A methylbenzimidazole derivative regulates mammalian circadian rhythms by targeting Cryptochrome proteins [version 2; peer review: 2 approved]

Moeri Yagi1,2*, Simon Miller1*, Yoshiko Nagai1*, Shinsuke Inuki3, Ayato Sato1, Tsuyoshi Hirota1,2

1Institute of Transformative Bio-Molecules, Nagoya University, Nagoya, 464-8601, Japan
2Division of Biological Sciences, Graduate School of Science, Nagoya University, Nagoya, 464-8601, Japan
3Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, 606-8501, Japan

* Equal contributors

Abstract

**Background:** Impairment of the circadian clock has been associated with numerous diseases, including sleep disorders and metabolic disease. Although small molecules that modulate clock function may form the basis of drug discovery of clock-related diseases, only a few compounds that selectively target core clock proteins have been identified. Three scaffolds were previously discovered as small-molecule activators of the clock protein Cryptochrome (CRY), and they have been providing powerful tools to understand and control the circadian clock system. Identifying new scaffolds will expand the possibilities of drug discovery.

**Methods:** A methylbenzimidazole derivative TH401 identified from cell-based circadian screens was characterized. Effects of TH401 on circadian rhythms were evaluated in cellular assays. Functional assays and X-ray crystallography were used to elucidate the effects of the compound on CRY1 and CRY2 isoforms.

**Results:** TH401 lengthened the period of circadian rhythms and stabilized both CRY1 and CRY2. The compound repressed Per2 reporter activity, which was reduced by Cry1 or Cry2 knockout and abolished by Cry1/Cry2 double knockout, indicating the dependence on CRY isoforms. Thermal shift assays showed slightly higher interaction of TH401 with CRY2 over CRY1. The crystal structure of CRY1 in complex with TH401 revealed a conformational change of the gatekeeper W399, which is involved in isoform-selectivity determination.

**Conclusions:** The present study identified a new small molecule TH401 that targets both CRY isoforms. This compound has expanded the chemical diversity of CRY activators, and will ultimately aid in the
development of therapeutics against circadian clock-related disorders.

**Keywords**
Circadian clock, Cryptochrome, Small-molecule compound

**Corresponding author:** Tsuyoshi Hirota (thirota@itbm.nagoya-u.ac.jp)

**Author roles:**
Yagi M: Formal Analysis, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing;
Miller S: Data Curation, Formal Analysis, Investigation, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing;
Nagai Y: Investigation, Validation, Writing – Review & Editing;
Inuki S: Resources, Writing – Review & Editing;
Sato A: Resources, Writing – Review & Editing;
Hirota T: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

**Grant information:** This work was supported in part by Japan Society for the Promotion of Science Grants 18H02402, 20K21269, and 21H04766; the Takeda Science Foundation; the Tokyo Biochemical Research Foundation; and the Hitachi Global Foundation (T.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Copyright:** © 2022 Yagi M et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**How to cite this article:** Yagi M, Miller S, Nagai Y et al. A methylbenzimidazole derivative regulates mammalian circadian rhythms by targeting Cryptochrome proteins [version 2; peer review: 2 approved] F1000Research 2022, 11:1016
https://doi.org/10.12688/f1000research.124658.2

**First published:** 07 Sep 2022, 11:1016 https://doi.org/10.12688/f1000research.124658.1
Introduction
Living organisms have a molecular clock, called the circadian clock, which drives the ~24-hour circadian rhythm. The circadian clock regulates daily rhythms of various physiological processes, such as sleep-wake behavior, body temperature, and metabolism.\(^1\) This clock is composed of a transcriptional regulatory network of clock genes, *Period (Per1 and Per2), Cryptochrome (Cry1 and Cry2), Clock and Bmal1*. In the core feedback loop of the mammalian circadian clock, transcription factors CLOCK and BMAL1 activate transcription of *Per* and *Cry* genes by forming a heterodimer. The translated PER and CRY proteins then repress the transcriptional activity of CLOCK-BMAL1 to close the loop, followed by the degradation of PER and CRY through the ubiquitin-proteasome pathway reactivating CLOCK-BMAL1.\(^2\)

Impairment of clock functions due to genetic mutations of clock genes or environmental factors, including shift work or chronic jet lag, has been shown to cause sleep disorders and increase the risk of numerous diseases, such as obesity and cancer.\(^3\) Thus, elucidating the circadian clock system is important for understanding how circadian clock dysfunction results in circadian-related diseases. Small-molecule compounds that control clock function provide a powerful and useful tool in drug discovery related to diseases that are impacted by circadian disruption.\(^4\)–\(^6\) Cell-based chemical screening has identified several synthetic small-molecule compounds that selectively target the core clock protein CRY. A carbazole-containing compound KL001 targets both CRY1 and CRY2 to inhibit their ubiquitin-dependent degradation, thus lengthening the circadian period.\(^7\) Several KL001 derivatives have been developed, including KL044 which is 10 times more potent than KL001,\(^8\) and a period-shortening compound GO044.\(^9\) Several other KL001 derivatives have shown potential application in the treatment of diabetes and glioblastoma. Compound 41 and compound 50 improved glucose clearance in diet-induced obese mice and *db/db* mice, respectively, indicating their antidiabetic efficacy.\(^10\),\(^11\) Treatment with KL001 and its derivative SHP656 inhibited proliferation and survival of patient-derived glioblastoma stem cells (GSCs), which cause a highly malignant primary brain tumor, and SHP656 prolonged the survival of mice implanted with GSCs.\(^12\) Furthermore, a new series of CRY activators that target either CRY1 or CRY2 in an isoform-selective manner were recently identified: phenylpyrazole-containing compounds KL101, TH301, and TH129,\(^13\),\(^14\) and a thienopyrimidine derivative KL201.\(^15\) In addition to these three scaffolds, the identification of novel scaffolds will expand the chemical diversity of CRY activators, as well as the possibility of drug discovery for the treatment of circadian clock-related diseases.

In this study, we revealed the effects of a new circadian clock modulator TH401, which contains a methylbenzimidazole moiety, on CRY isoforms by taking a target-based approach. TH401 showed stabilization and activation of CRY1 and CRY2. The repression of *Per2* reporter by TH401 was dependent on both CRY isoforms, indicating CRY-specific activity of the compound. TH401 directly interacted with CRY1 and CRY2, albeit with a slight preference to CRY2, and the X-ray crystal structure of a CRY1-TH401 complex revealed the binding mode of TH401.

Methods

**TH401 and derivatives**
TH401 powder was purchased from Vitas-M Laboratory (STK095604). TH403-TH411 were obtained from a 10 mM original stock of a compound library containing 20,000 small molecules used for primary screening of circadian clock modulators.

**Cell-based circadian assays**
U2OS cells expressing a *Bmal1-dLuc* and *Per2-dLuc* reporter\(^16\),\(^17\) were plated onto a white, solid-bottom 384-well plate at 30 μl (3,000 cells) per well as previously described.\(^18\) After 2 days, 40 μl of explant medium [DMEM (12800-017, Gibco) supplemented with 2% B27 (17504-001, Gibco), 10 mM HEPES, 0.38 mg/ml sodium bicarbonate, 0.29 mg/ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM luciferin; pH 7.2] was dispensed into each well, and 500 nl of compounds (final 0.7% dimethyl sulfoxide (DMSO)) were applied. The luminescence was recorded every 100 min for 5 days in a microplate reader (Infinite M200Pro, Tecan).

**Cell viability assay**
*Bmal1-dLuc* and *Per2-dLuc* U2OS cells were plated by following the cell-based circadian assay protocol as described above and cultured for 5 days. Then, CellTiter-Glo Reagent (G9242, Promega) was applied to each well, and luminescence corresponding to cellular ATP levels was recorded in a multi-mode reader (Cytation3, BioTek).
In vitro kinase assay

The effect of compounds on casein kinase Iδ (CKIδ) activity in vitro was analyzed as previously described. The reaction mixture containing 2 ng/μl CKIδ (14-520, Eurofins), 50 μM peptide substrate RKKKAEpSVASLTSQCSYSS corresponding to human PER2 Lys659-Ser674 (custom made), CKI buffer (40 mM Tris, 10 mM MgCl2, 0.5 mM DTT, 0.1 mg/ml BSA, pH 7.5), compound (final 5% DMSO), and 5 μM ATP was incubation at 30°C for 3 h. Kinase-Glo Luminescent Kinase Assay reagent (V6713, Promega) was used to determine the amount of remaining ATP.

Degradation assay

Stable HEK293 cells expressing a C-terminally luciferase-fused CRY1 (CRY1-LUC), CRY2-LUC or LUC reporter were plated onto a white, solid-bottom 96-well plate (30,000 cells per well) and treated with TH401 for 24 h as previously described. After 24 h treatment with compounds, luciferin (final 0.1 mM) and HEPES-NaOH (pH 7.2; final 10 mM) were added to the medium. After 1 h, it was further supplemented with cycloheximide (final 20 μg/ml), and luminescence was recorded every 10 min for 18 h in a microplate reader (Infinite M200Pro, Tecan).

Per2::Luc repression assay

Wild type, Cry1/Cry2 double knockout, Cry1 knockout, and Cry2 knockout fibroblasts expressing a Per2::Luc knock-in reporter were plated on a white, solid-bottom 384-well plate. They were cultured for 2 days to reach confluency, and 500 nl of compounds (final 0.7% DMSO) were added. After 2 days of treatment with the compounds, the medium was replaced with BrightGlo (E2620, Promega), and luminescence was recorded in a multi-mode reader (Cytation3, BioTek).

Cry rescue assay

Functional rescue of Cry1/Cry2 double knockout mouse embryonic fibroblasts with CRY expression vectors was performed as previously described with modifications: 15,000 cells were plated onto a white, solid-bottom 96-well plate, and after 24 h, transfected with 0.1 or 0.2 ng of CRY1 and CRY2 expression vectors and 100 ng of a Bmal1-Eluc reporter vector by Fugene 6 (E2691, Promega). After treatment with forskolin (final 10 μM) for 2 h, the medium was replaced with explant medium containing 0.2 mM luciferin, and 500 nl of compounds (final 0.4% DMSO) were applied. Luminescence was recorded every 36 min in a microplate reader (Infinite M200Pro, Tecan) for 5 days.

Cellular thermal shift assay

HEK293T cells were co-transfected with Flag-tagged CRY1 and HA-tagged CRY2 expression vectors as previously described. After 2 days, the cell pellet was suspended in serum-free DMEM with cOmplete EDTA-free Protease Inhibitor Cocktail (04693132001, Roche), treated with 0, 8, and 24 μM of compounds (final 0.7% DMSO) on a 96-well PCR plate, and incubated at 37°C for 1 h, followed by heat treatment for 3 min. The optimized temperatures for heat treatment of CRY1 and CRY2 were 55°C and 49°C, respectively. The cells were lysed by 2 cycles of freeze-thawing and centrifuged at 18,000 x g for 20 min at 4°C. The supernatants were analyzed by Western blotting with mouse monoclonal anti-Flag-HRP (A8592, Sigma; RRID: AB_439702) and rat monoclonal anti-HA-HRP (12013819001, Roche; RRID: AB_390917) antibodies.

Recombinant CRY expression and purification

His6-MBP-CRY1(PHR) and His6-MBP-CRY2(PHR) were expressed in Sf9 (Spodoptera frugiperda) insect cells via baculovirus infection as previously described. Cell pellets were resuspended in lysis buffer (1x PBS, 50 mM NaNO3, 1% (v/v) glycerol, 0.1% Triton X-100, and Complete Protease Inhibitor Cocktail (Roche); pH 7.4) and purified according to our previously determined method. Briefly, cells were sonicated on ice, centrifuged at 19,000 × g for 90 min at 4°C, and the supernatant, containing target CRY proteins, was purified via a high-performance liquid chromatography (HPLC) system using a HisTrap 5 ml column (GE Healthcare). After tobacco etch virus (TEV) protease cleavage of the His6-MBP tag, further purification was performed via a HiTrap Heparin HP column (GE Healthcare), amylase resin (E8021, New England Biolabs), and a gel filtration chromatography Superdex 75 16/60 column (GE Healthcare). Purified proteins were buffer-exchanged (see Protein crystallization and structure determination section) and concentrated using an Amicon Ultra (Merck) concentrator.

Thermal shift assay

CRY1(PHR) or CRY2(PHR) were diluted to 2 μM with differential scanning fluorimetry (DSF) buffer (20 mM HEPES-NaOH, 150 mM NaCl, 2 mM DTT; pH 7.5) and dispensed into a 384-well white PCR plate (Bio-Rad) at 17 μl per well. After the application of 1 μl of compounds (final 5% DMSO), the mixtures were incubated at room temperature with gentle shaking for 60 min. 2 μl of SYPRO Orange (S6650, Invitrogen) diluted with DSF buffer (final 5x SYPRO Orange) was added, and thermal denaturation was performed using a real-time PCR detection system (CFX384 Touch, Bio-Rad).
Protein crystallization and structure determination

CRY1(PHR) was buffer-exchanged into 100 mM Bis-Tris propane (B6755, Sigma), 100 mM NaCl, and 2 mM tris (2-carboxyethyl) phosphine (209-19861, Wako Pure Chemical Industries); pH 7.5, concentrated to 6 mg/ml, and crystallized via hanging-drop vapor diffusion at 20°C. CRY1(PHR) (1 μl) was mixed with 1 μl of precipitant solution containing 250 mM NH₄Cl, 21% (w/v) PEG 3350, 3% (v/v) ethylene glycol. Apo crystals grew over several days and were soaked overnight with 0.5 mM TH401 dissolved in mother liquor. The crystals were cryoprotected in mother liquor plus 30% (v/v) PEG 400, and flash-cooled in liquid nitrogen. In contrast, we were unable to obtain protein crystals of a CRY2-TH401 complex.

X-ray diffraction data for CRY1-TH401 was collected at the SPring-8 synchrotron radiation facility (beamline BL41XU) at a wavelength of 1.0 Å and a temperature of 100 K. The dataset was processed with DIALS/xia2 and SCALA in the CCP4 suite. The CRY1-TH401 structure was determined in space group P2₁2₁2₁ (1 molecule per asymmetric unit) by Phaser using CRY1-apo (PDB ID: 6KX4) as a molecular replacement (MR) template. Density modification was performed with PARROT. Model building was performed iteratively using Coot and refinement in REFMAC5. Final refinement was performed with PHENIX refine.

Quantification

A curve fitting program MultiCycle (Actimetrics) was utilized to determine the circadian period, and the luminescence intensity was calculated by averaging the intensity during the entire experiment. Due to transient changes in luminescence upon medium exchange, data from the first day was excluded from analysis. In degradation assays, half-life was obtained by one phase exponential decay fitting with Prism software (version 7.04, GraphPad Software; any open-access software can be used as an alternative, including the freely available R). In cellular thermal shift assays, band intensity was analyzed by ImageQuant TL software (version 8.1, GE Healthcare). In thermal shift assays, the highest peak of the dF/dT curve (the first derivative of the fluorescence intensity against temperature) was defined as the melting temperature.

Figure 1. TH401 lengthens circadian period. (A) The chemical structure of TH401. (B-D) Effects on circadian rhythms in Bmal1-dLuc and Per2-dLuc U2OS cells. Luminescence rhythms in the presence of various concentrations of TH401 (B, mean of n = 2) and changes in period (C) and luminescence intensity (D) compared to a dimethyl sulfoxide (DMSO) control are shown (n = 6 biologically independent samples). When arrhythmic, the period is not plotted. (E) Effect on cell viability in Bmal1-dLuc and Per2-dLuc U2OS cells. Cellular ATP levels after treatment with various concentrations of TH401 are plotted by setting a DMSO control to 1 (n = 4 biologically independent samples).
Results and discussion

TH401 lengthens circadian period

We discovered new small-molecule modulators of the circadian clock from cell-based screens of a library of ~20,000 uncharacterized compounds. In this study, we characterized a methylbenzimidazole derivative TH401 (Figure 1A). Treatment of human U2OS cells expressing either a Bmal1 promoter-luciferase (Bmal1-dLuc) reporter or a Per2-dLuc reporter with TH401 caused lengthening of the circadian period in a dose-dependent manner (Figure 1B and C). Furthermore, increasing the concentrations of TH401 suppressed the intensity of the Per2-dLuc reporter more than that of Bmal1-dLuc (Figure 1B and D), without affecting cellular viability (Figure 1E). These results indicate that TH401 is a new clock-modulating compound.

TH401 targets both CRY isoforms

We took a target-based approach to reveal how TH401 modulates circadian rhythms. Longdaysin is known to induce period lengthening by targeting the protein kinase CKIδ, but TH401 did not affect CKIδ activity in an in vitro kinase assay (Figure 2A), suggesting an alternative mechanism of action other than CKI. We next analyzed the effect of TH401 on CRY stability in a cell-based degradation assay. HEK293 cells expressing a CRY1-luciferase (CRY1-LUC) or CRY2-LUC fusion protein reporter were treated with the compound at various concentrations, and the half-life of luminescence signals were measured. TH401 stabilized both CRY1 and CRY2 (Figure 2B), suggesting that the compound targets CRY proteins.

The effect of TH401 on endogenous CRY1 and CRY2 activity was analyzed by using Cry knock-out fibroblasts from mice carrying a Per2::Luc knock-in reporter. Cry is a repressor of CLOCK-BMAL1, and CRY stabilization reduces the expression of CLOCK-BMAL1-target genes such as Per2. TH401 repressed the intensity of the Per2::Luc reporter in a dose-dependent manner in wild type cells with both CRY1 and CRY2 present (Figure 2C). Per2 repression was not

---

**Figure 2.** TH401 stabilizes and interacts with CRY1 and CRY2. (A) Effect of TH401 on casein kinase Iδ (CKIδ) activity in vitro. Kinase activity was analyzed in the presence of various concentrations of compounds (n = 1). Longdaysin is an inhibitor of CKIδ. (B) Effect of TH401 on Cryptochrome (CRY) degradation in HEK293 cells. The half-lives of CRY-luciferase fusion proteins (CRY1-LUC and CRY2-LUC) relative to LUC are plotted by setting a DMSO control to 1 (n = 2 biologically independent samples). (C) Effect on Per2::Luc knock-in reporter activity in wild type, CRY1/CRY2 double knockout, CRY1 knockout, and CRY2 knockout fibroblasts. Changes in luminescence intensity compared to a DMSO control are shown (n = 4–8 biologically independent samples). (D) Effect on cellular circadian period of Bmal1-Eluc reporter rhythms in CRY1/Cry2 double knockout fibroblasts rescued with CRY. Changes in period compared to a DMSO control are shown (n = 3–6 biologically independent samples). (E) Interaction with CRY proteins in HEK293T cells. The band intensities of Flag-tagged CRY1 and HA-tagged CRY2 proteins protected from thermal denaturation were plotted by setting a DMSO control to 1 (mean of n = 4 biologically independent samples). (F) Interaction with CRY(PHR) in vitro. Changes in denaturing temperatures of recombinant CRY(PHR) proteins in the presence of various concentrations of TH401, compared to a DMSO control are shown (n = 2 biologically independent samples).
observed in Cry1/Cry2 double knockout fibroblasts, indicating that the effect of TH401 was CRY-dependent. In Cry1 and Cry2 single knockout cells, Per2 repression by TH401 was reduced compared to wild type, which supports that TH401 targets both CRY1 and CRY2. We further evaluated its effect on the circadian period of a Bmal1-Eluc reporter in Cry1/Cry2 double knockout mouse fibroblasts rescued with CRY1 and CRY2. Period-lengthening by TH401 was enhanced when the dose of CRY1 or CRY2 was increased (Figure 2D).30

To assess the interaction of TH401 with CRY proteins, a cellular thermal shift assay was conducted using HEK293T cells expressing CRY1-Flag and CRY2-HA. Exposing proteins to a high temperature causes them to lose their tertiary structure. However, the binding of a ligand increases resistance to unfolding, leading to thermal stabilization of the bound protein.32 TH401 stabilized CRY1 and CRY2 against thermal denaturation in a dose-dependent manner (Figure 2E),30 suggesting that TH401 interacts with both CRY isoforms. The direct interaction of TH401 with recombinant CRY1 photolyase homology region (PHR) and CRY2(PHR) was further evaluated by performing an in vitro thermal shift assay. We found that TH401 interacted with both recombinant CRY(PHR) proteins with a slightly higher preference against CRY2 over CRY1 (Figure 2F).30 Together, these data indicate that TH401 induces circadian period lengthening by targeting and interacting with both CRY1 and CRY2 proteins.

Structural binding mechanisms of TH401 in CRY1

To obtain insights into the regulatory effects of TH401 on CRY proteins, we determined the crystal structure of CRY1(PHR) in complex with TH401 at a resolution of 2.05 Å (Table 1) (PDB ID: 7WVA). The overall protein fold was highly similar to previously published CRY1 structures.13-15,33-35 With regard to the binding mode of TH401, the 1-methylbenzimidazole moiety formed hydrophobic interactions with W292, R293 and W399, as well as additional offset π-stacking with W292 (Figure 3A). The trimethoxyphenyl moiety formed multiple hydrophobic interactions with residues R358, A362, F381, L385, A388, W397 and L400. Oxygen atoms in two methoxy groups (ortho and meta) formed hydrogen bonds with the guanidinium group of R358, while methyl groups in two methoxy groups (ortho and meta) formed C−H hydrogen bonds with N393 and S396 (Figure 3A). One notable difference in the binding mode of TH401, compared to almost all other CRY-interacting compounds, was the absence of a canonical H-bond between the linker (connecting the methylbenzimidazole and trimethoxyphenyl moieties) and S396. Instead, H359 interacted with the sulfanylacetohydrazide linker by forming two hydrogen bonds, one with the hydrazide carbonyl and the other with a hydrazide nitrogen (Figure 3A).

TH401 binding was compatible with the intrinsic conformations of most FAD (flavin adenine dinucleotide) pocket residues of CRY1; however, a notable difference was observed in the conformation of the gatekeeper W399, and steric restraint was imposed on the possible rotamer positions of H355 (Figure 3B). W399 underwent a sizeable conformational change from an intrinsic “out” position to a “middle” conformation to form a hydrophobic interaction with the methyl group of the 1-methylbenzimidazole moiety, and H355 adopted a forward-facing rotamer, similar to an alternate conformer that was observed in the CRY1-apo structure13 (PDB ID: 6KX4). The lid loop was disordered in CRY1-apo structures that was observed in the CRY1-apo structure13 (PDB ID: 6KX4). The lid loop was disordered in CRY1-apo structures and in CRY2 (Figure 3B), and the lid loop was disordered as a result of TH401-induced repositioning of W399 (Figure 3B). Overall, the binding mode of TH401 is not fully compatible with the intrinsic FAD pocket in CRY1-apo and induces conformational rearrangement of key pocket residues for a favorable interaction.

Our structural data showed that TH401 binding to CRY1 induced a sizeable conformational change in the gatekeeper W399. Isoform-specific gatekeeper conformations that mediate distinct gatekeeper–lid loop interfaces in CRY1 and CRY2 have been implicated in the potential regulation of compound isoform-selectivity.35,36 Interestingly, the TH401-induced “middle” gatekeeper conformation in the CRY1-TH401 structure would appear to be more energetically favorable in CRY2, because only a small conformational change (W417 from “in” to “middle”; CRY2 W417 corresponds to CRY1 W399) would be required (Figure 3B). In contrast, CRY1 W399 would need to rotate much further from an “out” to a “middle” conformation. Furthermore, the NH–aryl interaction between W399 and Q407 in CRY1 has more favorable free binding energy than the stacking interaction of W417 and F424 in CRY2,35 which may result in CRY1 W399 being less flexible than CRY2 W417. These structural observations correlate to the slightly lower preferential interaction of TH401 with CRY1 compared to CRY2 in thermal shift assays (Figure 2E and F). In contrast, however, TH401 displayed a very low level of isoform preference in functional assays (Figure 2C and D). This disparity may be due to higher repressor activity of CRY1 over CRY2,37-40 resulting in the similar functional effects of TH401 on both isoforms, despite its preferential interaction with CRY2. In addition to the gatekeeper and lid loop, a flexible region downstream of the PHR known as the CRY C-terminal tail (CCT) has been associated with compound selectivity.35 In Drosophila CRY, the residue H378, corresponding to mouse CRY1 H355, has been shown to regulate CCT interaction with the FAD pocket via a conformational change.41-43 Both W399 and H355 in CRY1-TH401 underwent large conformational changes, compared to CRY1-apo structures (Figure 3B), and the lid loop was disordered as a result of W399-repositioning. These changes could affect CCT interaction for functional changes.
CRYs form large complexes in both the cytoplasm and nucleus, and PER2, a primary CRY-interacting protein, changes the conformations of key FAD pocket residues, including the gatekeeper W399, as well as the lid loop (PDB ID: 4CT0). Interestingly, the conformations of the gatekeeper W399 and H355 in the CRY1-TH401 crystal structure are very similar to those in CRY1/2-PER2 complex structures (PDB IDs: 4CT0; and 4U8H). The conformation of W292 in CRY1-PER2 would form a steric clash with the methylbenzimidazole of TH401; however, W292 is very flexible and can accommodate compounds by easily adopting a different rotamer. Therefore, TH401 may be able to bind to CRY1 and CRY2 equally when they are complexed with PER2, resulting in similar potency against both isoforms.

To further characterize CRY-TH401 interactions, we searched for TH401 derivatives in the compound library used for primary screening of circadian clock modulators and checked their activity in the screen (Figure 4, blue). Because the derivatives TH403-TH411 showed almost no effect on circadian period in the screen at 7 μM (using 1 mM working stock compounds), we obtained these compounds from the original 10 mM stock of the library and analyzed their activity in a circadian assay using human Bmal1-dLuc U2OS cells at 24 or 8 μM (Figure 4, purple). Extension of the methyl group of 1-methylbenzimidazole together with replacement of the ortho-methoxy group of trimethoxyphenyl to meta (TH403)

### Table 1. Data collection and refinement statistics. Values in parentheses are for the highest resolution shell. Root mean square (R.m.s.); correlation coefficient (CC).

| Data collection            | CRY1-TH401 (7WVA) |
|----------------------------|-------------------|
| Space group                | P2_12_21          |
| Cell dimensions            |                   |
| a, b, c (Å)                | 44.9, 78.2, 132.8 |
| α, β, γ (°)                | 90, 90, 90        |
| Resolution (Å)             | 2.05 (2.16-2.05)  |
| Rmerge                     | 0.077 (0.475)     |
| I/σ(I)                     | 13.7 (3.0)        |
| CC1/2                      | 0.999 (0.891)     |
| Completeness (%)           | 99.9 (99.7)       |
| Redundancy                 | 7.4 (7.2)         |

| Refinement                 |                   |
| Resolution (Å)             | 66.41-2.051       |
| No. reflections [unique]   | 221735 [30079]    |
| Rwork/Rfree               | 0.1897/0.2204     |
| No. atoms                  | 3840              |
| Protein                    | 3640              |
| Ligand/ion                 | 29                |
| Water                      | 171               |
| R.m.s. deviations          |                   |
| Bond lengths (Å)           | 0.007             |
| Bond angles (°)            | 0.779             |
| Ramachandran               |                   |
| Favored (%)                | 97.67             |
| Allowed (%)                | 2.33              |
| Outliers (%)               | 0                 |
| Average B-factors          |                   |
| Protein                    | 37.08             |
| Ligand                     | 33.38             |
| Solvent                    | 37.42             |
caused a loss of activity, consistent with the interactions of the methyl group with W399, and the \textit{ortho}-methoxy group with R358 and S396 (Figure 3A). Modifications to the trimethoxy groups of the trimethoxyphenyl resulted in either weak activity (TH404-TH406) or inactivity (TH407-TH411), supporting their interactions with R358, S396, and N393, as well as A362, F381, L385, A388, W397, and L400. The weak activities of TH404-TH406 suggested that an interaction of the \textit{ortho}-hydroxy group with R358 can support activity. Therefore, CRY-TH401 interactions in the crystal structure are consistent with activity in cells.
Conclusion
We have discovered that TH401 provides a new chemical scaffold, methylbenzimidazole, for CRY regulation by targeting both CRY1 and CRY2. Cell-based phenotypic screens of circadian clock modulators resulted in the identification of small-molecule activators of CRY proteins. In addition to this approach, CRY inhibitors have been identified through a cell-based screen of E-box-mediated transcription. 2-ethoxypropanoic acid derivatives target both CRY isoforms and inhibit their repressive function, enhancing E-box-mediated transcription.46,47 Furthermore, a recent study showed that structure-based drug design could be another useful approach to find CRY1 modulators.48 In order to obtain further insights into the mechanisms of action of CRY-modulating small molecules, it is necessary to determine complex crystal structures. The identification and characterization of new CRY modulators will facilitate the understanding and regulation of CRY protein functions in gene expression,49 metabolism,7,10,11,13,50,51 cancer,12,52–54 and sleep-wake rhythms,55–57 ultimately leading to the discovery of therapeutic agents for circadian clock-related diseases.

Data availability
Underlying data
The X-ray crystal structure of CRY1-TH401 was deposited into the Protein Data Bank with the accession number 7WVA.

Figshare: Yagi et al. Figure data. https://doi.org/10.6084/m9.figshare.20431692.30
This project contains the following underlying data:

- Figure 1B. csv (Luminescence rhythms of Bmal1-dLuc and Per2-dLuc U2OS cells in the presence of various concentrations of TH401 (n = 2))
- Figure 1C. csv (Changes in period (n = 6))
- Figure 1D. csv (Changes in luminescence intensity (n = 6))
- Figure 1E. csv (Changes in cellular ATP levels after treatment with various concentrations of TH401 (n = 4))
- Figure 2A. csv (Inhibitory effect of Longdaysin and TH401 on CKIδ activity in vitro (n = 1))
- Figure 2B. csv (Changes in the half-lives of CRY-luciferase fusion proteins (CRY1-LUC and CRY2-LUC) relative to LUC in the presence of various concentrations of TH401 (n = 2))
- Figure 2C. csv (Changes in Per2::Luc knock-in reporter activity in wild type, Cry1/Cry2 double knockout, Cry1 knockout, and Cry2 knockout fibroblasts (n = 4–8))
- Figure 2D. csv (Changes in the cellular circadian period of Bmal1-Eluc reporter rhythms in Cry1/Cry2 double knockout fibroblasts rescued with CRY1 and CRY2 (n = 3–6))
- Figure 2E. csv (Changes in the protection of CRY1 and CRY2 proteins from thermal denaturation in HEK293T cells (n = 4))
- Figure 2F. csv (Changes in denaturing temperatures of recombinant CRY1(PHR) and CRY2(PHR) in vitro (n = 2))
- Figure 4. csv (Changes in the circadian period in primary screening (tested at 7 μM; n = 1) and a secondary assay (tested at 24 or 8 μM; n = 3))

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Accession numbers**

Protein Data Bank:

- Crystal structure of mouse Cryptochrome 1 in complex with TH401 compound. Accession number: 7WVA. [https://doi.org/10.2210/pdb7WVA/pdb](https://doi.org/10.2210/pdb7WVA/pdb).
- Crystal structure of mouse CRY1 with bound cryoprotectant. Accession number: 7D0M. [https://doi.org/10.2210/pdb7D0M/pdb](https://doi.org/10.2210/pdb7D0M/pdb).
- Crystal structure of mouse CRY2 apo form. Accession number: 7D0N. [https://doi.org/10.2210/pdb7D0N/pdb](https://doi.org/10.2210/pdb7D0N/pdb).
- Crystal Structure of Mouse Cryptochrome1 in Complex with Period2. Accession number: 4CT0. [https://doi.org/10.2210/pdb4CT0/pdb](https://doi.org/10.2210/pdb4CT0/pdb).
- Crystal Structure of Mammalian Period-Cryptochrome Complex. Accession number: 4U8H. [https://doi.org/10.2210/pdb4U8H/pdb](https://doi.org/10.2210/pdb4U8H/pdb).
- Crystal structure of mouse Cryptochrome 1 apo form. Accession number: 6KX4. [https://doi.org/10.2210/pdb6KX4/pdb](https://doi.org/10.2210/pdb6KX4/pdb).

**Acknowledgements**

We thank Natsuko Ono, Dr. Kaori Goto, Naoya Kadofusa, and Dr. Kazuya Hasegawa for technical assistance, Dr. Shinya Oishi for technical assistance and helpful discussion, and Dr. Hiroki R. Ueda for Cry1/Cry2 double knockout cells and...
pMU2-P (Cry1)-FLAG-IRR-E-Cry1 plasmid. X-ray diffraction data collection and preliminary experiments were carried out at beamlines BL41XU and BL44XU of SPring-8 synchrotron facility (proposals 2019A6942 and 2019B6942) and BL-17A of Photon Factory (proposal 2019G024). Recombinant CRY expression, beamline experiments, and supply of library compounds were supported in part by Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS) from Japan Agency for Medical Research and Development support numbers JP20am0101074-0055, JP20am0101071-I-0529, and JP22ama121034.

References

1. Green CB, Takahashi JS, Bass J: The meter of metabolism. Cell 2008; 134: 728-742. PubMed Abstract | Publisher Full Text
2. Takahashi JS: Transcriptional architecture of the mammalian circadian clock. Nat. Rev. Genet. 2017; 18: 164-179. PubMed Abstract | Publisher Full Text
3. Takahashi JS, et al.: The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat. Rev. Genet. 2008; 9: 764-775. PubMed Abstract | Publisher Full Text
4. Chen Z, You SH, Takahashi JS: Development and Therapeutic Potential of Small-Molecule Modulators of Circadian Systems. Annu. Rev. Pharmacol. Toxicol. 2018; 58: 231-252. PubMed Abstract | Publisher Full Text
5. Miller S, Hirota T: Pharmacological Interventions to Circadian Clocks and Their Molecular Bases. J. Mol. Biol. 2020; 432: 3498-3514. PubMed Abstract | Publisher Full Text
6. Rasmussen ES, Takahashi JS, Green CB: Time to target the circadian clock for drug discovery. Trends Biochem. Sci. 2022; 47: 745-758. PubMed Abstract | Publisher Full Text
7. Hirota T, et al.: Identification of small molecule activators of cryptochrome. Science 2012; 337: 1094-1097. PubMed Abstract | Publisher Full Text
8. Lee JW, et al.: Development of Small-Molecule Cryptochrome Stabilizer Derivatives as Modulators of the Circadian Clock. ChemMedChem 2015; 10: 1489-1497. PubMed Abstract | Publisher Full Text
9. Oshima T, et al.: C-H activation generates period-shortening molecules that target cryptochrome in the mammalian circadian clock. Angew. Chem. Int. Ed. Engl. 2015; 54: 7193-7197. PubMed Abstract | Publisher Full Text
10. Humphries PS, et al.: Carbazole-containing sulfonamides and sulfamides: Discovery of cryptochrome modulators as antidiabetic agents. Bioorg. Med. Chem. Lett. 2016; 26: 757-760. PubMed Abstract | Publisher Full Text
11. Humphries PS, et al.: Carbazole-containing amidines and ureas: Discovery of cryptochrome modulators as antihyperglycemic agents. Bioorg. Med. Chem. Lett. 2018; 28: 293-297. PubMed Abstract | Publisher Full Text
12. Dong Z, et al.: Targeting Glioblastoma Stem Cells through Disruption of the Circadian Clock. Cancer Discov. 2019; 9: 1556-1573. PubMed Abstract | Publisher Full Text
13. Miller S, et al.: Isoform-selective regulation of mammalian cryptochromes. Nat. Chem. Biol. 2020; 16: 676-685. PubMed Abstract | Publisher Full Text
14. Kalaraki D, et al.: Photopharmacological Manipulation of Mammalian CRY1 for Regulation of the Circadian Clock. J. Am. Chem. Soc. 2021; 143: 2078-2087. PubMed Abstract | Publisher Full Text
15. Miller S, et al.: An Isoform-Selective Modulator of Cryptochrome 1 Regulates Circadian Rhythms in Mammals. Cell Chem. Biol. 2020; 27: 1192-1198.e5. PubMed Abstract | Publisher Full Text
16. Hirota T, et al.: A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta. Proc. Natl. Acad. Sci. U. S. A. 2008; 105: 20746-20751. PubMed Abstract | Publisher Full Text
17. Zhang EE, et al.: A genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 2009; 139: 199-210. Publisher Full Text
18. Hatomi M, Hirota T: Cell-Based Phenotypic Screens to Discover Circadian Clock-Modulating Compounds. Methods Mol. Biol. 2022; 2482: 95-104. PubMed Abstract | Publisher Full Text
19. Hirota T, et al.: High-throughput chemical screen identifies a novel potent modulator of cellular circadian rhythms and reveals CK1α as a clock regulatory kinase. PloS Biol. 2010; 8: e1000559. PubMed Abstract | Publisher Full Text
20. Liu AC, et al.: Intercellular coupling confers robustness against mutations in the SCN circadian clock network. Cell 2007; 129: 605-616. PubMed Abstract | Publisher Full Text
21. Ode KL, et al.: Knockout-Rescue Embryonic Stem Cell-Derived Mouse Reveals Circadian-Period Control by Quality and Quantity of CRY1. Mol. Cell 2017; 65: 176-190. Publisher Full Text
22. Winter G: xia2: an expert system for macromolecular crystallography data reduction. J. Appl. Crystallogr. 2010; 43: 186-190. Publisher Full Text
23. Evans P: Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 2006; 62: 72-82. Publisher Full Text
24. Winn MD, et al.: Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 2011; 67: 235-242. PubMed Abstract | Publisher Full Text
25. McCoy AJ, et al.: Phaser crystallographic software. J. Appl. Crystallogr. 2007; 40: 658-674. PubMed Abstract | Publisher Full Text
26. Cowtan K: Recent developments in classical density modification. Acta Crystallogr. D Biol. Crystallogr. 2010; 66: 470-478. PubMed Abstract | Publisher Full Text
27. Emsley P, et al.: Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 2010; 66: 486-501. PubMed Abstract | Publisher Full Text
28. Murshudov GN, et al.: REFMAC5 for the refinement of macromolecular crystal structures. Acta Crystallogr. D Biol. Crystallogr. 2011; 67: 355-367. PubMed Abstract | Publisher Full Text
29. Afonine PV, et al.: Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr. D Biol. Crystallogr. 2012; 68: 352-367. PubMed Abstract | Publisher Full Text
30. Yagi M, et al.: Figure data. Figsare 2022. Publisher Full Text
31. Yoo SH, et al.: PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. U. S. A. 2004; 101: 5339-5346. PubMed Abstract | Publisher Full Text
32. Martinez Molina D, et al.: Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. Science 2013; 341: 84-87. PubMed Abstract | Publisher Full Text
33. Czarna A, et al.: Structures of Drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function. Cell 2013; 153: 1394-1405. PubMed Abstract | Publisher Full Text
34. Michael AK, et al.: 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. 2017; 114: 1560-1565. PubMed Abstract | Publisher Full Text
46. Chun SK, et al.: Identification and validation of cryptochrome inhibitors that modulate the molecular circadian clock. ACS Chem. Biol. 2014; 9: 709-710. PubMed Abstract | Publisher Full Text
47. Jeong YU, et al.: Development of Non-Ethoxypropanoic Acid Type Cryptochrome Inhibitors with Circadian Molecular Clock-Enhancing Activity by Biosynthetic Replacement. Pharmaceuticals (Basel) 2021; 14: 496. PubMed Abstract | Publisher Full Text
48. Guo S, et al.: Structure-based design and classifications of small molecules regulating the circadian rhythm period. Sci. Rep. 2021; 11: 18510. PubMed Abstract | Publisher Full Text
49. Koike N, et al.: Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 2012; 338: 349-354. PubMed Abstract | Publisher Full Text
50. Zhang EE, et al.: Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. Nat. Med. 2010; 16: 1152-1156. PubMed Abstract | Publisher Full Text
51. Lamia KA, et al.: Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature 2011; 480: 552-556. PubMed Abstract | Publisher Full Text
52. Huber AL, et al.: CYR2 and FBXL3 Cooperatively Degrade c-MYC. Mol. Cell 2016; 64: 774-789. PubMed Abstract | Publisher Full Text
53. Chan AB, et al.: CYR2 missense mutations suppress PS3 and enhance cell growth. Proc. Natl. Acad. Sci. U. S. A. 2021; 118: e2101416118. PubMed Abstract | Publisher Full Text
54. Shafi AA, et al.: The circadian cryptochrome, CR1, is a protumorigenic factor that rhythmically modulates DNA repair. Nat. Commun. 2021; 12: 401. PubMed Abstract | Publisher Full Text
55. Hirano A, et al.: A Cryptochrome 2 mutation yields advanced sleep phase in humans. elife 2016; 5: e16695. PubMed Abstract | Publisher Full Text
56. Patke A, et al.: Mutation of the Human Circadian Clock Gene CRY1 in Familial Delayed Sleep Phase Disorder. Cell 2017; 169: 203-215. e19. PubMed Abstract | Publisher Full Text
57. Iida M, et al.: Effects of cryptochrome-modulating compounds on circadian behavioural rhythms in zebrafish. J. Biochem. 2022; 171: 501-507. PubMed Abstract | Publisher Full Text
Moeri Yagi and colleagues report the identification of a chemical stabilizer of CRY1 and CRY2 based on a methylbenzimidazole scaffold, which is different from the scaffolds of previously reported CRY-stabilizing compounds. They present well designed experiments that persuasively show that this compound, TH401, interacts directly with both CRY1 and CRY2 at their FAD-binding...
pockets and thereby leads to their stabilization and lengthening of circadian period in cells.

I have one minor suggestions to improve clarity: In the introduction, the sentence “…compounds that control clock function provide a powerful and useful tool in the drug discovery of such diseases” is unclear. Do the authors mean “… compounds that control clock function provide a powerful and useful tool in drug discovery related to diseases that are impacted by circadian disruption”?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** circadian biology, cryptochrome proteins

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

Author Response 30 Sep 2022

Tsuyoshi Hirota, Nagoya University, Nagoya, Japan

**Reviewer #2:**

Moeri Yagi and colleagues report the identification of a chemical stabilizer of CRY1 and CRY2 based on a methylbenzimidazole scaffold, which is different from the scaffolds of previously reported CRY-stabilizing compounds. They present well designed experiments that persuasively show that this compound, TH401, interacts directly with both CRY1 and CRY2 at their FAD-binding pockets and thereby leads to their stabilization and lengthening of circadian period in cells.

I have one minor suggestions to improve clarity: In the introduction, the sentence “…compounds that control clock function provide a powerful and useful tool in the drug discovery of such
"diseases" is unclear. Do the authors mean “... compounds that control clock function provide a powerful and useful tool in drug discovery related to diseases that are impacted by circadian disruption”?

We thank the reviewer for the insightful comment. We are very pleased to hear that the reviewer approved the publication of this manuscript. We agree with the reviewer and modified the sentence accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Report 21 September 2022

https://doi.org/10.5256/f1000research.136879.r149802

© 2022 Zhang E. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Eric Zhang
National Institute of Biological Sciences (NIBS), Beijing, China

In this article, Moeri Yagi and co-authors describe the characterization of a small molecule TH401 which stabilizes both isoforms of the clock protein Cryptochrome (CRY1 and CRY2) and lengthens the period of circadian rhythms in cellular models. The co-crystal structure of CRY1 in complex with TH401 was obtained and compared with the structure of CRY1 and CRY2, explaining the slight preference of TH401 to CRY2. Several TH401 derivatives were further tested for their period-lengthening activities, confirming the proposed mechanism of CRY-TH401 interaction. As a methylbenzimidazole derivative, TH401 provides a new scaffold for CRY modulators, which will not only enable a better understanding of the structure of CRY, but also contribute to the discovery of therapeutics against circadian clock-related diseases. In general, this manuscript is well-written, and I recommend its publication after a minor revision.

Specific comments:
1. In the first paragraph on page 9, the conclusion “the binding mode of TH401 appears less compatible with the FAD pocket in CRY1 than CRY2” is drawn before the structure of FAD pocket in CRY2 is elucidated, which is somehow confusing.

2. In the third paragraph on page 10, the authors state “To further characterize the CRY2-TH401 interaction...” at the beginning and “CRY2-TH401 interactions in the crystal structure are consistent with activity in cells” in the end. However, the experiments using Bmal1-dLuc U2OS cells can not reflect the interaction of TH401 derivatives with CRY1 and CRY2 separately. It might be better to change “CRY2-TH401” into “CRY-TH401”.

3. Although several TH401 derivatives have been tested for their period-lengthening activities, no mutants of CRY1 or CRY2 were tested for their interactions with TH401. Rescue assay in
Cry1/Cry2 double knockout fibroblasts with CRY mutants carrying point mutations in TH401 binding residues may further confirm the proposed mechanism of CRY-TH401 interaction and is therefore recommended.

4. The hyphen should not be added between “small molecule” when it is used as a noun. This has appeared three times: “Although small-molecules that...” in Abstract, “…identified a new small-molecule TH401 that...” in Abstract and “…action of these small-molecules” in Conclusion.

5. Table 1 legend: “R,m.s.” should be “R.m.s.”

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Circadian clock

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 30 Sep 2022
Tsuyoshi Hirota, Nagoya University, Nagoya, Japan

Reviewer #1:
In this article, Moeri Yagi and co-authors describe the characterization of a small molecule TH401 which stabilizes both isoforms of the clock protein Cryptochrome (CRY1 and CRY2) and lengthens the period of circadian rhythms in cellular models. The co-crystal structure of CRY1 in complex with TH401 was obtained and compared with the structure of CRY1 and CRY2, explaining the slight preference of TH401 to CRY2. Several TH401 derivatives were further tested for their period-
lengthening activities, confirming the proposed mechanism of CRY-TH401 interaction. As a methylbenzimidazole derivative, TH401 provides a new scaffold for CRY modulators, which will not only enable a better understanding of the structure of CRY, but also contribute to the discovery of therapeutics against circadian clock-related diseases. In general, this manuscript is well-written, and I recommend its publication after a minor revision.

We thank the reviewer for the insightful comments. We are very pleased to hear that the reviewer recommends the publication of this manuscript. Our point-by-point responses are listed below. We believe that the changes have clarified our manuscript.

**Comment 1:** In the first paragraph on page 9, the conclusion “the binding mode of TH401 appears less compatible with the FAD pocket in CRY1 than CRY2” is drawn before the structure of FAD pocket in CRY2 is elucidated, which is somehow confusing.

We agree with the reviewer and modified the sentence to "Overall, the binding mode of TH401 is not fully compatible with the intrinsic FAD pocket in CRY1-apo and induces conformational rearrangement of key pocket residues for a favorable interaction."

**Comment 2:** In the third paragraph on page 10, the authors state “To further characterize the CRY2-TH401 interaction...” at the beginning and “CRY2-TH401 interactions in the crystal structure are consistent with activity in cells” in the end. However, the experiments using Bmal1-dLuc U2OS cells can not reflect the interaction of TH401 derivatives with CRY1 and CRY2 separately. It might be better to change “CRY2-TH401” into “CRY-TH401”.

Thank you for raising this point. We changed “CRY2-TH401” to “CRY-TH401”.

**Comment 3:** Although several TH401 derivatives have been tested for their period-lengthening activities, no mutants of CRY1 or CRY2 were tested for their interactions with TH401. Rescue assay in Cry1/Cry2double knockout fibroblasts with CRY mutants carrying point mutations in TH401 binding residues may further confirm the proposed mechanism of CRY-TH401 interaction and is therefore recommended.

This is an interesting point that we would like to address in our future studies. Thank you for your suggestion.

**Comment 4:** The hyphen should not be added between “small molecule” when it is used as a noun. This has appeared three times: "Although small-molecules that...” in Abstract, “...identified a new small-molecule TH401 that...” in Abstract and “...action of these small-molecules” in Conclusion.

Thank you for pointing this out. We removed the hyphen.

**Comment 5:** Table 1 legend: “R,m.s.” should be “R.m.s.”

Thank you for pointing this out. It was fixed.
**Competing Interests:** No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com