments are shown ( $n = 3$ ). E, 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  $\pm$  S.D. (error bar) of two independent experiments are shown ( $n = 3$ ).

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 (supplemental Fig. S5). These results are consistent with reporter activities observed in kinetic recordings (Figs. 1–3). 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 (29), we determined the rhythm amplitude of *Cry*-rescued circadian oscillations in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts (Fig. 4D). 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 with *Cry1*, *Cry1*( $\Delta$ CTD) 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. 4E). 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.

## 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 (1, 10), in which transcriptional repression by the CRYs lies at the heart of this mechanism (16, 38, 39). 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 pri-

marily the E/E'-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene. This allowed us to engineer a synthetic composite promoter that is both necessary and sufficient for establishing the *Cry1*-phase. Importantly, we demonstrated, through genetic complementation in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts, 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 (45, 47, 49, 50), 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 with the stronger CMV promoter, SV40-driven *Cry1* and *Cry2* exhibited a more noticeable difference in transcriptional repression (Fig. 4A). 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. 4). 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 with wild type CRY1, the CRY1 chimera harboring the CTD of CRY2 (A1B1C1D2) shortened the period length (Fig. 3), indicating its role in period length regulation. Compared with CRY1, CRY\* (A2B1C2D2) displayed slightly reduced, but by and large similar repression activity. Interestingly, however, compared with 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 the 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 (48), 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 (1, 9, 10), and feedback repression is mediated primarily by CRYs, not PERs (16, 17). 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 (38). 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 (29). Here we further demonstrate that *Cry1*-specific repression is the key to generating circadian rhythms; *Cry1* was able to rescue the rhythms in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts, but *Cry2* failed to do so. In addition, *Cry1*<sup>-/-</sup> cells are largely arrhythmic, suggesting that endogenous *Cry2* alone is unable to support clock function (8, 27). Thus, experimental data from both gain-of-function (this study) and loss-of-function studies in cellular clock models (8, 51), as well as in circadian behavior of composite knock-out mice (14, 28), establish that *Cry1* plays a more prominent role in clock function than *Cry2*. Despite the essential role of *Cry1* in cell-autonomous models, *Cry1*<sup>-/-</sup> mice, nevertheless, display persistent free-running rhythms (14, 15, 28). Therefore, there exists a gap in knowledge as to how transient rhythms in individual *Cry1*<sup>-/-</sup> 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 (52). 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 BMAL1-CLOCK 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 CK1 $\epsilon$ , GSK3 $\beta$ , MAP kinase, and AMP-activated protein kinase (44, 45, 47, 49, 50, 53–56), 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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## Supplemental Data

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Identification of a Novel Cryptochrome Differentiating Domain Required for Feedback Repression in  
Circadian Clock Function

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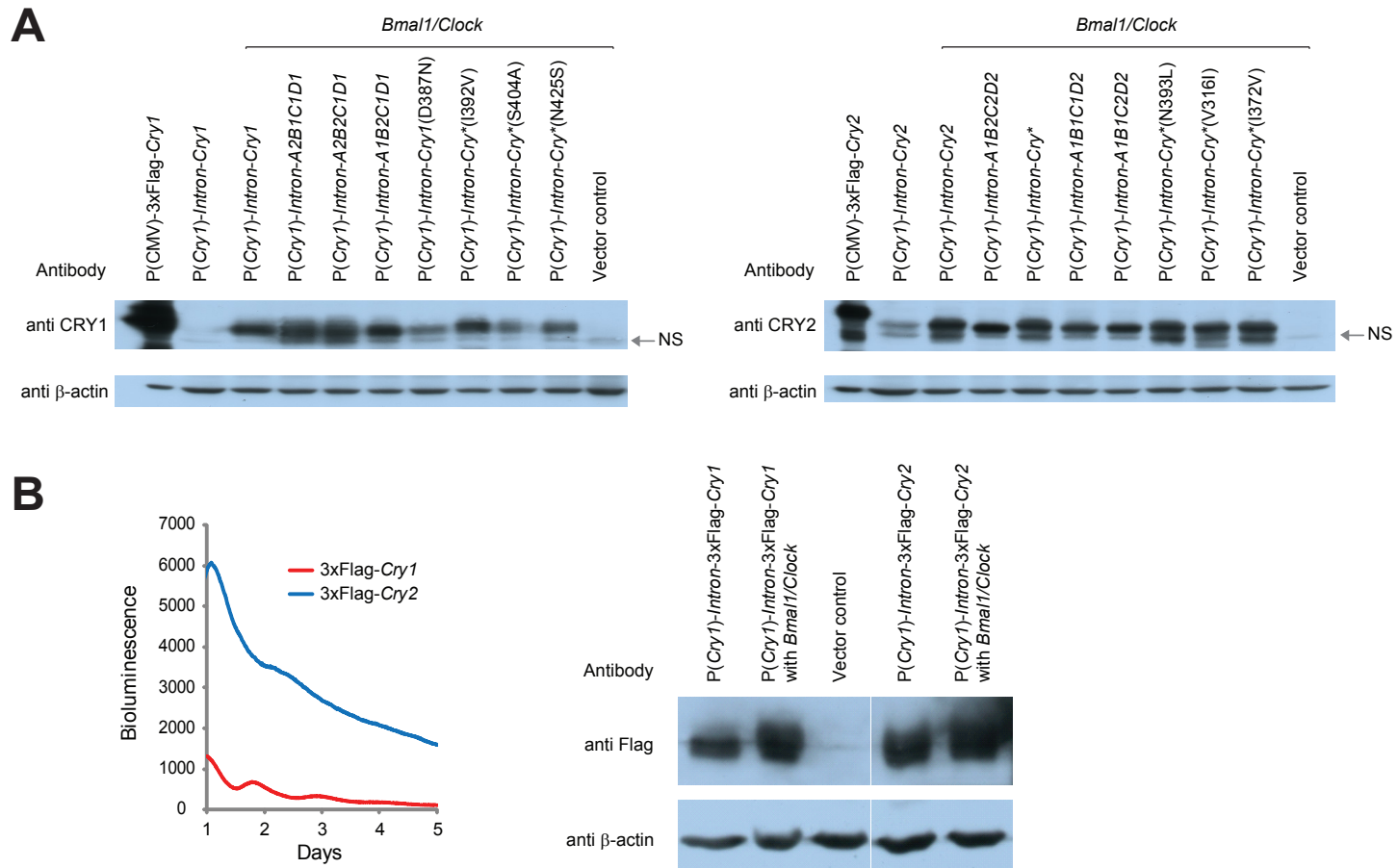
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**Table S1. Primer list for generation of domain swapped chimeric constructs.**

Name of the construct	Round of PCR		Primer sequence
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A1B2C2D2	First	Reaction 1	f-primer: ggtagagctgtcaagattac r-primer: ctggatgcagatggggtccctccattttgtcaaa
		Reaction 2	f-primer: ttgacaaaatggaagggaaccccatctgcatccag r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A1B1C2D2	First	Reaction 1	f-primer: ggtagagctgtcaagattac r-primer: gcctttcagtttgggcagataacgcctaataatagtc
		Reaction 2	f-primer: gactatattaggcggttatctgcccactgaaaggc r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A1B1C1D2	First	Reaction 1	f- primer: ggtagagctgtcaagattac r- Primer: caatagacagagtccccggtaccgggaaagctgctgatagat
		Reaction 2	f-primer: atctatcagcagctttcccggtaccggggactctgtctattg r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A2B1C1D1	First	Reaction 1	f-primer: ggtagagctgtcaagattac r-Primer: ctgaacacagatggggtccctccattcggtcaaa
		Reaction 2	f-primer: ttgaccgaatggagggaaccccatctgtgttcag r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A2B2C1D1	First	Reaction 1	f-primer: ggtagagctgtcaagattac r-Primer: gcctcttaggacaggaagtaccgccgatgtagtc
		Reaction 2	f-primer: gactacatccggcggtacttacctgtcctaagaggc r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A2B1C2D2	First	Reaction 1	f-primer: ggtagagctgtcaagattac r-Primer: ctgaacacagatggggtccctccattcggtcaaa
		Reaction 2	f-primer: ttgaccgaatggagggaaccccatctgtgttcag r-Primer: gcctttcagtttgggcagataacgcctaataatagtc
		Reaction 3	f-primer: gactatattaggcggttatctgcccactgaaaggc r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg
pMU2-P( <i>Cry1</i> )- <i>Cry1</i> intron- A1B2C1D1	First	Reaction 1	f-primer: ggtagagctgtcaagattac r-Primer: ctggatgcagatggggtccctccattttgtcaaa
		Reaction 2	f-primer: ttgacaaaatggaagggaaccccatctgcatccag r-Primer: gcctcttaggacaggaagtaccgccgatgtagtc
		Reaction 3	f-primer: gactacatccggcggtacttacctgtcctaagaggc r-Primer: gtatcttatcatgtctgctcg
	Second		f-primer: ggtagagctgtcaagattac r-Primer: gtatcttatcatgtctgctcg

# Figure S1

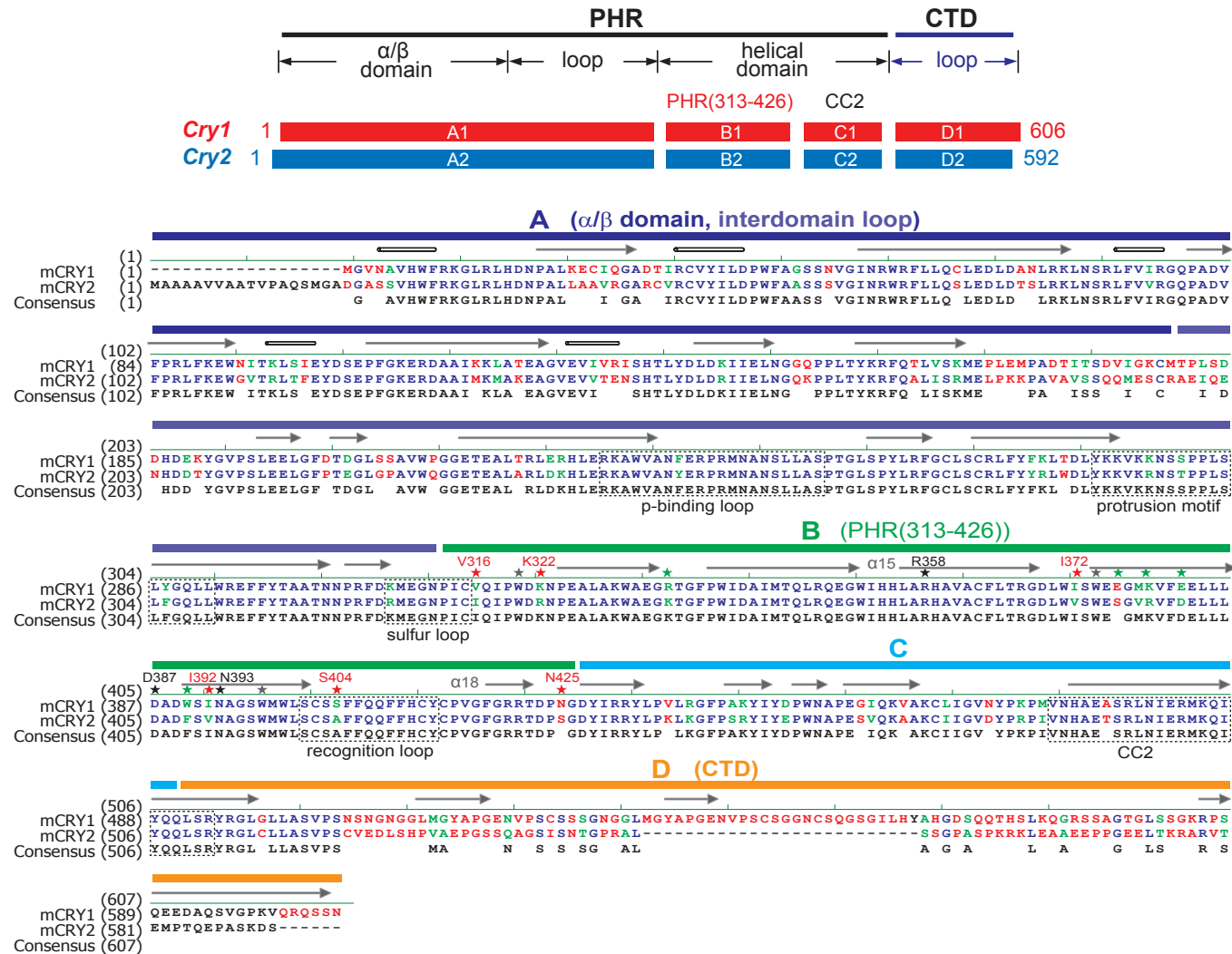


**Figure S1. Western blot analysis of CRY proteins.**

(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*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts in the rescue assay. *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts were transfected with 3xFlag-*Cry1* or 3xFlag-*Cry2* constructs in the absence or presence of *Bmal1/Clock* as indicated. 3xFlag-*Cry1* and 3xFlag-*Cry2* are functionally comparable to *Cry1* and *Cry2*, respectively, in the rescue assay (left panel). Western blot analysis with anti-Flag antibody shows that CRY1 and CRY2 are expressed to similar levels in these cells (right panel).

**Figure S2**



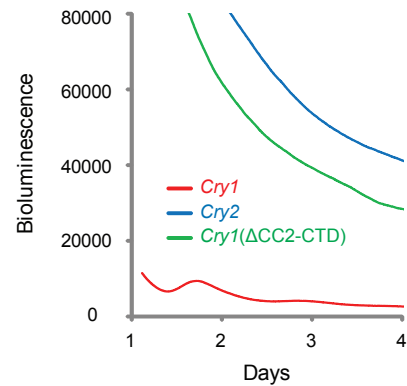
**Figure S2. Sequence alignment and key structural features of mCRY1 and mCRY2.**

mCRY1 and mCRY2 share ~70/80% sequence identity/similarity. Amino acid sequence alignment was generated using Vector NTI (Invitrogen). Above the sequence alignment, a schematic diagram of domain structure of CRY proteins, as in Figure 2A. Amino acid sequence alignment: blue, identical; green, similar; red, divergent. Domain assignment: region A (blue), B (green), C (cyan), and D (yellow). Secondary structures: arrow, alpha helix (numbers are assigned based on *Arabidopsis* UVR3); barrel, beta strand; CC2: coiled coil 2. Key amino acid residues: black star, FAD-binding residues; gray star, Trp triad; red star, arrhythmic when mutated in *Cry\**; green star, rhythmic when mutated.

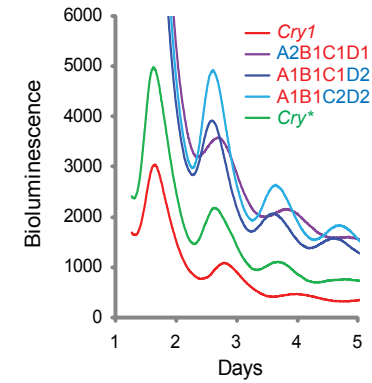
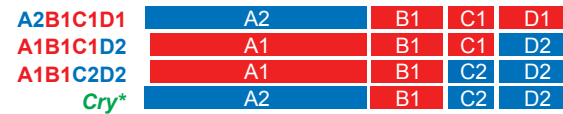


## Figure S3

**A**



**B**

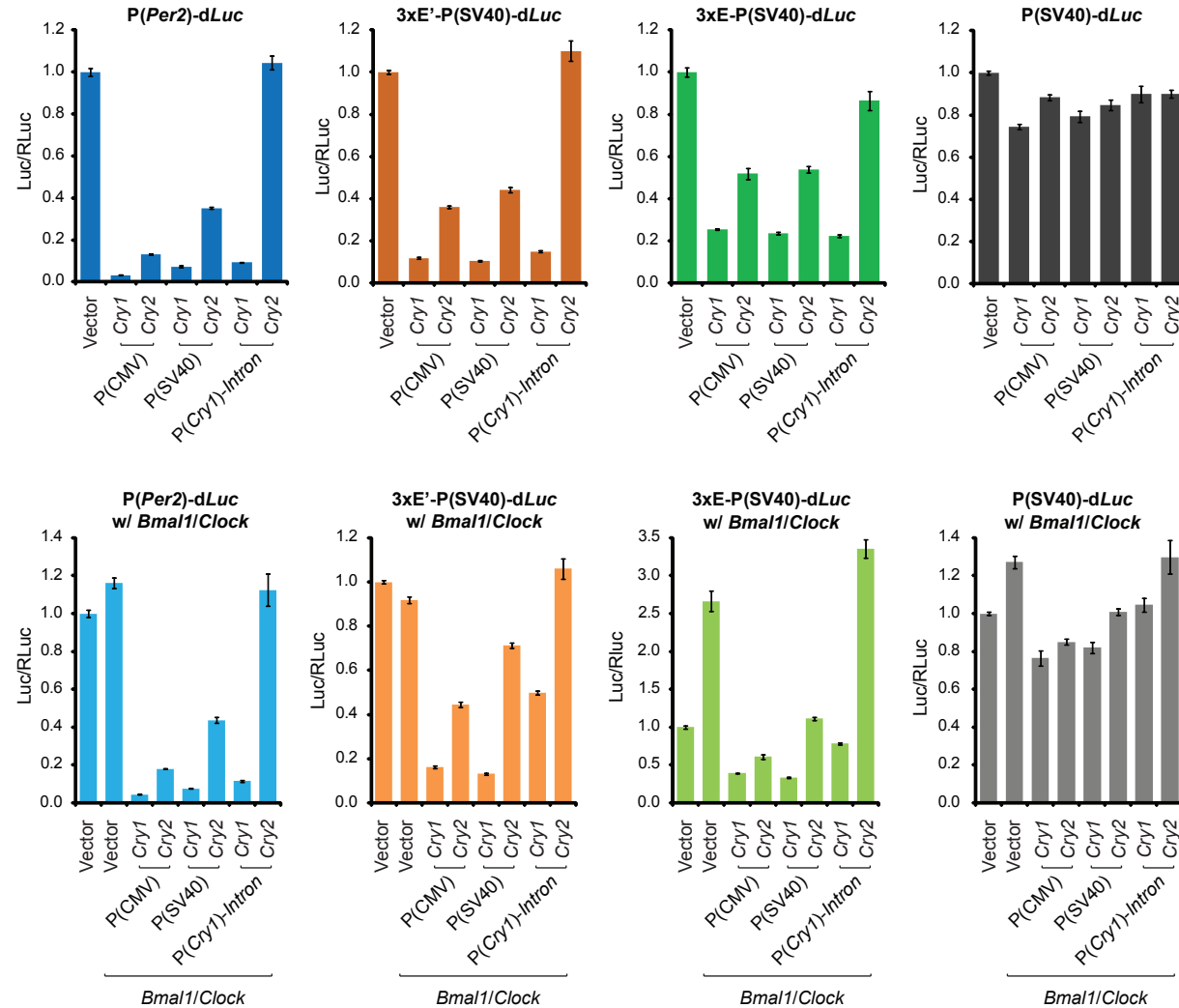


**Figure S3. Roles of coiled-coil 2 (CC2)-containing C region and the CTD of CRY in clock function.**

**(A)** In the truncation construct, *Cry1(ΔCC2-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*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts (bottom panel; representative bioluminescence records).

**(B)** Representative bioluminescence records from *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts expressing various *Cry* chimeras. The *Cry* rescue assay was performed as in Figure 1B. While the chimeras that contain the CTD from CRY1 (*Cry1* and A2B1C1D1) restored rhythms with a period length of ~27 hr (Figure 3A), those that contain the CTD from CRY2 (A1B1C1D2, A1B1C2D2 and *Cry\**) restored rhythms with a period length of ~24 hr (Figure 3A), implicating the CTD in regulating period length.

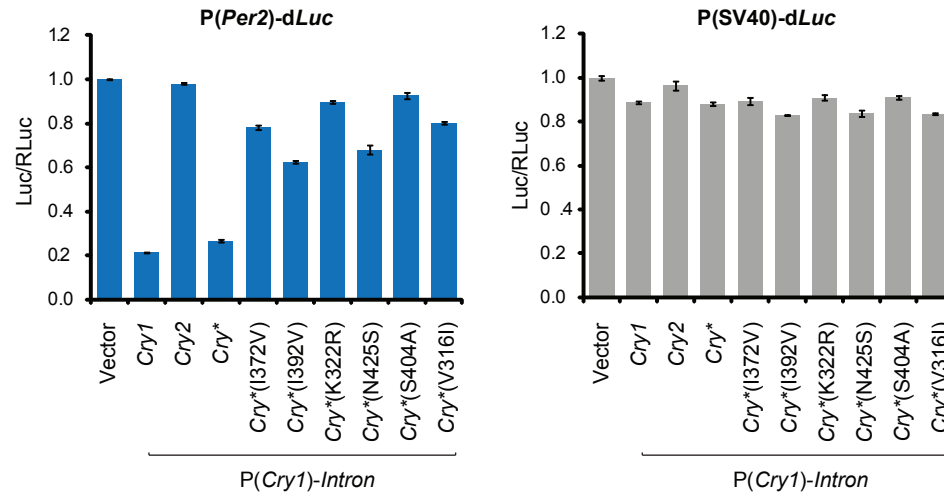
# Figure S4



**Figure S4. Cry1 and Cry2 display differential repression activity.**

Dual Luciferase reporter assay in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts was performed to test repression activity of *Cry1* and *Cry2* in the absence (top panel) or presence (bottom panel) 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)-dLuc (control) or the P(*Per2*)-dLuc reporter. A Renilla *Luciferase* (RLuc) 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. Similar results were obtained using 3x E'-box-P(SV40) or 3xE-box-P(SV40) promoter, and when *Bmal1* and *Clock* are ectopically over-expressed. Mean and SD (error bar) of two independent experiments are shown (n = 3 for each experiment).

# Figure S5



**Figure S5. Mutation of each of the six critical residues within the CRY1-PHR(313-426) of CRY\* abolishes transcriptional repression activity.**

Dual Luciferase reporter assay in *Cry1*<sup>-/-</sup>:*Cry2*<sup>-/-</sup> fibroblasts 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 2D). All mutants displayed weak repression (similar to *Cry2*), corroborating circadian phenotypes as shown in Figure 2D.



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