

# The Arg-293 of Cryptochrome1 is responsible for the allosteric regulation of CLOCK-CRY1 binding in circadian rhythm

Received for publication, May 12, 2020, and in revised form, October 1, 2020. Published, Papers in Press, October 7, 2020, DOI 10.1074/jbc.RA120.014333

Seref Gul<sup>1</sup> , Cihan Aydin<sup>2</sup>, Onur Ozcan<sup>3</sup> , Berke Gurkan<sup>3</sup>, Saliha Surme<sup>3</sup> , Ibrahim Baris<sup>3</sup> ,  
and Ibrahim Halil Kavakli<sup>1,3,\*</sup> 

From the <sup>1</sup>Department of Chemical and Biological Engineering, Koc University, Istanbul, Turkey, <sup>2</sup>Department of Molecular Biology and Genetics, Istanbul Medeniyet University, Istanbul, Turkey, and <sup>3</sup>Department of Molecular Biology and Genetics, Koc University, Istanbul, Turkey

Edited by John M. Denu

Mammalian circadian clocks are driven by transcription/translation feedback loops composed of positive transcriptional activators (BMAL1 and CLOCK) and negative repressors (CRYPTOCHROMES (CRYs) and PERIODs (PERs)). CRYs, in complex with PERs, bind to the BMAL1/CLOCK complex and repress E-box-driven transcription of clock-associated genes. There are two individual CRYs, with CRY1 exhibiting higher affinity to the BMAL1/CLOCK complex than CRY2. It is known that this differential binding is regulated by a dynamic serine-rich loop adjacent to the secondary pocket of both CRYs, but the underlying features controlling loop dynamics are not known. Here we report that allosteric regulation of the serine-rich loop is mediated by Arg-293 of CRY1, identified as a rare *CRY1* SNP in the Ensembl and 1000 Genomes databases. The p.Arg293His *CRY1* variant caused a shortened circadian period in a *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> double knockout mouse embryonic fibroblast cell line. Moreover, the variant displayed reduced repressor activity on BMAL1/CLOCK driven transcription, which is explained by reduced affinity to BMAL1/CLOCK in the absence of PER2 compared with CRY1. Molecular dynamics simulations revealed that the p.Arg293His *CRY1* variant altered a communication pathway between Arg-293 and the serine loop by reducing its dynamicity. Collectively, this study provides direct evidence that allosterism in CRY1 is critical for the regulation of circadian rhythm.

The circadian clock modulates numerous behavioral and physiological processes through rhythmic transcriptional regulation (1, 2). In humans, physiological parameters such as alertness, memory, heart rate, blood pressure, and immune responses are clock-controlled (3–6). Additionally, genetic and epidemiological studies have linked clock disruption to various adverse metabolic phenotypes (7). Circadian rhythms at the metabolic and transcriptional level are conserved across phyla, influencing physiology in cyanobacteria, fungi, plants, and virtually all animals (8).

The cellular circadian clock is composed of positive and negative transcriptional feedback loops. Transcriptional factors BMAL1 and CLOCK, having basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domains, are central to circadian

biology. They form heterodimer and bind to E-box elements (CACGTG), where they initiate the transcription of clock-controlled genes including the *Period* (*Per*) and *Cryptochrome* (*Cry*) (9–11). PER and CRY form a heterodimer which translocates into the nucleus upon phosphorylation by casein kinase IΔ, and inhibits BMAL1/CLOCK-mediated transcription. Ultimately, PER and CRY act as negative regulators of their own transcription (12–15). Although the initial transcriptional activation step generates the positive arm of the circadian clock, the latter inhibitory step generates the negative arm of the feedback loop (9, 16). F-box proteins, β-transducin repeat containing protein 1 (beta-TrCP1), and F-box and leucine rich repeat protein 3 (FBXL3) ubiquitinate PER and CRY, and thus initiate their proteasomal degradation, respectively (17, 18). Degradation of CRY and PER proteins relieves the repression on BMAL1/CLOCK, restarting the cycle anew (19).

There are two *Cry* genes in mammals: *Cry1* and *Cry2*. They code for proteins that belong to the photolyase/cryptochrome family and act as transcriptional repressors (12). Several studies suggest that CRYs might have different roles in the circadian clock. First, studies with *Cry1*<sup>-/-</sup> and *Cry2*<sup>-/-</sup> mice suggest that each *Cry* plays a different role in animal behavior. *Cry1*<sup>-/-</sup> animals exhibit shorter free-running circadian rhythm, whereas *Cry2*<sup>-/-</sup> animals display a longer free-running circadian rhythm (14, 20). *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> animals show an arrhythmic phenotype under constant dark conditions (14, 15). Second, CRY1 and CRY2 are present at different circadian times on chromosomal DNA (21). Third, when compared with CRY2, CRY1 is a more potent repressor of BMAL1/CLOCK transactivation on E-boxes (22, 23). Finally, CRY1 specifically interacts with cystathionine β-synthase by regulating one-carbon and trans-sulfuration pathways (24). All these studies show functional differences between the CRYs in the circadian clock mechanism.

CRYs contain a photolyase homology region (PHR) consisting of primary (FAD binding) and secondary pockets and extended C-terminal region (25, 26). PHR and C-terminal regions of the CRYs are required to maintain rhythmicity and amplitude of circadian rhythm, respectively (22, 27). CRY1 interacts directly with CLOCK and BMAL1 through two distinct regions on the PHR domain (28). C-terminal regions of CRYs interact with the transactivation domain (TAD) of BMAL1 (29, 30). Biochemical and computational modeling

\* For correspondence: Ibrahim Halil Kavakli, [hkavakli@ku.edu.tr](mailto:hkavakli@ku.edu.tr).

This is an open access article under the CC BY license.

analyses show that the HI loop on the PAS-B domain of CLOCK docks into the secondary pocket of CRYs (28). Moreover, mutational analysis of CRYs indicated that residues around the secondary pocket of CRY1 and CRY2 are critical for the CLOCK binding and their repression activity (31). In fact, a human gain-of-function CRY1 variant (exon 11 skipping mutation in C-tail of CRY1) found in people suffering from familial delayed sleep phase disorder and attention deficit/hyperactivity disorder exhibits high affinity to BMAL1/CLOCK (32, 33).

Coiled-coil helix in the C-terminal of CRYs, which interacts with PER2 and plays a role in CRY nuclear shuttling, inhibits the binding of FBXL3 and subsequent proteasomal degradation (34, 35). FBXL3 competes with PER2 to interact with CRYs by wrapping around the coiled-coil helix via  $\beta$ -sheet domain. In addition, the last five C-terminal residues of FBXL3 penetrate to the FAD binding pocket which, in turn, facilitates the ubiquitination of CRYs (36, 37). Crystal structure of CRY2 PHR-PER2 C-terminal CRY-binding domain (CBD) revealed an unusual binding mode of PER2-CBD onto CRY2 (34). The N-terminal half of PER2-CBD reaches the rim of secondary pocket of the CRY2-PHR. This interaction allows the serine loop, located adjacent to the secondary pocket, to adopt different conformations. A recent study indicates that PER2 remodels the serine loop of CRY2 and, in turn, enhances its affinity toward BMAL1/CLOCK (38). In the same study, replacement of amino acid residues in the serine loop of CRY1 reduced its affinity to BMAL1/CLOCK. However, communication and allosteric regulation between primary and secondary pockets of CRY1 have not been reported so far.

Here, we characterized three SNPs (p.Gly144Val, p.Arg293His, and p.Arg348Cys) identified from 1000 Genomes project and Ensembl databases. Among them, analysis of the p.Arg293His CRY1 variant indicated that it had a longer  $t_{1/2}$  and weaker repression activity and caused shorter period of circadian rhythm. Further biochemical analysis showed that this variant had a weaker affinity toward the BMAL1/CLOCK complex in the absence of PER2. Interestingly, a comparison of CRY2 and the p.Arg293His CRY1 variant showed very similar molecular characteristics in terms of  $t_{1/2}$  and binding properties to BMAL1/CLOCK. However, in rescue assays using *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> mouse embryonic fibroblasts CRY2 and p.Arg293His CRY1 showed some differences. The period length of rhythm observed in p.Arg293His CRY1 rescue cell lines were shorter than WT CRY1, but longer than CRY2. To understand all these changes mediated by Arg-293 within the structural context of CRY1, we performed MD simulations. The MD studies of both mutant and WT CRY1 suggest that Arg-293 is involved in allosteric regulation of CLOCK binding by regulating the dynamicity of the serine loop adjacent to the secondary pocket.

Collectively, this study provides new insights into the circadian clock mechanism. In particular, our study suggests potential new targets for drug discovery by identifying novel amino acids affecting the repressor and binding activities of the CRY1 and the possible allosteric regulation in the CRY1 secondary pocket.

## Results

### Selection of CRY1 SNPs

Several SNPs with either low or high frequencies are associated with different types of diseases. However, the functional significance of rare missense mutations deduced from large-scale genomic studies and how these mutations affect protein function are largely unknown. Because of that, we investigated the effect of missense variants of the human *CRY1* gene. The SNPs for *CRY1* were selected from the 1000 Genomes project and Ensembl databases (39, 40) based on *in silico* analyses and their location within the CRY1 structure. Among 12,000 *CRY1* SNPs, a majority of them are located within noncoding regions. Of the SNPs reported within the *CRY1* coding region (116 missense mutations are detected), we have focused on missense mutations affecting the PHR domain of the CRY1, which is crucial for its function (22). Three of these variants predicted to be detrimental to protein structure or function were selected based on their SIFT, PolyPhen, and Proven scores (Table 1). These variants were interpreted as “disease-causing” by MutationTaster (41). Additionally, VarSome-Verdict and ClinVar were used to understand the clinical effects of these variants. Although these variants were classified as “variant of uncertain significance-VUS”, and all of them meet two pathogenic American College of Medical Genetics and Genomics criteria (guidelines for interpretation of sequence variants) (42). None of these variants were reported in ClinVar. The p.Arg293His and p.Arg348Cys CRY1 variants were detected as heterozygous; the genotype of the p.Gly144Val was not reported in Ensembl. To analyze the degree of conservation of these SNPs, we performed multiple sequence alignment (Fig. 1A). The Arg-293 in CRY1 is conserved in all CRY1, whereas Gly-144 is conserved in all CRY1 proteins with the exception of mosquito CRY1. Finally, Arg-348 is conserved in CRY1 from most organisms, but not in CRY1 from sponge, mosquito, housefly, green sea turtle, blowfly, and stony coral. WT amino acids in all three SNPs are highly conserved in the CRY1 across different organisms, which suggests a potential role in protein function (Fig. 1A). Thus, we aimed to investigate the effects of these variants at the molecular level. To do that, we generated mutant constructs of mouse *Cry1* (*mCry1*) by site-directed mutagenesis, and these variants were subjected to further functional analysis.

### The effect of SNPs on CRY1 $t_{1/2}$ and molecular clock function

The functional characteristics of the selected rare missense variations were studied in detail using biochemical and cell-based assays. Mapping of SNPs onto the crystal structure of CRY1 indicated that these SNPs are located on functionally important domains. Gly-144 and Arg-348 are both exposed to solvent and are located on the loop between helices  $\alpha 5$  and  $\alpha 6$ , or in helix  $\alpha 14$ , respectively (Fig. 1B). Arg-293 is located within the FAD binding pocket and FBXL3 interaction site which is adjacent to the secondary pocket (Fig. 1, B and C). Because FBXL3 is the E3 ligase responsible for ubiquitination of CRY1, mutations in this region of FBXL3 might change the stability of CRY1 (Fig. 1D). We, therefore, investigated the effect of these mutations on the  $t_{1/2}$  of CRY1 by monitoring the degradation of CRY1 fused to LUC (CRY1::LUC) at the C-terminal. After

**Table 1**

The clinical features of selected CRY1 SNPs

| dbSNP       | Position in CDS (NM_004075.4) | Position in protein (NP_004066.1) | SIFT/PolyPhen/Provean | ACMG           | gnomAD exom      | ClinVar |
|-------------|-------------------------------|-----------------------------------|-----------------------|----------------|------------------|---------|
| rs78310335  | c.431G>T                      | p.Gly144Val                       | 0/0.999/damaging      | VUS (PM2, PP3) | not detected     | —       |
| rs772585429 | c.878G>A                      | p.Arg293His                       | 0/1/damaging          | VUS (PM2, PP3) | $f = 0.00000797$ | —       |
| rs749506981 | c.1042C>T                     | p.Arg348Cys                       | 0/0.989/damaging      | VUS (PM2, PP3) | not detected     | —       |

generating *Cry1* variants, plasmids were transiently transfected into HEK293T cells, which were then treated with cycloheximide to inhibit protein synthesis. Luminescence was monitored to determine the  $t_{1/2}$  of the proteins. Stability of the p.Arg348Cys CRY1 and p.Gly144Val CRY1 variants were found to be similar to WT CRY1 (Fig. 2A). However, the  $t_{1/2}$  of the p.Arg293His CRY1 variant was significantly longer than that of WT CRY1 and shorter than luciferase. We further confirm this result with biochemical assays. After transfection of HEK293T cells with proper plasmids, cells were treated with cycloheximide and harvested at the 4th and 8th h. Analysis of samples with Western blotting indicated that p.Arg293His CRY1 is indeed more stable than the WT CRY1 (Fig. 2A, right panel). These results suggested that although mutations distant from FBXL3 interaction sites did not change the stability of CRY1, mutation in the binding region of FBXL3 (i.e. p.Arg293His) increased the  $t_{1/2}$  of CRY1 probably because of the attenuated interaction between CRY1 and FBXL3.

We next determined the effect of these variants on circadian clock function. To do this, we employed a complementation assay using *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> mouse embryonic fibroblast cells (DKO-MEF). Plasmid constructs carrying WT *Cry1* were shown to rescue circadian rhythms in DKO-MEF when expressed under the control of m*Cry1* promoter containing E/E'-box and D-box elements and the ROR/REV-ERB-binding element in the first intron (43). Thus, we introduced the p.Gly144Val, p.Arg293His, and p.Arg348Cys variants into the pMU2-*Cry* plasmid. The *Cry* expression vector carrying either the variants or WT *Cry1* and the *mPer2*-dLuc reporter plasmid were transiently expressed in DKO-MEF cells. We monitored bioluminescence for 5 days. Although WT and variants were able to restore the circadian rhythm, they had varying effects on period length. The period length for WT CRY1 was calculated as 26.3 h (Fig. 2B). The p.Gly144Val and p.Arg348Cys CRY1 variants increased the period length to 27.8 and 27 h, respectively. Despite the longer  $t_{1/2}$  of the p.Arg293His CRY1 variant, the mutant caused a significantly shorter period length (23.8 h) and displayed weaker repressor activity based on higher luminescence intensities compared with WT CRY1. Because rhythm is generated as a result of interaction between core clock proteins, e.g. CRY1, PER2, BMAL1, and CLOCK, this intriguing observation on p.Arg293His mutant led us to analyze binding properties of these mutants to the core clock proteins.

#### p.Arg293His CRY1 binding to BMAL1/CLOCK is differentially disrupted

To assess binding of each CRY1 variants to CLOCK and BMAL1, HEK293T cells were transiently transfected with expression plasmids coding for *CLOCK*, *Bmal1*, and either WT or *Cry1* variants. The CRY1-myc-His (variants or WT)–containing protein complexes were then precipitated using

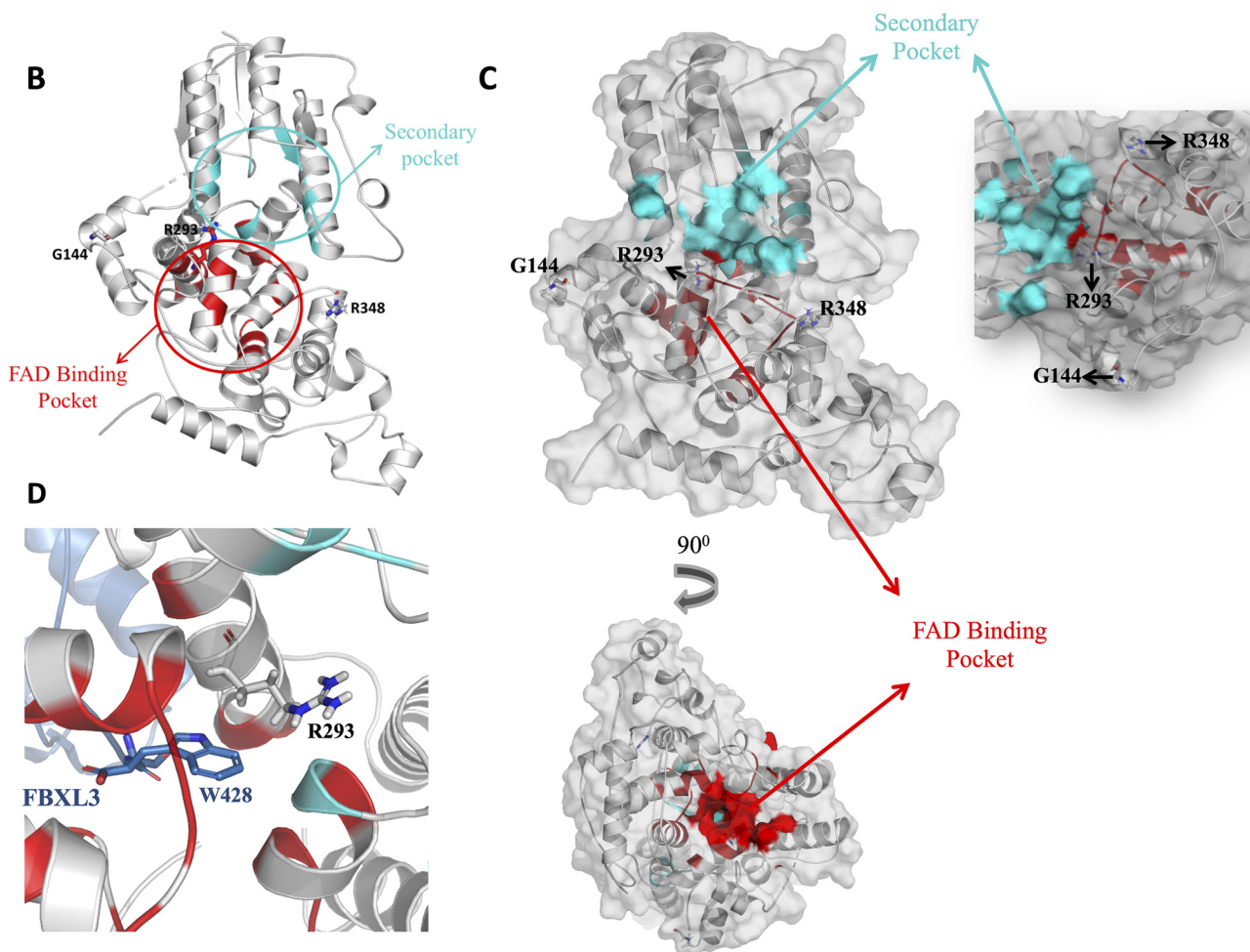
Ni-NTA agarose, and the samples were probed for the presence of CLOCK and BMAL1 by Western blot analysis as described in Ref. 31. To assess the binding of CLOCK properly, comparable amount of the CRYs were used in pulldown assay. The p.Gly144Val and p.Arg348Cys CRY1 variants exhibited similar affinities to BMAL1/CLOCK compared with WT CRY1, whereas the p.Arg293His CRY1 variant exhibited significantly less affinity to BMAL1/CLOCK (Fig. 3A). A similar experiment was performed in the presence and absence of PER2 to investigate the possibility that PER2 is required for proper binding. All three CRY1 variants and WT CRY1 bound similar amounts of PER2. Nevertheless, the p.Arg293His CRY1 variant was to bind less BMAL1/CLOCK in the absence of PER2 (Fig. 3B). Biochemical binding analysis showed that reduced repression by the p.Arg293His variant of CRY1 is most likely caused by attenuated binding to the BMAL1/CLOCK dimer. However, the p.Gly144Val and p.Arg348Cys variants retained binding activity to the BMAL1/CLOCK dimer. Overall, the p.Arg293His CRY1 variant had longer  $t_{1/2}$ , rescued circadian rhythms with shorter period in a weakly repressed state in DKO-MEF cells (Fig. 3B) and exhibited reduced affinity for the BMAL1/CLOCK dimer even in the presence of PER2 (Fig. 3). These features of the p.Arg293His CRY1 variant reported here are very similar to those reported for CRY2 (22, 31), which led us to perform additional studies.

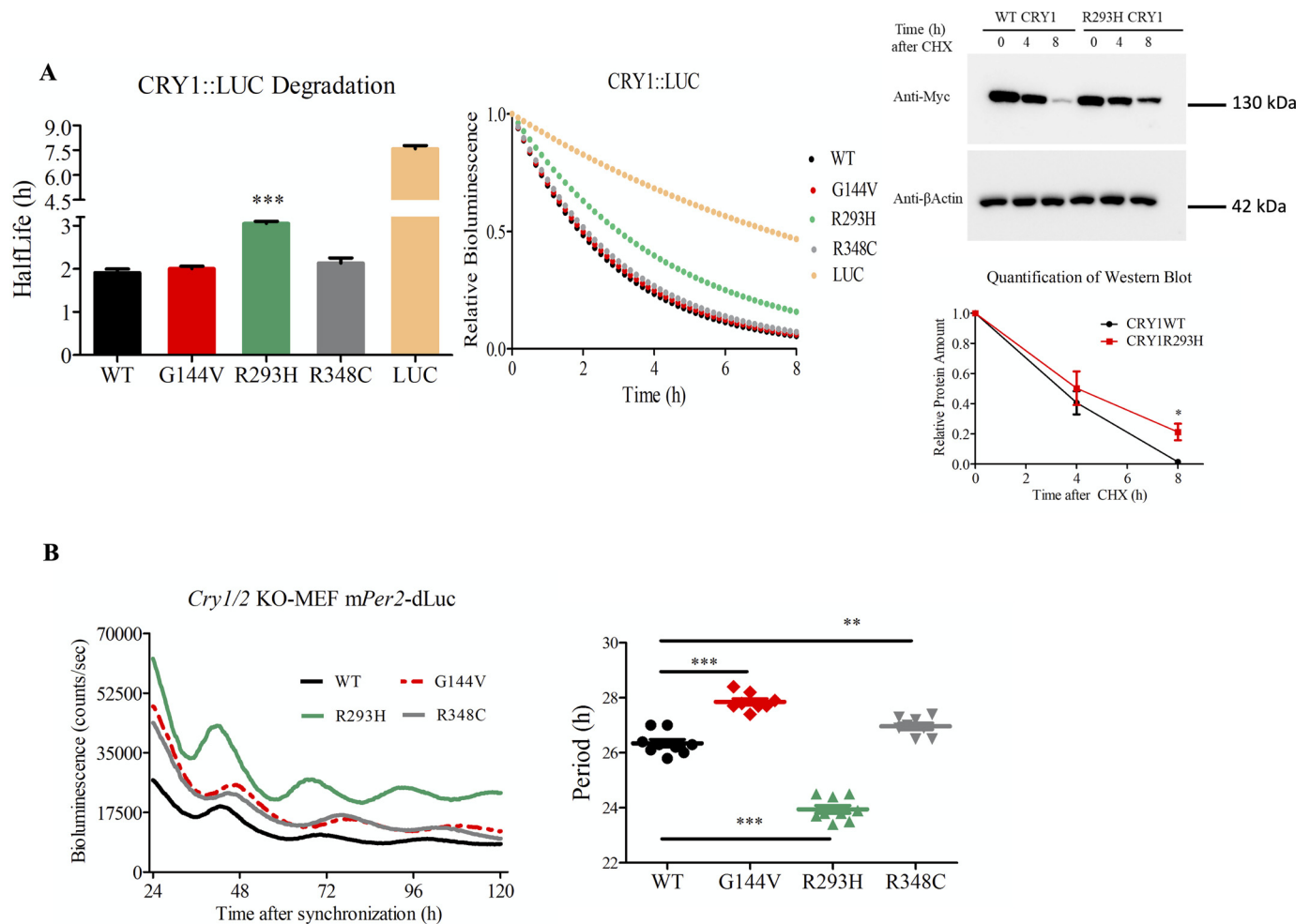
#### p.Arg293His CRY1 variant exhibits similar characteristics to CRY2

The secondary pocket of CRY1 is critical for its interaction with CLOCK. Subtle changes in this pocket can result in substantial differences in periodicity in cycling cells (28, 38). For example, differential rhythms driven by CRY1 and CRY2 are the result of these changes (23). To directly compare p.Arg293His CRY1 and WT CRY2, we measured their half-lives and effects on circadian rhythms using DKO-MEF cells. Both the pArg293His CRY1 variant and WT CRY2 exhibited similar half-lives (Fig. 4A). It has been shown that CRY2 can rescue circadian rhythms when the pMU2 plasmid with *Cry2* cDNA is transfected in DKO-MEFs (31, 43). Therefore, we cloned *Cry2* cDNA into the pMU2 plasmid and transfected this construct into DKO-MEF to see whether it rescues circadian rhythms. Compared with CRY1, CRY2 was able to rescue circadian rhythms with a shorter period, which was consistent with a previous study (31). Our results showed that cells expressing CRY2 had higher overall luminescence intensity than cells expressing CRY1, suggesting higher BMAL1/CLOCK activity, again consistent with a previously published report (22). Interestingly, cells expressing the p.Arg293His CRY1 or CRY2 had similar luminescence level (Fig. 4B). In the complementation assay, DKO-MEF cells expressing WT CRY1 or CRY2 had



| A                                       | G144                                 | R293                              | R348                                  |
|-----------------------------------------|--------------------------------------|-----------------------------------|---------------------------------------|
| <i>Amphimedon queenslandica</i> CRY2    | HTLYSPTELIKLN--GCKPILTFKDFRTLLSK...  | LVQEVSSKLLQREFYFLVSSQVNFPTD...    | SGMTGYFPWIDAAIRQMKEGWIHNLRESIATFLTR   |
| <i>Amphimedon queenslandica</i> CRY1    | HTLYNPEDILIRAN--NGRFPHSYKELRLLFL...  | LFAELTKNLLMREFAYMVGLTVPKFDTM...   | EGRTGYFPWIDAAIRQIVRDGWSHYSTRQSIATFLTR |
| <i>Danaus plexippus</i> Cry1            | HTLWNPDTVIKAN--GGIPPLTYQMFLHTVETI... | TQFTTGQLIWRREFFYTMSVNNPNYAQM...   | EGRTGFPWIDAAIRQLRTEGWLHVVNRVTASFLTR   |
| <i>Helicoverpa armigera</i> CRY1        | HTLWPEPTVIKAN--GGIPPLTYQMFLHTVAT...  | ASHFITGQLIWRREFFYTMSVNNPNYQGM...  | EGRTGFPWIDAAIRQLRTEGWLHHAARNTASFLTR   |
| <i>Anopheles gambiae</i> Cry1           | HTLWNPPIEVQTN--GDIPPLTYQMFLHTVNI...  | GGHHITGQLIWRREFFYTMSVNNPNHYGEM... | EGRTGFPWIDAAIRQLLAEGWLHLLRNITATFLTR   |
| <i>Drosophila melanogaster</i> Cry      | HTLWDPQLVIETN--GGIPPLTYQMFLHTVQI...  | GGAHITGQLIWRREFFYTMSVNNPNYDRM...  | LGQTGFPLIDGAMRQLLAEGWLHLLRNITATFLTR   |
| <i>Lucilia cuprina</i> CRY1             | HTLWDPKKVIDTN--GGIPPLTYQMFLHTVQI...  | GGAHITGQLIWRREFFYTMSVNNPNYDRM...  | MGQTGFPLIDAAIRQLLAEGWLHLLRNITATFLTR   |
| <i>Musca domestica</i> CRY1             | HTLWDPQTVIDTN--GGIPPLTYQMFLHTVQV...  | GGAHITGQLIWRREFFYTMSVNNPNYDRM...  | EGQTGFPLIDAAIRQLLAEGWLHLLRNITATFLTR   |
| <i>Acropora millepora</i> CRY2          | HTLYDPRIILRGLS--SGIVPLLFDEFKESVLQ... | PPSELVFKLLWRREFFVVGQIPDAHEM...    | KGTTGFPWIDAIMRQLRTEGWIHNIARRAVASFLTR  |
| <i>Danio rerio</i> CRY4                 | HTLYDVKRIVKAN--GGSPPLTYKRFHLVLSV...  | PPVSLQGQLLWRREFFYTVASATPNFTKM...  | TAQTGFPWIDAIMTQLLQEGWIHLLARHAVACFLTR  |
| <i>Xenopus tropicalis</i> CRY4          | HTLYDIKTIILN--CGKPPLTYKRFHLVLSM...   | PPVSLQGQLLWRREFFYTVASATPNFTKM...  | EAKTGFPWIDAIMTQLHNEGWIHLLARHAVACFLTR  |
| <i>Chelonia mydas</i> CRY1              | HTLYDIRILDMN--GGTPPLTYKRFHLVLSV...   | PPVSLQGQLLWRREFFYTVASATPNFTKM...  | MAQTGFPWIDAIMTQLHNEGWIHLLARHAVACFLTR  |
| <i>Gallus gallus</i> CRY4               | HTLYNPQRILELN--GGTPPLTYKRFHLVLSL...  | PPVSLQGQLLWRREFFYTVASATPNFTKM...  | TAQTGFPWIDAIMTQLRQEGWIHLLARHAAACFLTR  |
| <i>Drosophila melanogaster</i> (6-4)PHR | HTIYNPELVIAKN--LGKAPITYKQFLGIVEQ...  | PPVSLIGQLMWRREFFYTVAEEPNFDRM...   | HGRTGYFPWIDAIMRQLRQEGWIHLLARHAVACFLTR |
| <i>Acropora millepora</i> CRY1          | HTLYDPQLIIKHN--SGTAPLTYKRFALVRS...   | PPVSLIGQLMWRREFFYTVAEEPNFDRM...   | CGQTGFPWIDAIMIQLKQEGWIHLLARHAVACFLTR  |
| <i>Danio rerio</i> Cry2a                | HTLYNLDKIIELN--GGQPPLTYKRFQTLISR...  | PPISLYGQLLWRREFFYTVAANNPNFDQM...  | EGKTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Danio rerio</i> Cry1a                | HTLYDLDKIIELN--GGQSPLTYKRFQTLISR...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Xenopus laevis</i> CRY1              | HTLYDLDKIIELN--GGQPPLTYKRFQTLISK...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Rattus norvegicus</i> CRY1           | HTLYDLDKIIELN--GGQPPLTYKRFQTLISK...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Homo sapiens</i> CRY1                | HTLYDLDKIIELN--GGQPPLTYKRFQTLISK...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Mus musculus</i> CRY1                | HTLYDLDKIIELN--GGQPPLTYKRFQTLISK...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Sylvia borin</i> CRY1b               | HTLYDLDKIIELN--GGQPPLTYKRFQTLISR...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Gekko japonica</i> CRY1              | HTLYDLDKIIELN--GGQPPLTYKRFQTLISR...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Gallus gallus</i> CRY1               | HTLYDLDKIIELN--GGQPPLTYKRFQTLISR...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Alligator sinensis</i> CRY1          | HTLYDLDKIIELN--GGQPPLTYKRFQTLISR...  | PPISLYGQLLWRREFFYTAAATNNPRFDM...  | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Danio rerio</i> Cry3                 | HTLYNPDRIIEMN--NHSPPPLTYKRFQAIVNR... | PPISLFGQLLWRREFFYTAGTNNPNFDM...   | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Cyprinus carpio</i> CRY              | HTLYSPDRIIEMN--NHSPPPLTYKRFQAIVNR... | PPISLFGQLLWRREFFYTAGTNNPNFDM...   | EGRTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Xenopus laevis</i> CRY2              | HTLYSDSRVIELN--GHSPPPLTYKRFQAIISR... | PPISLYGQLLWRREFFYTAAATNNPNFDM...  | EGKTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Gallus gallus</i> CRY2               | HTLYNLDRIIELN--GNKPPLTYKRFQAIISR...  | PPISLYGQLLWRREFFYTAAATNNPNFDM...  | EGKTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Homo sapiens</i> CRY2                | HTLYDLDRIIELN--GOKPPLTYKRFQAIISR...  | PPISLFGQLLWRREFFYTAAATNNPNFDM...  | EGKTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Mus musculus</i> CRY2                | HTLYDLDRIIELN--GOKPPLTYKRFQAIISR...  | PPISLFGQLLWRREFFYTAAATNNPNFDM...  | EGKTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Rattus norvegicus</i> CRY2           | HTLYDLDRIIELN--GOKPPLTYKRFQAIISR...  | PPISLFGQLLWRREFFYTAAATNNPNFDM...  | EGKTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Tribolium castaneum</i> CRY          | HTLYHLQDIIDRN--GGKAPLTYHQFLAIAC...   | PPISLHGQLLWRREFFYTCAATNNPNFDM...  | NGQTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Apis mellifera</i> CRY2              | HTLYKLDEIERN--GDKPPLTYHQFQVIVAS...   | PPISLHGQLLWRREFFYTCAATNNPNFDM...  | NGQTGFPWIDAIMTQLRQEGWIHLLARHAVACFLTR  |
| <i>Antheraea pernyi</i> CRY2            | HTLYKLDKIIERN--GCKAPLTYHQFQALIAS...  | PPISLHGQLLWRREFFYTCAATNNPNFDM...  | NGQTGFPWIDAIMIQLREEGWIHLLARHAVACFLTR  |
| <i>Helicoverpa armigera</i> CRY2        | HTLYKLDKIIERN--GCKAPLTYHQFQALIAS...  | PPISLHGQLLWRREFFYTCAATNNPNFDM...  | SGQTGFPWIDAIMIQLREEGWIHLLARHAVACFLTR  |
| <i>Spodoptera littoralis</i> CRY2       | HTLYKLDKIIERN--GCKAPLTYHQFQALIAS...  | PPISLHGQLLWRREFFYTCAATNNPNFDM...  | SGQTGFPWIDAIMIQLREEGWIHLLARHAVACFLTR  |
| <i>Escherichia coli</i> PHR CPD         | SVILPPGAVMTGNHEMYKVFPPFNKAWLKLRL...  | AGSVLNLIELWRREFFYRHLITYHPSLCKH... | EGKTGYPIVDAAIRQLNSTGWMHNRNITATFLVK    |
| <i>Arabidopsis thaliana</i> CRY1        | DLLEYPEWVTEDEL--GRPFSPFAFWERCLS...   | SVNLFLKSIGLREYSR-YISFNHPYSHE...   | QGRTGYPVLVDAGMRELWATGLHDIRVVVSSFFVK   |
| <i>Arabidopsis thaliana</i> CRY2        | DLLEYPEWIEYCEK--GKPFSTSNYSWKCLD...   | SADLFLRGIGLREYSR-YICFNPFTHE...    | QGRTGYPVLVDAGMRELWATGWMHNRNITATFLVK   |





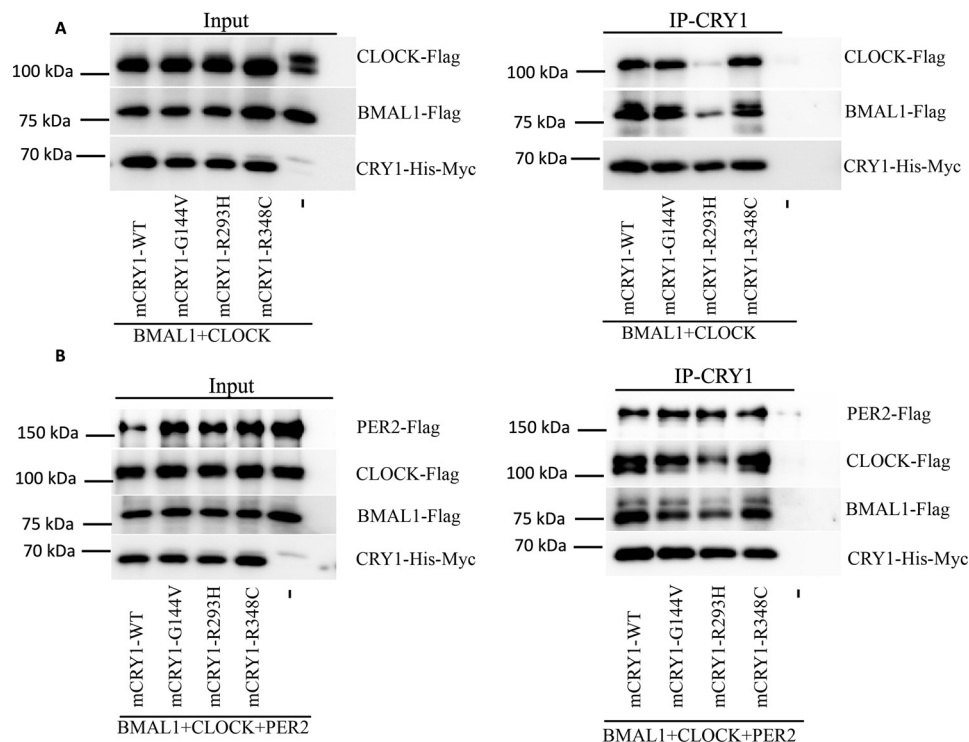
**Figure 2.  $t_{1/2}$  and rescue analysis of the WT and CRY1 variants.** A, the half-lives of WT and variant CRY1 were determined by monitoring CRY1::LUC degradation. Half-lives were calculated using a one-phase exponential decay function. Average  $t_{1/2}$  values  $\pm$  S.E. from three independent experiments (with quadruplicate samples) relative to WT CRY1::LUC were reported. Statistical significance was determined as in (B) (G144V,  $p = 0.427$ ; R293H,  $p = 0.013$ ; R348C,  $p = 0.227$ ). Statistical analysis for Western blot was performed with student's  $t$ -test (\*,  $p < 0.0297$ ). B, WT or variant CRY1 rescued circadian rhythms in *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> MEF cells with varying effects on period and amplitude. DKO MEF cells transiently transfected with pGL3-Per2-dLuc and *Cry* rescue plasmids (WT or variant). After 72 h, cells were synchronized with dexamethasone (0.1  $\mu$ M) and luminescence values were recorded for 5 days. CRY1 rhythm was representative bioluminescence of four independent experiments with eight plates. The circadian period from all plates were determined. Statistical analysis was performed using an unpaired  $t$  test with a Welch's correction (\*\*\*,  $p < 0.0001$ ; \*\*,  $p < 0.005$ ; \*,  $p < 0.05$ ; G144V,  $p < 0.0001$ ; R293H,  $p < 0.0001$ ; R348C,  $p < 0.0044$ ).

circadian periods of 26.2 and 22.9 h, respectively. Cells expressing the p.Arg293His CRY1 variant had a period length of 24.1 h, which is shorter than that of WT CRY1 and longer than CRY2 (Fig. 4B). Finally, the amplitude of the circadian rhythm was higher in cells expressing p.Arg293His CRY1 variant and CRY2 than WT CRY1 (Fig. 4B). To compare the ability of p.

Arg293His CRY1 and CRY2 to repress BMAL1/CLOCK trans-activation, we used an *mPer1*-dLuc assay using *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> MEF cells. These results indicated that both CRY2 and p.Arg293His CRY1 had comparable levels of repressor activity that was dose-dependent and statistically significantly different from that of WT CRY1 (Fig. 4C). Additionally, binding of

**Figure 1. Three rare SNPs affect highly conserved amino acid residues in CRY1.** A, alignments of selected each class of the cryptochrome/photolyase family proteins from the phylogenetic tree recently constructed by Kavakli *et al.* (66). Human CRY1 was indicated highlighted in gray color and in bold. Protein accession number organisms are as follows: *Amphimedon queenslandica* CRY1 (accession number: XP\_003386582.1), *Danaus plexippus* Cry1 (accession number: AAX58599.1), *Helicoverpa armigera* CRY1 (accession number: XP\_021184243.1), *Anopheles gambiae* CRY1 (accession number: NP\_034093), *Drosophila melanogaster* Cry1 (accession number: BAA05042.1), *Lucilia cuprina* CRY1 (accession number: XP\_023306440.1), *Musca domestica* CRY1 (accession number: XP\_005178207), *Drosophila melanogaster* (6-4)PHR (accession number: NP\_001260633.1), *Acropora millepora* CRY1 (accession number: ABP97098.1), *Danio rerio* Cry1a (accession number: BAA96846.1), *Xenopus laevis* CRY1 (accession number: NP\_001081129.1), *Rattus norvegicus* CRY1 (accession number: NP\_942045.2), *Homo sapiens* CRY1 (accession number: NP\_004066.1  $\times$ ), *Mus musculus* CRY1 (accession number: NP\_031797.1), *Sylvia borin* CRY1b (accession number: ABH03083.1), *Gekko japonica* CRY1 (accession number: XP\_015283074.1), *Gallus gallus* Cry1 (accession number: NP\_989576.1), *Alligator sinensis* CRY1 (accession number: XP\_006024339.1), *Tribolium castaneum* CRY1 (accession number: NP\_001076794.1), *Escherichia coli* PHR CPD (accession number: WP\_032176081.1), *Arabidopsis thaliana* CRY1 (accession number: NP\_567341.1), and *Solanum lycopersicum* CRY1 (accession number: AAF72555.1). B, positions of SNPs were visualized in the mCRY1 (PDB ID: 4K0R) structure using Pymol (RRID:SCR\_000305). The mCRY1 protein structure is shown as a ribbon diagram (left) or surface (right), and the mutant residues are shown as sticks. C, surface representation of CRY1 residues in the FAD binding pocket and in the secondary pocket were colored red and cyan, respectively, as described in Rosensweig *et al.* (31). Gly-144 and Arg-348 are exposed to solvent; Arg-293 is located in the FAD binding pocket facing the secondary pocket. D, Arg-293 is contacting FBXL3. CRY1 structure was superimposed to CRY2-FBXL3 complex in PDB ID 4I6J.





**Figure 3. Pulldown analysis.** A and B, physical interaction between WT/p.Arg293His CRY1 variant with (A) BMAL1/CLOCK and (B) BMAL1/CLOCK/PER2. HEK293T cells were transfected with plasmids expressing *Bmal1*-FLAG, *CLOCK*-FLAG, and WT or variant *Cry1*-His-Myc. Proteins were isolated with a Ni-NTA agarose resin and analyzed by Western blotting. The blot shown is representative of three independent experiments. Multiple bands in BMAL1 and CLOCK are a result of posttranslational modifications. Results are shown here are representations of three independent experiments.

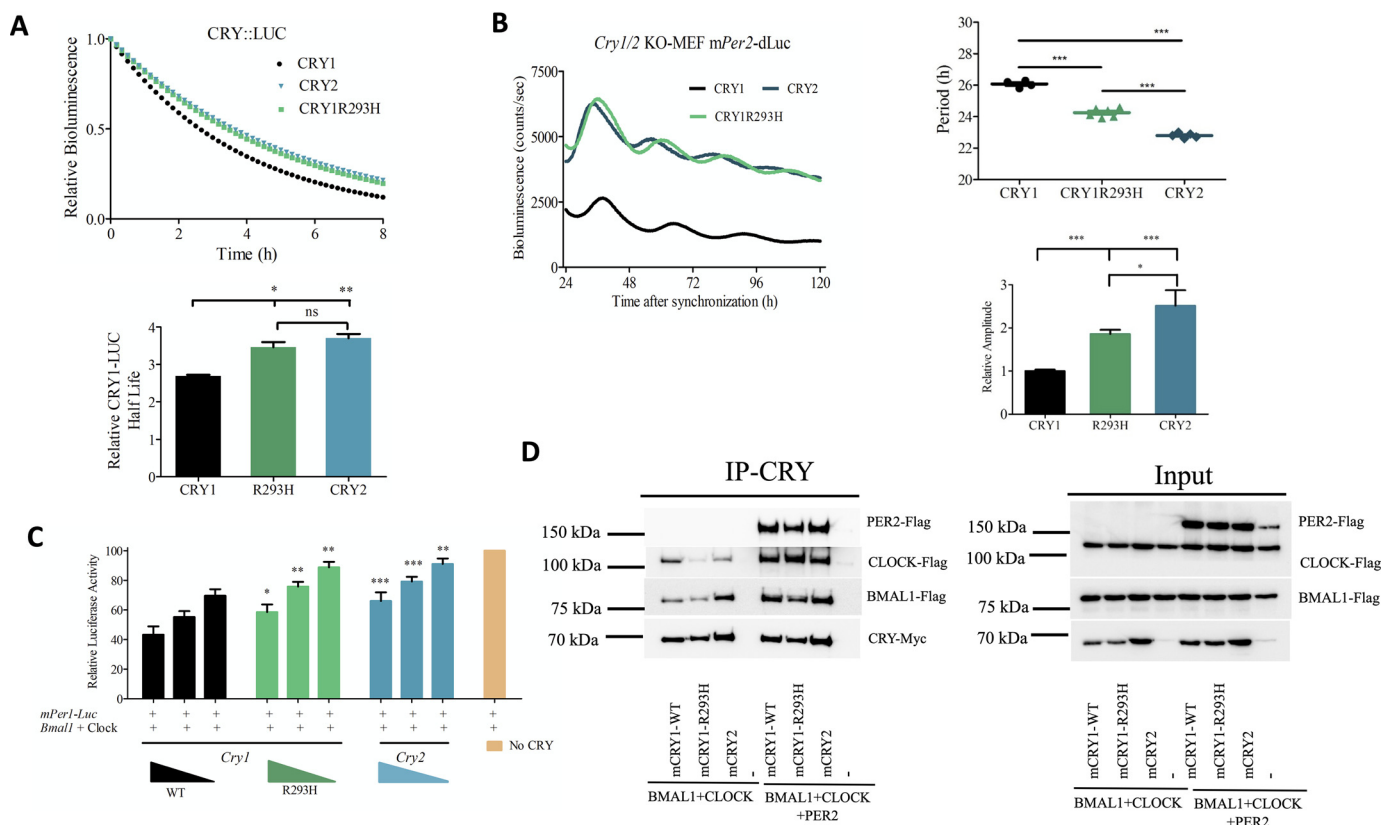
WT and p.Arg293His CRY1 and CRY2 to BMAL1, CLOCK, and PER2 were analyzed side by side (Fig. 4D). We performed pulldown assay in the absence and presence of PER2 to assess the binding ability of WT CRY1, CRY2, and p.Arg293His CRY1 to CLOCK. All CRYs tested in this study had comparable binding to CLOCK in the presence of PER2 (Fig. 4D). However, CRY2 and p.Arg293His CRY1 had attenuated binding to CLOCK compared with WT CRY1 in the absence of the PER2.

Collectively, our results suggested that the p.Arg293His CRY1 variant possesses similar properties to CRY2 in terms of stability, binding to core clock components, repression activity and rescuing circadian rhythms in DKO-MEF, except p.Arg293His CRY1 generated longer rhythm than CRY2.

#### Understanding the role of Arg-293 in CRY1 function

Although p.Arg293His CRY1 variant exhibits CRY2-like *in vitro* properties, the conservation of this amino acid residue in all mammalian CRY1s suggests that it might play an important role, such as regulation of CLOCK binding to the secondary pocket of CRY1. To investigate this, we employed MD simulations using CRY1 structural data. Recently, it has been shown that CRYs bind to the CLOCK PASB domain through the secondary pocket, and this is regulated by the serine loop (38). We, therefore, explored possible dynamical differences between WT CRY1 and p.Arg293His CRY1 around the secondary pocket via MD simulations. p.Gly144Val CRY1 and p.Arg348Cys CRY1 structures were also simulated as controls. For this purpose, we utilized mouse CRY1 crystal structure to generate a complete PHR domain of the individual

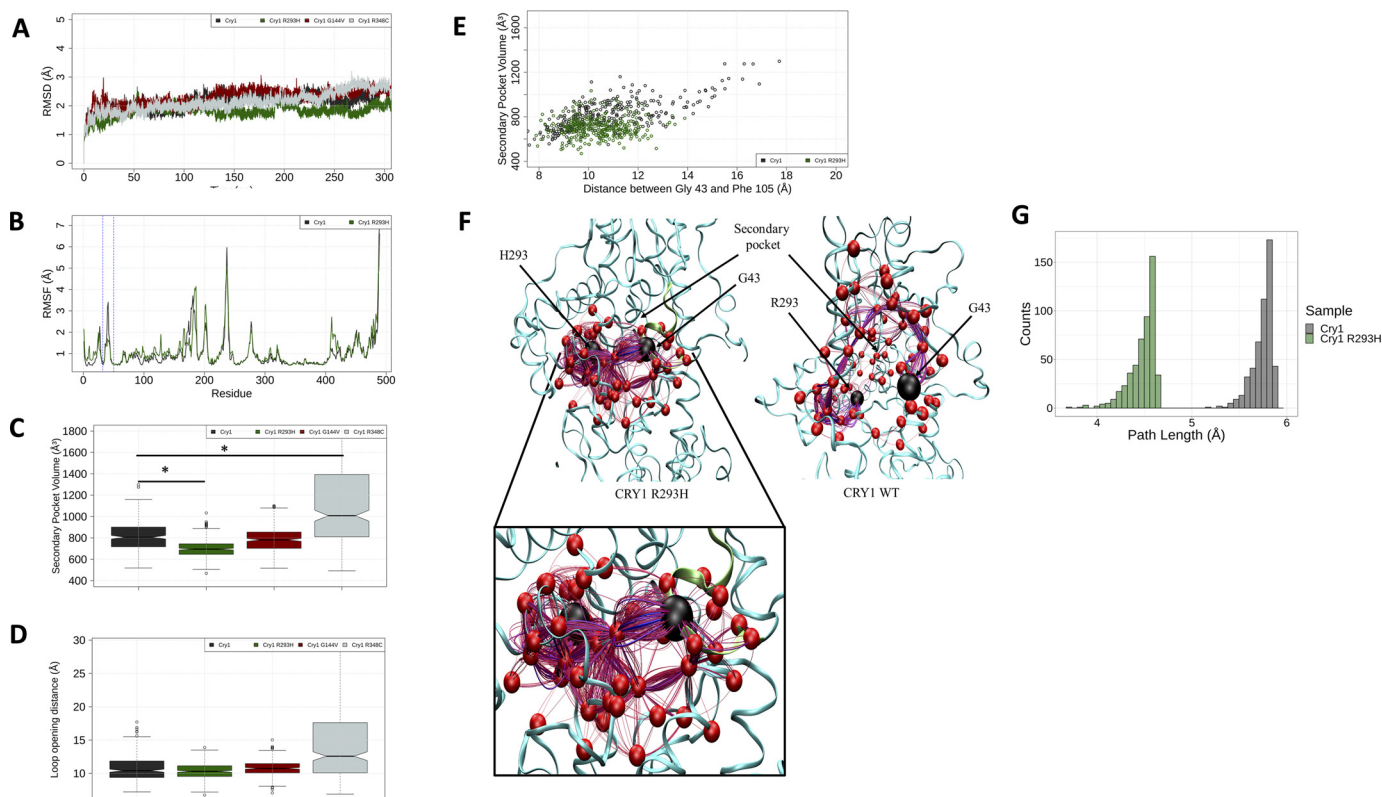
variants. Each protein was sampled for 300 ns, and the root mean square deviation (RMSD) values were determined to show that simulations reached equilibrium (Fig. 5A). To investigate the dynamics of each residue, root mean square fluctuation (RMSF) values were assessed (Fig. 5B). We observed differences in the RMSF values of residues in the serine loop (residues 38–48). When compared with WT CRY1, the residues in p.Arg293His CRY1 simulations showed minimal dynamism/fluctuation (Fig. 5B). Because the serine loop regulates the volume and access to the secondary pocket, we analyzed the volume of the secondary pocket using POVME 3.0 software. Analysis showed that simulations of p.Arg293His CRY1 sampled significantly smaller secondary pocket volume than WT CRY1 (Fig. 5C). Secondary pocket volume of p.Gly144Val CRY1 was comparable to WT CRY1. Interestingly, p.Arg348Cys CRY1 simulation sampled a larger secondary volume pocket. Because secondary pocket volume of WT CRY1 allows CLOCK binding, we do not expect larger pocket volume will affect CLOCK binding to p.Arg348Cys CRY1. In fact, rescue assays (Fig. 2B) and BMAL1/CLOCK binding analysis to CRY1 variants (Fig. 3) confirmed that p.Arg348Cys mutation did not change the binding of CRY1 to CLOCK. To further examine the relative internal dynamics of the loop, we analyzed the distance between the  $C_{\alpha}$  atoms of Gly-43 located on the loop and Phe-105 (Fig. 5, D and E). The average loop distances of CRY1 and p.Arg293His CRY1 were not significantly different. However, the variance of loop distances sampled throughout the simulations were significantly different (Levene's test). To compare the scatter of loop distance and its correlation with the volume of the secondary



**Figure 4. Comparison of protein  $t_{1/2}$  and analysis of rescue of CRY1 and CRY2.** A, The half-lives of WT, R293H CRY1, and CRY2 were determined as in Fig. 2. Average  $t_{1/2}$  values  $\pm$  S.E. from three independent experiments (with triplicate samples) relative to WT CRY1::dLUC were reported (left panel). Statistical analysis was performed using an unpaired *t* test with a Welch's correction (\*\*\*,  $p < 0.0001$ ; \*\*,  $p < 0.005$ ; \*,  $p < 0.05$ ; WT CRY1 versus R293H,  $p = 0.0226$ ; WT CRY1 versus CRY2,  $p = 0.0034$ ; R293H CRY1 versus CRY2,  $p = 0.4854$ ). Degradation of WT CRY1 and R293H CRY1 was analyzed by immunoblot using anti-Myc for CRYs and anti- $\beta$ -actin for loading controls (right panel). B, pArg293His CRY1 and CRY2 rescued the rhythm similarly, although periods are still significantly different. Rescue assays were performed as described in Fig. 3. Representative rhythms of three independent experiments are shown. Periods and amplitudes of all plates were reported (4-WT, 6-R293H CRY1, and 6-CRY2). Significance analysis was performed using unpaired *t* test with Welch's correction (\*\*\*,  $p < 0.0001$ ; \*\*,  $p < 0.005$ ; \*,  $p < 0.05$ ; WT CRY1 versus R293H,  $p < 0.0001$ ; WT CRY1 versus CRY2,  $p < 0.0001$ ; R293H CRY1 versus CRY2,  $p < 0.0001$ ). C, repression analysis. *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> MEF cells were transfected with plasmids encoding *Bmal1* (50 ng), *Clock* (125 ng), and different amounts of WT *Cry1*, *Cry2*, and *Cry1R293H*, along with the pGL3-*mPer1:luc* (50 ng) reporter plasmid and pRL-TK Renilla (4 ng) as a transfection control. Comparisons were made between WT CRY1 and CRY1R293H and WT CRY1 and CRY2 (\*\*\*,  $p < 0.0001$ ; \*\*,  $p < 0.005$ ; \*,  $p < 0.05$ ). D, pull-down analysis of WT or pArg293His CRY1, CRY2 with BMAL1, CLOCK, and PER2. HEK293T cells were transfected with plasmids expressing *Bmal1*-FLAG, *CLOCK*-FLAG, and *Cry*-His-Myc. Proteins were pulled down with Ni-NTA agarose resin and analyzed with Western blotting. This blot is the representation of three independent experiments.

pocket, we plotted loop distance against the secondary pocket volume (Fig. 5E). Our analysis of secondary pocket volume and loop distance showed that p.Arg293His CRY1 behaved similarly to WT CRY1 (Fig. 5, C–E). Despite this similarity between CRY1 and p.Arg293His CRY1 simulations, p.Arg293His CRY1 only sampled a fraction of the space explored by WT CRY1 (Fig. 5E). In line with secondary pocket volume analysis, p.Gly144Val sampled similar loop distance to WT CRY, p.Arg348Cys sampled longer loop distance. These observations in MD simulations implied that the dynamics of the serine loop in CRY1 and p.Arg293His CRY1 are regulated differently. Although the serine loop in WT CRY1 is more dynamic, reduced dynamicity of this loop in p.Arg293His CRY1 might explain the attenuated binding of p.Arg293His CRY1 to CLOCK. To explain this differential regulation on the serine loop in detail, we analyzed possible communication pathways between Arg-293 and the serine loop by using Weighted Implementation of the Suboptimal Paths (WISP) software as described in Ref. 44. The WISP algorithm can calculate both optimal and suboptimal (longer) communication

pathways between nodes (residues). Residue 293 was used as a source residue, and several possible residues on the serine loop (Gly-43, Ser-44, Ser-45, Asn-46) were used as a sink to determine promising pairs for communicating pathways. A total of 500 pathways between residues Arg-293 and Gly-43 for each WT and p.Arg293His CRY1 were calculated. The shortest pathway length for p.Arg293His CRY1 was calculated as 3.26 Å. The shortest communicating pathway involves the His-293, Ile-392, Ser-391, Trp-390, Gly-43 residues. However, the length of shortest pathway for WT CRY1 was calculated as 5.19 Å, which involves the Arg-293, Phe-296, Ala-300, Pro-104, Gly-106, Arg-109, Trp-8, Ile-36, Asp-38, Gly-43 residues (Fig. 5, F and G). Not only is the shortest pathway changed, but also the suboptimal pathways sampled longer lengths in p.Arg293His CRY1 (Fig. 5G). Residues involved more than 200 suboptimal paths between Arg-293/His-293 and Gly-43 are Asp-38, Pro-104, Gly-106, Ile-36, Ala-300, Arg-109, Phe-296, Trp-8, and Ala-299 for WT CRY1; Glu-382, Phe-296, Gln-393, and Ile-392 for p.Arg293His CRY1. Both representation of pathways and histogram of



**Figure 5. Molecular dynamic simulation studies of mutant and WT CRY1.** A, RMSD of CRY1 WT, G144V, R293H, and R348C CRY1 simulations. B, RMSF of CRY1 WT and pArg293His CRY1. Residue numbering is according to CRY1. Residues between two dashed lines belong to the serine loop. C, box plot representation of the secondary pocket volume sampled throughout the simulations. Wilcoxon rank sum test was applied (\*,  $p < 0.0001$ ). D, box plot representation of the loop distance produced by measuring the distance between C $\alpha$  atoms of Gly-43 and Phe-105. Distance between Gly-43 (on loop) and Phe-105 was calculated with a custom Python script. The same multi-frame PDB files extracted from the trajectories were used to calculate the secondary pocket volume and loop distances. Statistical analysis between variances of loop distances of CRY1 and CRY1R293H simulations was calculated by the Levene's test ( $p < 0.001$ ). E, scatter plot of the loop distance against the secondary pocket volume in WT and R293H CRY1 simulations. F, WISP analysis of the 500 pathways for CRY1 WT and CRY1R293H simulations. Red spheres represent the nodes participating in the communication pathway between residues 293 and 43 in CRY1. Each node corresponds to a residue. Residues Arg-293 or His-293 and Gly-43 were shown as black spheres. G, histogram representation of the 500 pathways' lengths calculated by WISP.

pathway lengths showed that the mutation introduced new communicating pathways between His-293 and Gly-43. Taken together, these computational findings suggest that the serine loop is allosterically regulated by residue Arg-293 in CRY1 and reduces the dynamicity of the serine loop, keeping it in a closed conformation. Our search in the ClinVar database revealed that there was no disease phenotype associated with this variant.

## Discussion

Mammalian CRYs are integral components of the circadian clock mechanism and are strong transcriptional repressors of BMAL1/CLOCK transactivation (45–47). Several genetic studies have highlighted the relationship between CRY variants and different types of diseases. For example, CRY1 variants have been associated with depression and mood disorders (48–50), elevated blood pressure and hypertension (49), and insulin resistance (51, 52). This clinical heterogeneity implies a variety of different functions for CRY1 potentially caused by different functional domains of the protein. Multiple sequence alignment shows that CRYs have variable extended C-terminal domains that range from 30 to 300 amino acids depending on the species (25, 26). N-terminal domain exhibits high homology to photolyases and is called the PHR domain.

The C terminus of mammalian CRY possesses a highly conserved helix that has a periodic spacing of nonpolar residues common to coiled-coil and is called the coiled-coil helix (53). In mammals, this region is competitively bound by FBXL3 (54) and PER2 (35) to regulate the stability of CRYs, and to tune the circadian clock machinery. The PHR domain essentially consists of two important regions, an  $\alpha$ -helical domain (primary pocket), and an  $\alpha/\beta$  domain (secondary pocket). The  $\alpha/\beta$  domain (secondary pocket) has been shown to be important for the interaction with the CLOCK PAS B domain and maintains the BMAL1/CLOCK transcription factor in a repressed state to close the circadian feedback loop (28).

Although rare CRY1 variants have been previously shown to be associated with several disorders, the underlying mechanisms are yet to be characterized. Here, we not only investigated the potential functional impact of rare nonsynonymous SNPs on the function of CRY1 but also revealed the allosteric regulation between primary and secondary pockets of CRY1 by characterizing one of the rare mutants. The three variants, which map to important domains of the CRY1 and are predicted to affect its function, were selected from Ensembl and the 1000 Genomes project. Arg-348 and Gly-144 residues map to the surface; Arg-293 is located within the primary pocket,



FAD-binding domain, and FBXL3 binding site of CRY1 (Fig. 1). We initially analyzed the effect of mutations on the  $t_{1/2}$  CRY1 protein using the CRY1::dLUC degradation assay. p.Arg348Cys and p.Gly144Val CRY1 variants had no effect on the stability of CRY1, whereas the p.Arg293His variant significantly increased the stability compared with WT CRY1 (Fig. 2A). Next, we utilized a cell-based rescue assay in *Cry1*<sup>-/-</sup>*Cry2*<sup>-/-</sup> MEF cells with a special *Cry1* rescue plasmid to identify their effects on the circadian rhythm. Surprisingly, p.Arg293His CRY1 variant had longer  $t_{1/2}$  than the WT CRY1 and caused shorter period length with enhanced luminescence. This implies less repression activity (Fig. 2B), which is the indicator of weak binding of mutant CRY1 to BMAL1/CLOCK. Other two variants caused slight increase in the period length, which is consistent with previous findings indicating that mutation of residues on the surface of the  $\alpha$ -helical domain have a tendency to change the repressor activity of mammalian CRYs (55). Despite earlier studies suggested that as the stability of CRY increases by either mutation or chemicals, period of circadian rhythm becomes longer; however, recent findings unveiled that not the stability but the ability of CRY to bind BMAL1/CLOCK is the primary factor in determining the period length (30, 31, 33, 56).

To test binding of CRY to core clock proteins, pulldown experiment was performed with CRY1 and BMAL1/CLOCK in the presence and absence of PER2. As rescue assay results suggested, p.Arg293His CRY1 has deficiency to bind CLOCK, even in the presence of PER2 (Fig. 3). On the other hand, p.Gly144Val and p.Arg348C mutants showed comparable binding to core clock proteins with WT CRY1. Interestingly, functional characterization of p.Arg293His, which is located in the  $\alpha$ 12-helix and possibly interacts with the secondary pocket, revealed different properties than the other CRY1 variants. That is to say,  $t_{1/2}$  of the p.Arg293His CRY1 was longer than WT CRY1. Second, functional complementation with this variant in DKO-MEF resulted in a short circadian period phenotype with increased luminescence. Third, biochemical studies showed that this variant had reduced affinity to CLOCK. Interestingly, these features are more similar to CRY2 than CRY1 (31). Thus, we tested WT and p.Arg293His CRY1 and WT CRY2 in the same experimental set-up to determine the differences and similarities among these proteins. Although p.Arg293His CRY1 and WT CRY2 had comparable  $t_{1/2}$  (Fig. 4A), repression capacity, and binding to BMAL1/CLOCK in pulldown assays (Fig. 4, C and D), p.Arg293His caused a longer rhythm than WT CRY1 but shorter than WT CRY2 (Fig. 4B). Despite similarities between p.Arg293His CRY1 variant and WT CRY2 in various aspects, rescue assay exhibited that they have slight functionality differences in the circadian clock mechanism (Fig. 4B).

To understand the dramatic change in the function of CRY1 upon Arg-293 to His-293 mutation and to assess the role of Arg-293 in CRY1, we employed molecular dynamics simulations. Computational analysis suggested that Arg-293 might be a critical residue for the regulation of the serine loop in CRY1, which is known to be important for CLOCK binding to the secondary pocket (38). Replacing Arg-293 with His changes a highly dynamic serine loop into a less dynamic state and closes the gate of the secondary pocket (Fig. 5, B–E), which may be re-

sponsible for the reduced binding of CLOCK to the mutant CRY1. Further analysis of our MD simulations of both variant and WT CRY1 in terms of the communication pathways suggested that mutation of Arg-293 to His changes the path to serine loop, which shows the allosteric regulation of secondary pocket availability by the primary pocket (Fig. 5, F and G).

The rare variants characterized in this study were selected from published human SNPs, which are all in heterozygous state and, to our knowledge, have not yet been associated with any disease phenotype. It is possible that these variants may exhibit a disease phenotype in recessive state. Their effects can be further analyzed in mouse models by reverse phenotyping studies. In conclusion, we characterized the effect of rare human CRY1 SNPs on circadian clock function at the molecular level. Our findings suggest that Arg-293 is important for the allosteric regulation in CRY1 and has impact on the molecular clock.

## Experimental Procedures

### Site-directed mutagenesis

For site-directed mutagenesis, Phusion polymerase-based quick-change method was used. Primers were designed using the recommended guidelines (57). The PCR reaction mixtures contained 0.3 mM dNTP, 5  $\mu$ l of 10 $\times$  Phusion GC Buffer (Thermo Scientific), 3% DMSO, 1  $\mu$ M each primer, 30 ng of the template plasmid (*mCry1* in pcDNA4A), and 1 unit of Phusion DNA polymerase in a 50  $\mu$ l of final volume. The reaction conditions were set to 98°C for 15 s, 66°C for 30 s, and 72°C for 4 min, 20 cycles. PCR products were visualized in 1% agarose gel. The samples with correct-sized bands were digested with 1 unit of FastDigest DpnI enzyme (Thermo Scientific) for 1 h at 37°C and then 5  $\mu$ l of sample was transformed to *Escherichia coli* DH5 $\alpha$  cells. Colonies were picked to culture and then plasmids were isolated via MiniPrep (Macherey Nagel). Sanger sequencing (Macrogen) was used to confirm the mutations.

### Cry-Luc construct generation

To form *Cry*-Luc constructs, coding sequence of firefly luciferase in pG5luc plasmid (Addgene) was amplified with primers having NotI and XhoI flanking sites. Product size was verified by visualizing in agarose gel and product was isolated from the gel by using NucleoSpin PCR and Gel Purification Kit (Macherey Nagel). Purified fragments were cloned to pJET1.2/blunt vector (Thermo Scientific) using CloneJET PCR Cloning Kit (Thermo Scientific). 5  $\mu$ l of sample was transformed to *E. coli* DH5 $\alpha$  cells. Colonies were picked to culture and then plasmids were isolated via MiniPrep. *Luc* plasmid was double digested with NotI and XhoI; *Cry* pcDNA plasmids (WT or variant) were double digested with EcoRV and NotI FastDigest enzymes (Thermo Scientific). *Cry* plasmids were treated with FastAP (Thermo Scientific) to prevent self-annealing. After gel isolation the inserts and the destination vectors were cleaned and ligated by using T4 DNA ligase (Thermo Scientific). 5  $\mu$ l of sample was transformed to *E. coli* DH5 $\alpha$  cells. Colonies were picked to culture and then plasmids were isolated via MiniPrep. Presence of *Cry* and *Luc* were verified by double digestions with EcoRV/NotI and NotI/XhoI, respectively.

## Allosteric regulation in Cryptochrome1

### Repression assay of BMAL1/CLOCK-dependent transactivation

Plasmids used in this study were gifted to us from Professor John Hogenesch from Cincinnati Children's Hospital. A mixture of 50 ng pSport6-*Bmal1*, 125 ng pSport6-*CLOCK*, 50 ng pGL3-*mPer1::dLuc*, 1 ng pRL-TK, 2.5 ng or lower doses of pcDNA4A-*Cry1* (WT or variant), and empty pSport6 (to equalize the transfected amount of DNA) was reverse transfected to  $4 \times 10^4$  HEK293T cells/well in a 96-well opaque plate via PEI transfection reagent. To determine the activity of BMAL1/CLOCK-dependent transactivation without a repressor, the mixture was supplemented with empty pcDNA4/myc-His A instead of *Cry1* construct. In each independent experiment, transfections were triplicated for each condition. The plates were incubated for 24 h at 37°C, 5% CO<sub>2</sub>. Firefly luciferase and renilla luciferase expression was determined using the Dual-Glo Luciferase Assay System (Promega) using manufacturer's protocol.

### Pulldown assay

$4 \times 10^5$  HEK293T cells (per well) were seed to 6-well tissue plate 24 h before the transfection. Cells were transfected with *Cry1*-His-Myc (WT or variants) and *Cry2*-His-Myc in pcDNA4-A with FLAG-CMV-*Bmal1*, FLAG-CMV-*CLOCK* via PEI transfection reagent for IP with BMAL1, CLOCK, and CRY1 as described in Ref. 57. FLAG-*PER2*-CMV plasmid was also transfected along with *BMAL1*, *CLOCK*, and *Cry1* plasmids to pull down four clock proteins. For negative control *BMAL1*, *CLOCK* or *BMAL1*, *CLOCK*, and *PER2* were transfected with empty pSport6 plasmid instead of *Cry1*. 24 h after transfection, cells were harvested via ice-cold PBS. After centrifugation pellets were lysed in 300  $\mu$ l passive lysis buffer (PLB) (15 mM HEPES, 300 mM NaCl, 5 mM NaF, 1% Nonidet P-40 supplemented with fresh protease inhibitor) for 20 min on ice. To get rid of cell debris, samples were centrifuged for 15 min at  $13,000 \times g$  at +4°C. 10% of the supernatant was saved as input. 15  $\mu$ l Ni-NTA Agarose resin (Qiagen) per sample was equilibrated by washing two times with TBS-300 (15 mM Tris, 300 mM NaCl) supplemented with 25 mM imidazole and two times with PLB. Remaining supernatant was added on to equilibrated resins with 25 mM imidazole. Cell lysates and resins were mixed for ~2 h at 4°C to pull down CRYs. Resins were washed four times with TBS-300 (300  $\mu$ l) having 35 mM imidazole. Proteins were isolated from resins by boiling in Laemmli Buffer (31.5 mM Tris-HCl, pH 6.8, buffer 10% glycerol, 1% SDS, 0.005% Bromphenol Blue, freshly added  $\beta$ -mercaptoethanol (5%)).

Anti-FLAG antibody (Sigma A9469) was used to detect BMAL1, CLOCK, and PER2. Blots were stripped (Advansta Strip-It Buffer, R03722-D50) and incubated with anti-Myc antibody (Abcam, ab18185) and anti-CRY1 (Bethyl, catalog no. A302-614A) to detect CRYs as described in Ref. 57. In all Western blot analyses, blots were incubated overnight with primary antibody. Mouse IgG  $\kappa$  binding protein (m-IgG $\kappa$  BP) conjugated to horseradish peroxidase (HRP) (sc-516102) was used as the secondary antibody. Anti-beta Actin (Cell Signaling Technology, 8H10D10) was used to detect  $\beta$ -actin protein. To cap-

ture the chemiluminescent signals, WesternBright ECL HRP Substrate (Advansta K-12045-D20) and Bio-Rad ChemiDoc™ Imaging System were used.

### CRY-LUC degradation assay

40 ng of expression vector (*Cry-Luc* plasmid) was reverse transfected to  $4 \times 10^4$  HEK293T cells on opaque 96-well plate with flat bottom via PEI transfection reagent. 48 h of post-transfection cells were treated with luciferin (0.4 mM final) and HEPES (10 mM final and pH 7.2). After 2 h, cycloheximide (20  $\mu$ g/ml final) was added to wells to stop the protein synthesis. Plate was sealed with optically clear film and placed to Synergy H1. Luminescence readings were recorded every 10 min at 32°C for 24 h.  $t_{1/2}$  of protein was calculated via one-phase exponential decay fitting function in GraphPad Prism5 software.

### CRY degradation assay

500 ng of WT CRY1 or p.Arg293His CRY1 plasmids in pcDNA4A-His-Myc vector were transfected to HEK293T cells on a 6-well plate with PEI transfection reagent. 24 h after transfection cells were treated with cycloheximide (100  $\mu$ g/ml final) to stop protein synthesis. Cells were harvested at the time of cycloheximide treatment (0 h) after 4 and 8 h. Levels of proteins were analyzed via Western blotting by using anti-Myc or anti- $\beta$ -actin to detect CRY and  $\beta$ -actin levels, respectively. Details of antibody treatments are as explained in the pulldown assay section above.

### Real-time bioluminescence rescue assay

$3 \times 10^5$  *Cry1*<sup>-/-</sup>/*Cry2*<sup>-/-</sup> mouse embryonic fibroblasts (DKO-MEFs) were seeded onto 35-mm clear tissue culture plates (cells were obtained from Prof. Ueda's group). Cells were transfected with 4000 ng pGL3-*Per2*-dLuc (luciferase reporter) and 150 ng of the *Cry1* expression vector (pMU2-P(*CRY1*)-(intron 336), obtained from the Ueda group) (WT or variant), using FuGENE6 transfection reagent according to the manufacturer's protocols. After 72 h, cells were synchronized by treatment with 0.1  $\mu$ M dexamethasone for 2 h. Then, the medium was replaced with bioluminescence recording medium (1% DMEM powder (w/v), 0.035% sodium bicarbonate, 0.35% D(+) glucose powder, 0.01 M HEPES buffer, 0.25% penicillin/streptomycin, 5% FBS), in which luciferin was freshly added (0.1 mM final concentration). Plates were sealed with vacuum grease and placed into a LumiCycle (Actimetrics). Bioluminescence was monitored for 70 s every 10 min for 5 days. Luminescence values were recorded and processed using the LumiCycle Analysis software. The first 20 h, data were discarded from the analysis because of high transient luminescence upon medium change. Period and amplitude were calculated for each sample. Each independent experiment was carried out with duplicate samples.

### Molecular dynamics simulations

The protein structures of CRY1 and CRY2 were obtained via homology modeling. Complete PHR domain sequences of CRY1 and CRY2 were obtained from UniProt (mouse CRY1



(P97784), mouse CRY2(Q9R194)). Sequences were submitted to the RaptorX web server to obtain protein structures for CRY1 and CRY2 (58). The structure of the CRY1 mutant R293H was also obtained by the same procedure. All structures were protonated with PROPKA using the PDB2PQR webserver (59). The CHARMM36m force field was used for simulations (60). All three structures were solvated in a rectangular box using TIP3P water molecules and neutralized with the appropriate amount of sodium and chloride ions in visual molecular dynamics (VMD) (61). Energy minimization was performed for 100,000 steps. Systems were gradually heated and equilibrated in NPT ensemble for 140 ps. Throughout equilibration, constraints on the proteins were gradually removed. After equilibration, a production run of 300 ns for WT, G144V, R293H, and R348C CRY1 was performed. Total of 1200 ns consisting of two independent production runs were performed for each protein. All production runs were performed at 310 K and under 1 atm pressure using a Langevin thermostat and a Langevin barostat, respectively. All simulations were performed using NAMD software on in-house, local machines (62). To prepare trajectories for analysis, all trajectories were aligned to the initial structure, and the first 10 ns of all simulations were discarded. RMSD and RMSF calculations were performed using VMD. Each of the refined trajectories were saved as two multi-frame PDB files with 1 ns and 10-ps intervals for POVME and WISP, respectively, for further analysis. Calculation of the secondary pocket was performed using POVME 3.0 software (63). For all trajectories, the volume of the secondary pocket was calculated with a 1-ns interval. The Wilcoxon rank sum test was used to determine significance of the difference between the pocket volumes implemented in R (64). Allosteric pathway analysis was performed using command line WISP 1.1 software (65). Visualizations of pathways were performed using VMD software.

## Data Availability

All data are contained within the manuscript.

**Acknowledgments**—We thank Emma Harris from the University of Edinburgh for critical reading of the manuscript. We are grateful to Prof. Hiroki Ueda and Andrew Liu for the generous gift of the Cry1<sup>-/-</sup>/Cry2<sup>-/-</sup> mouse embryonic fibroblasts and the Cry1 rescue vector. Finally, we thank Prof. Hogenesch for the generous gifts of clock-related plasmids and the support.

**Author contributions**—S. G. and I. H. K. conceptualization; S. G., C. A., O. O., B. G., S. S., and I. B. data curation; S. G., C. A., O. O., and I. H. K. methodology; S. G., I. B., and I. H. K. writing—original draft; C. A., O. O., and S. S. formal analysis; C. A. and I. H. K. investigation; I. B. and I. H. K. funding acquisition; I. H. K. resources; I. H. K. supervision; I. H. K. writing—review and editing.

**Funding and additional information**—This work was supported by TUBITAK Grant 114Z879 and Istanbul Development Agency Grant ISTKA-TR/14/EVK/0039 (to I. H. K.).

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: PAS, PER-ARNT-SIM domains; PHR, photolyase homology region; CBD, CRY-binding domain; MD, molecular dynamics; DKO-MEF, double KO mouse embryonic fibroblast cells; Ni-NTA, nickel-nitrilotriacetic acid; RMSD, root mean square deviation; RMSF, root mean square fluctuation; WISP, Weighted Implementation of the Suboptimal Paths software; VMD, visual molecular dynamics.

## References

- Gachon, F., Nagoshi, E., Brown, S. A., Ripperger, J., and Schibler, U. (2004) The mammalian circadian timing system: From gene expression to physiology. *Chromosoma* **113**, 103–112 [CrossRef Medline](#)
- Green, C. B., Takahashi, J. S., and Bass, J. (2008) The meter of metabolism. *Cell* **134**, 728–742 [CrossRef Medline](#)
- Cermakian, N., Lange, T., Golombek, D., Sarkar, D., Nakao, A., Shibata, S., and Mazzocchi, G. (2013) Crosstalk between the circadian clock circuitry and the immune system. *Chronobiol. Int.* **30**, 870–888 [CrossRef Medline](#)
- Degaute, J. P., van de Borne, P., Linkowski, P., and Van Cauter, E. (1991) Quantitative analysis of the 24-hour blood pressure and heart rate patterns in young men. *Hypertension* **18**, 199–210 [CrossRef Medline](#)
- May, C. P., Hasher, L., and Stoltzfus, E. R. (1993) Optimal time of day and the magnitude of age differences in memory. *Psychol. Sci.* **4**, 326–330 [CrossRef](#)
- Petros, T. V., Beckwith, B. E., and Anderson, M. (1990) Individual differences in the effects of time of day and passage difficulty on prose memory in adults. *Br. J. Psychol.* **81**, 63–72 [CrossRef](#)
- Sahar, S., and Sassone-Corsi, P. (2009) Metabolism and cancer: The circadian clock connection. *Nat. Rev. Cancer* **9**, 886–896 [CrossRef Medline](#)
- McIntosh, B. E., Hogenesch, J. B., and Bradfield, C. A. (2010) Mammalian Per-Arnt-Sim proteins in environmental adaptation. *Annu. Rev. Physiol.* **72**, 625–645 [CrossRef Medline](#)
- Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**, 1564–1569 [CrossRef Medline](#)
- Hogenesch, J. B., Gu, Y. Z., Jain, S., and Bradfield, C. A. (1998) The basic helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5474–5479 [CrossRef Medline](#)
- King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., Steeves, T. D., Vitaterna, M. H., Kornhauser, J. M., Lowrey, P. L., Turek, F. W., and Takahashi, J. S. (1997) Positional cloning of the mouse circadian clock gene. *Cell* **89**, 641–653 [CrossRef Medline](#)
- Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., and Reppert, S. M. (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* **98**, 193–205 [CrossRef Medline](#)
- Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H., and Reppert, S. M. (2000) Interacting molecular loops in the mammalian circadian clock. *Science* **288**, 1013–1019 [CrossRef Medline](#)
- van der Horst, G. T., Muijtens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J. H., and Yasui, A. (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* **398**, 627–630 [CrossRef Medline](#)
- Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikawa, T., Miyazaki, J., Takahashi, J. S., and Sancar, A. (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12114–12119 [CrossRef Medline](#)



16. Aryal, R. P., Kwak, P. B., Tamayo, A. G., Gebert, M., Chiu, P. L., Walz, T., and Weitz, C. J. (2017) Macromolecular assemblies of the mammalian circadian clock. *Mol. Cell* **67**, 770–782. [CrossRef Medline](#)
17. Reischl, S., Vanselow, K., Westermarck, P. O., Thierfelder, N., Maier, B., Herzel, H., and Kramer, A. (2007)  $\beta$ -TrCP1-mediated degradation of PERIOD2 is essential for circadian dynamics. *J. Biol. Rhythms* **22**, 375–386 [CrossRef Medline](#)
18. Siepka, S. M., Yoo, S. H., Park, J., Song, W., Kumar, V., Hu, Y., Lee, C., and Takahashi, J. S. (2007) Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* **129**, 1011–1023 [CrossRef Medline](#)
19. Takahashi, J. S. (2016) Molecular architecture of the circadian clock in mammals. in *A Time for Metabolism and Hormones* (Sassone-Corsi, P., and Christen, Y., eds.), pp. 13–24, Springer, New York [CrossRef](#)
20. Thresher, R. J., Vitaterna, M. H., Miyamoto, Y., Kazantsev, A., Hsu, D. S., Petit, C., Selby, C. P., Dawut, L., Smithies, O., Takahashi, J. S., and Sancar, A. (1998) Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. *Science* **282**, 1490–1494 [CrossRef Medline](#)
21. Koike, N., Yoo, S. H., Huang, H. C., Kumar, V., Lee, C., Kim, T. K., and Takahashi, J. S. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* **338**, 349–354 [CrossRef Medline](#)
22. Khan, S. K., Xu, H. Y., Ukai-Tadenuma, M., Burton, B., Wang, Y. M., Ueda, H. R., and Liu, A. C. (2012) Identification of a novel cryptochrome differentiating domain required for feedback repression in circadian clock function. *J. Biol. Chem.* **287**, 25917–25926 [CrossRef Medline](#)
23. Rosensweig, C., and Green, C. B. (2020) Periodicity, repression, and the molecular architecture of the mammalian circadian clock. *Eur. J. Neurosci.* **51**, 139–165 [CrossRef Medline](#)
24. Cal-Kayitmazbatir, S., Kulkoyluoglu-Cotul, E., Growe, J., Selby, C. P., Rhoades, S. D., Malik, D., Oner, H., Asimgil, H., Francey, L. J., Sancar, A., Kruger, W. D., Hogenesch, J. B., Weljie, A., Anafi, R. C., and Kavakli, I. H. (2020) CRY1-CBS binding regulates circadian clock function and metabolism. *FEBS J* [Online ahead of print] [CrossRef Medline](#)
25. Kavakli, I. H., Baris, I., Tardu, M., Gül, Ş., Öner, H., Çal, S., Bulut, S., Yarpavvar, D., Berkel, Ç., Ustaoglu, P., and Aydın, C. (2017) The photolyase/cryptochrome family of proteins as DNA repair enzymes and transcriptional repressors. *Photochem. Photobiol.* **93**, 93–103 [CrossRef Medline](#)
26. Partch, C. L., Clarkson, M. W., Özgür, S., Lee, A. L., and Sancar, A. (2005) Role of structural plasticity in signal transduction by the cryptochrome blue-light photoreceptor. *Biochemistry* **44**, 3795–3805 [CrossRef Medline](#)
27. Gao, P., Yoo, S. H., Lee, K. J., Rosensweig, C., Takahashi, J. S., Chen, B. P., and Green, C. B. (2013) Phosphorylation of the Cryptochrome 1 C-terminal tail regulates circadian period length. *J. Biol. Chem.* **288**, 35277–35286 [CrossRef Medline](#)
28. Michael, A. K., Fribourgh, J. L., Chelliah, Y., Sandate, C. R., Hura, G. L., Schneidman-Duhovny, D., Tripathi, S. M., Takahashi, J. S., and Partch, C. L. (2017) Formation of a repressive complex in the mammalian circadian clock is mediated by the secondary pocket of CRY1. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 1560–1565 [CrossRef Medline](#)
29. Czarna, A., Breitkreuz, H., Mahrenholz, C. C., Arens, J., Strauss, H. M., and Wolf, E. (2011) Quantitative analyses of Cryptochrome-mBMAL1 interactions mechanistic insight into the transcriptional regulation of the mammalian circadian clock. *J. Biol. Chem.* **286**, 22414–22425 [CrossRef Medline](#)
30. Xu, H. Y., Gustafson, C. L., Sammons, P. J., Khan, S. K., Parsley, N. C., Ramanathan, C., Lee, H. W., Liu, A. C., and Partch, C. L. (2015) Cryptochrome 1 regulates the circadian clock through dynamic interactions with the BMAL1 C terminus. *Nat. Struct. Mol. Biol.* **22**, 476–484 [CrossRef Medline](#)
31. Rosensweig, C., Reynolds, K. A., Gao, P., Laothamatas, I., Shan, Y. L., Ranganathan, R., Takahashi, J. S., and Green, C. B. (2018) An evolutionary hotspot defines functional differences between CRYPTOCHROMES. *Nat. Commun.* **9**, 1138 [CrossRef Medline](#)
32. Onat, O. E., Kars, M. E., Gül, Ş., Bilguvar, K., Wu, Y., Özhan, A., Aydın, C., Başak, A. N., Trusso, M. A., Goracci, A., Fallerini, C., Renieri, A., Casanova, J.-L., Itan, Y., Atbaşoğlu, C. E., et al. (2020) Human CRY1 variants associated with attention deficit/hyperactivity disorder. *J. Clin. Invest.* **130**, 3885–3900 [CrossRef Medline](#)
33. Patke, A., Murphy, P. J., Onat, O. E., Krieger, A. C., Özçelik, T., Campbell, S. S., and Young, M. W. (2017) Mutation of the human circadian clock gene CRY1 in familial delayed sleep phase disorder. *Cell* **169**, 203–215 [CrossRef Medline](#)
34. Nangle, S. N., Rosensweig, C., Koike, N., Tei, H., Takahashi, J. S., Green, C. B., and Zheng, N. (2014) Molecular assembly of the period-cryptochrome circadian transcriptional repressor complex. *Elife* **3**, e03674 [CrossRef Medline](#)
35. Ozber, N., Baris, I., Tatlici, G., Gur, I., Kilinc, S., Unal, E. B., and Kavakli, I. H. (2010) Identification of two amino acids in the C-terminal domain of mouse CRY2 essential for PER2 interaction. *BMC Mol. Biol.* **11**, 69 [CrossRef Medline](#)
36. Busino, L., Bassermann, F., Maiolica, A., Lee, C., Nolan, P. M., Godinho, S. I., Draetta, G. F., and Pagano, M. (2007) SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* **316**, 900–904 [CrossRef Medline](#)
37. Godinho, S. I., Maywood, E. S., Shaw, L., Tucci, V., Barnard, A. R., Busino, L., Pagano, M., Kendall, R., Quwillid, M. M., Romero, M. R., O'Neill, J., Chesham, J. E., Brooker, D., Lallan, Z., Hastings, M. H., et al. (2007) The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* **316**, 897–900 [CrossRef Medline](#)
38. Fribourgh, J. L., Srivastava, A., Sandate, C. R., Michael, A. K., Hsu, P. L., Rakers, C., Nguyen, L. T., Torgrimson, M. R., Parico, G. C. G., Tripathi, S., Zheng, N., Lander, G. C., Hirota, T., Tama, F., and Partch, C. L. (2020) Dynamics at the serine loop underlie differential affinity of cryptochromes for CLOCK:BMAL1 to control circadian timing. *Elife* **9**, e55275 [CrossRef](#)
39. 1000 Genomes Project Consortium, Auton, A., Brooks, L. D., Durbin, R. M., Garrison, E. P., Kang, H. M., Korbel, J. O., Marchini, J. L., McCarthy, S., McVean, G. A., and Abecasis, G. R. (2015) A global reference for human genetic variation. *Nature* **526**, 68–74 [CrossRef Medline](#)
40. Zerbino, D. R., Achuthan, P., Akanni, W., Amode, M. R., Barrell, D., Bhai, J., Billis, K., Cummins, C., Gall, A., Girón, C. G., Gil, L., Gordon, L., Haggerty, L., Haskell, E., Hourlier, T., et al. (2018) Ensembl 2018. *Nucleic Acids Res.* **46**, D754–D761 [CrossRef Medline](#)
41. Schwarz, J. M., Cooper, D. N., Schuelke, M., and Seelow, D. (2014) MutationTaster2: Mutation prediction for the deep-sequencing age. *Nat. Methods* **11**, 361–362 [CrossRef Medline](#)
42. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., Reh, H. L., and ACMG Laboratory Quality Assurance Committee. (2015) Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* **17**, 405–424 [CrossRef Medline](#)
43. Ukai-Tadenuma, M., Yamada, R. G., Xu, H. Y., Ripberger, J. A., Liu, A. C., and Ueda, H. R. (2011) Delay in feedback repression by Cryptochrome 1 is required for circadian clock function. *Cell* **144**, 268–281 [CrossRef Medline](#)
44. Ozdemir, E. S., Jang, H., Gursoy, A., Keskin, O., Li, Z., Sacks, D. B., and Nussinov, R. (2018) Unraveling the molecular mechanism of interactions of the Rho GTPases Cdc42 and Rac1 with the scaffolding protein IQGAP2. *J. Biol. Chem.* **293**, 3685–3699 [CrossRef Medline](#)
45. Kavakli, I. H., and Sancar, A. (2002) Circadian photoreception in humans and mice. *Mol. Interv.* **2**, 484–492 [CrossRef Medline](#)
46. Sato, T. K., Yamada, R. G., Ukai, H., Baggs, J. E., Miraglia, L. J., Kobayashi, T. J., Welsh, D. K., Kay, S. A., Ueda, H. R., and Hogenesch, J. B. (2006) Feedback repression is required for mammalian circadian clock function. *Nat. Genet.* **38**, 312–319 [CrossRef Medline](#)
47. Ueda, H. R., Hayashi, S., Chen, W. B., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005) System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat. Genet.* **37**, 187–192 [CrossRef Medline](#)
48. Hua, P., Liu, W., Chen, D., Zhao, Y., Chen, L., Zhang, N., Wang, C., Guo, S., Wang, L., Xiao, H., and Kuo, S. H. (2014) Cry1 and Tef gene polymorphisms are associated with major depressive disorder in the Chinese population. *J. Affect. Disord.* **157**, 100–103 [CrossRef Medline](#)

49. Kovanen, L., Donner, K., Kaunisto, M., and Partonen, T. (2015) CRY1, CRY2 and PRKCDBP genetic variants in metabolic syndrome. *Hypertens. Res.* **38**, 186–192 [CrossRef Medline](#)
50. Soria, V., Martínez-Amorós, E., Escaramís, G., Valero, J., Pérez-Egea, R., García, C., Gutiérrez-Zotes, A., Puigdemont, D., Bayés, M., Crespo, J. M., Martorell, L., Vilella, E., Labad, A., Vallejo, J., Pérez, V., *et al.* (2010) Differential association of circadian genes with mood disorders: CRY1 and NPAS2 are associated with unipolar major depression and CLOCK and VIP with bipolar disorder. *Neuropsychopharmacology* **35**, 1279–1289 [CrossRef Medline](#)
51. Dashti, H. S., Smith, C. E., Lee, Y. C., Parnell, L. D., Lai, C. Q., Arnett, D. K., Ordovás, J. M., and Garaulet, M. (2014) CRY1 circadian gene variant interacts with carbohydrate intake for insulin resistance in two independent populations: Mediterranean and North American. *Chronobiol. Int.* **31**, 660–667 [CrossRef Medline](#)
52. Kovanen, L., Donner, K., Kaunisto, M., and Partonen, T. (2016) CRY1 and CRY2 genetic variants in seasonality: A longitudinal and cross-sectional study. *Psychiatry Res.* **242**, 101–110 [CrossRef Medline](#)
53. Chaves, I., Yagita, K., Barnhoorn, S., Okamura, H., van der Horst, G. T. J., and Tamanini, F. (2006) Functional evolution of the photolyase/cryptochrome protein family: Importance of the C terminus of mammalian CRY1 for circadian core oscillator performance. *Mol. Cell Biol.* **26**, 1743–1753 [CrossRef Medline](#)
54. Xing, W., Busino, L., Hinds, T. R., Marionni, S. T., Saifee, N. H., Bush, M. F., Pagano, M., and Zheng, N. (2013) SCF(FBXL3) ubiquitin ligase targets cryptochromes at their cofactor pocket. *Nature* **496**, 64–68 [CrossRef Medline](#)
55. McCarthy, E. V., Baggs, J. E., Geskes, J. M., Hogenesch, J. B., and Green, C. B. (2009) Generation of a novel allelic series of cryptochrome mutants via mutagenesis reveals residues involved in protein-protein interaction and CRY2-specific repression. *Mol. Cell Biol.* **29**, 5465–5476 [CrossRef Medline](#)
56. Hirano, A., Shi, G., Jones, C. R., Lipzen, A., Pennacchio, L. A., Xu, Y., Hallows, W. C., McMahon, T., Yamazaki, M., Ptáček, L. J., and Fu, Y.-H. (2016) A Cryptochrome 2 mutation yields advanced sleep phase in humans. *Elife* **5**, e16695 [CrossRef Medline](#)
57. Doruk, Y. U., Yarpavar, D., Akyl, Y. K., Gul, S., Taskin, A. C., Yilmaz, F., Baris, I., Ozturk, N., Türkay, M., Ozturk, N., Okyar, A., and Kavakli, I. H. (2020) A CLOCK-binding small molecule disrupts the interaction between CLOCK and BMAL1 and enhances circadian rhythm amplitude. *J. Biol. Chem.* **295**, 3518–3531 [CrossRef Medline](#)
58. Källberg, M., Wang, H. P., Wang, S., Peng, J., Wang, Z. Y., Lu, H., and Xu, J. B. (2012) Template-based protein structure modeling using the RaptorX web server. *Nat. Protoc.* **7**, 1511–1522 [CrossRef Medline](#)
59. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A., and Baker, N. A. (2004) PDB2PQR: An automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* **32**, W665–W667 [CrossRef Medline](#)
60. Huang, J., Rauscher, S., Nawrocki, G., Ran, T., Feig, M., de Groot, B. L., Grubmüller, H., and MacKerell, A. D., Jr. (2017) CHARMM36m: an improved force field for folded and intrinsically disordered proteins. *Nat. Methods* **14**, 71–73 [CrossRef Medline](#)
61. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38 [CrossRef Medline](#)
62. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802 [CrossRef Medline](#)
63. Wagner, J. R., Sørensen, J., Hensley, N., Wong, C., Zhu, C., Perison, T., and Amaro, R. E. (2017) POVME 3.0: Software for mapping binding pocket flexibility. *J. Chem. Theory Comput.* **13**, 4584–4592 [CrossRef Medline](#)
64. Wickham, H. (2009) *ggplot2: Elegant Graphics for Data Analysis*, 2nd Ed. Springer, New York
65. Van Wart, A. T., Durrant, J., Votapka, L., and Amaro, R. E. (2014) Weighted Implementation of Suboptimal Paths (WISP): An optimized algorithm and tool for dynamical network analysis. *J. Chem. Theory Comput.* **10**, 511–517 [CrossRef Medline](#)
66. Kavakli, I. H., Ozturk, N., and Gul, S. (2019) DNA repair by photolyases. *Adv. Protein Chem. Struct. Biol.* **115**, 1–19 [CrossRef Medline](#)