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Identification of Small Molecule Activators of Cryptochrome

Tsuyoshi Hirota^{1,7}, Jae Wook Lee², Peter C. St. John³, Mariko Sawa¹, Keiko Iwaisako⁴, Takako Noguchi⁵, Pagkapol Y. Pongsawakul¹, Tim Sonntag¹, David K. Welsh^{5,6}, David A. Brenner⁴, Francis J. Doyle III³, Peter G. Schultz^{2,*}, and Steve A. Kay^{1,7,*}

¹Division of Biological Sciences and Center for Chronobiology, University of California San Diego, La Jolla, CA 92093, USA.

²Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA.

³Department of Chemical Engineering, University of California Santa Barbara, Santa Barbara, CA 93106, USA.

⁴Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA.

⁵Department of Psychiatry and Center for Chronobiology, University of California San Diego, La Jolla, CA 92093, USA.

⁶Veterans Affairs San Diego Healthcare System, San Diego, CA 92161, USA.

⁷San Diego Center for Systems Biology, University of California San Diego, La Jolla, CA 92093, USA.

Abstract

Impairment of the circadian clock has been associated with numerous disorders, including metabolic disease. Although small molecules that modulate clock function might offer therapeutic approaches to such diseases, only a few compounds have been identified that selectively target core clock proteins. From an unbiased cell-based circadian screen, we identified KL001, a small molecule that specifically interacts with cryptochrome (CRY). KL001 prevented ubiquitin-dependent degradation of CRY, resulting in lengthening of the circadian period. In combination with mathematical modeling, KL001 revealed that CRY1 and CRY2 share a similar functional role in the period regulation. Furthermore, KL001-mediated CRY stabilization inhibited glucagon-induced gluconeogenesis in primary hepatocytes. KL001 thus provides a tool to study the regulation of CRY-dependent physiology and aid development of clock-based therapeutics of diabetes.

The circadian clock is an intrinsic time-keeping mechanism that controls the daily rhythms of numerous physiological processes, such as sleep/wake behavior, body temperature, hormone secretion, and metabolism (1-3). Circadian rhythms are generated in a cell-autonomous manner through transcriptional regulatory networks of clock genes. In the core feedback loop, the transcription factors CLOCK and BMAL1 activate expression of

*To whom correspondence should be addressed. Steve A. Kay, Ph.D., Division of Biological Sciences, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA., Tel: 858-822-7781; Fax: 858-822-7780, skay@ucsd.edu, Peter G. Schultz, Ph.D., Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA., Tel: 858-784-9273; Fax: 858-784-9440, schultz@scripps.edu.

Supplementary Materials

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Materials and Methods

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Period (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. After translation and nuclear localization, PER and CRY proteins inhibit CLOCK-BMAL1 function, resulting in rhythmic gene expression (1-3). Rate-limiting steps in many physiological pathways, including hepatic processes, are under the control of the circadian clock (1-3). The gluconeogenic genes *phosphoenol pyruvate carboxykinase* (*Pck1*) and *glucose 6-phosphatase* (*G6pc*) are controlled by CRY and the nuclear receptor REV-ERB (4-6).

Perturbations to clock function by genetic mutations or environmental factors (for example, shift work and jet lag) have been implicated in sleep disorders, cancer, cardiovascular and metabolic diseases (1-3). Thus, identification of clock-modulating small molecules may prove useful for the treatment of circadian-related disorders. Through cellbased high-throughput chemical screening approaches, a number of compounds that affect circadian rhythms have been discovered, including casein kinase I (CKI) inhibitors such as longdaysin (7-11). Synthetic ligands for the nuclear receptors REV-ERB and ROR have also been used to regulate the clock and metabolism (12, 13). Here we report the identification and characterization of a small molecule that specifically acts on CRY proteins and, as a result, regulates hepatic gluconeogenesis.

To identify small molecule modulators of the circadian clock, we analyzed the effect of a library of ~60,000 compounds on circadian rhythms of human osteosarcoma U2OS cell lines harboring a *Bmal1-dLuc* luciferase reporter (14). Among molecules that lengthened the period of luminescence rhythms, three carbazole derivatives (KL001-003, Fig. 1A) had pronounced effects. Continuous treatment with these compounds caused period lengthening and amplitude reduction in a dose-dependent manner in stable U2OS reporter cell lines harboring *Bmal1-dLuc* or *Per2-dLuc* (Figs. 1B, 1C, and S1). Additionally, treatment of cells with these compounds lowered basal reporter activity in *Per2-dLuc* cells compared with that of *Bmal1-dLuc* cells, whereas longdaysin had equivalent effects on both reporter cells (Figs. 1B, 1C, and S1). High concentrations of KL001 (>50 μ M) exhibited cytotoxicity against U2OS cells (Fig. S2). We further tested the effect of KL001 on transiently transfected *Bmal1-dLuc* and *Per2-dLuc* reporters in mouse NIH-3T3 fibroblasts (Fig. S3) and on a *mPer2^{Luc}* knock-in reporter (15) in mouse SCN and lung explants (Figs. 1D and 1E). KL001 caused dose-dependent lengthening of the period as well as signal reduction of *Per2* reporters at the transcription (*Per2-dLuc*) and protein (*mPer2^{Luc}*) levels in all assays. The compound had reduced potency in the SCN explant (Fig. 1D), suggesting a difference between SCN and peripheral clocks. Although period lengthening has been linked to the inhibition of casein kinase (CK) I δ , CKI α or CK2 (9, 16), the carbazole derivatives did not affect their activities *in vitro* (Fig. S4), suggesting an alternative mechanism of action.

To identify the molecular target(s) of KL001, we used an affinity-based proteomic approach. A limited structure-activity relationship study identified a derivative of KL001 with an ethylene glycol substituent at the methanesulfonyl position that maintained period lengthening activity (KL001-linker, Fig. S5A). We therefore prepared an agarose-conjugate of KL001-linker and incubated it with U2OS cell lysate in the presence of 0, 20 or 50 μ M KL001. Proteins that bound to the affinity resin and were released in the presence of free KL001 were analyzed by liquid chromatography-tandem mass spectrometry. In two independent experiments, only CRY1 was identified as a candidate of KL001-binding protein (Table S1). Protein immunoblotting with CRY1 specific antibody (5) (Fig. S6) confirmed both the binding of CRY1 to the affinity resin and decreased binding in the presence of 20 and 50 μ M free KL001 (Fig. 2A). We further used antibodies against other core clock proteins and detected interaction of the affinity resin with CRY2, and to a much lower extent PER1, but not CLOCK. β -actin showed nonspecific binding that was not displaced by free KL001 (Fig. 2A). In extracts of human embryonic kidney HEK293T cells transiently overexpressing Flag-tagged core clock proteins, the KL001-agarose conjugate

interacted with CRY1 and CRY2, but not PER1, PER2, CLOCK or BMAL1 (Figs. 2B and S7). Purified CRY1 proteins also directly bound to the affinity resin (Fig. S8). KL001 and KL002 similarly displaced CRY1 from the affinity resin (Fig. 2C), and KL004, an analog with a weak period effect (Fig. S5B), blocked CRY1 binding less effectively (Fig. 2C). Flavin adenine dinucleotide (FAD), a cofactor of CRY, inhibited CRY1 interaction with the affinity resin when added in excess (500–5000 μ M, Figs. 2C and S9). Moreover, CRY1 proteins with mutations in the FAD binding sites (CRY1D387N and CRY1N393D) (17) interacted very weakly with the affinity resin (Fig. 2D). Thus, KL001 selectively interacts with CRY.

We analyzed the effect of KL001 on the *mPer2^{Luc}* knock-in reporter in *Cry* deficient mouse fibroblasts. KL001-mediated reduction of the *mPer2^{Luc}* intensity in wild type cells was abolished in the *Cry1/2* double knockout cells (Fig. 3A). Similarly, siRNA-mediated depletion of CRY1 and CRY2 diminished the KL001-dependent reduction of *Per2-dLuc* intensity in U2OS cells (Fig. S10). Furthermore, a mutation of the CLOCK-BMAL1-binding site, the E2 enhancer element (18), abrogated the *Per2* reporter response to KL001 in an NIH-3T3 transient transfection assay (Fig. S11). Thus, the compound likely enhances the repressive activity of CRY on the *Per2* reporter in a CRY and E2 enhancer-dependent manner.

Treatment of unsynchronized U2OS cells with KL001 reduced amounts of endogenous *Per2* mRNA in a dose-dependent manner and had almost no effect on *Bmal1* (Fig. 3B). Other CLOCK-BMAL1 target genes *Per1*, *Cry1*, *Cry2* and *Dbp* exhibited a pattern of suppression similar to *Per2* (Fig. 3B), consistent with KL001 enhancing CRY. Although amounts of PER1 protein decreased in parallel with *Per1* mRNA expression after KL001 treatment, amounts of CRY1 and CRY2 did not correlate with their mRNA expression and were increased and sustained, respectively (Fig. 3C). Thus KL001 may stabilize CRY proteins. We therefore analyzed the effect of KL001 on the half-life of CRY1 by transiently expressing a CRY1-luciferase fusion protein (CRY1-LUC) in HEK293T cells. Treatment of cells with KL001 led to a dose-dependent increase in the half-life of CRY1-LUC, but did not affect the stability of the FAD binding site mutant CRY1D387N (Fig. 3D). In HEK293 stable cell lines expressing CRY1-LUC, CRY2-LUC or LUC, treatment with KL001 and KL002 increased the half-life of CRY1 and CRY2, whereas the same concentrations of KL004 showed almost no effect (Figs. 3E and S12). The effect of each compound on CRY stability is consistent with their effects on the circadian period (Figs. 1C and S5B) and CRY1 interaction (Fig. 2C), connecting the CRY stabilization with period lengthening. Because CRY proteins are targets of an E3 ubiquitin ligase FBXL3 and degraded through the ubiquitin-proteasome pathway (19-21), we tested the effect of KL001 on CRY1 ubiquitination *in vitro* in a lysate of HEK293T cells transiently overexpressing CRY1-Flag. The compound (50 μ M) inhibited ubiquitination of CRY1 and showed only a little effect on the CRY1D387N mutant (Fig. S13). Moreover, siRNA-mediated depletion of FBXL3 in U2OS reporter cells diminished the effects of KL001 on the period and *Per2* reporter intensity without affecting longdaysin effects (Figs. 3F and S14). These results indicate that KL001 inhibits FBXL3 and ubiquitin-dependent degradation of CRY proteins and further support the selectivity of the compound.

We then utilized KL001 in combination with mathematical modeling to explore how KL001-mediated CRY stabilization results in period lengthening and to define the roles of the seemingly redundant CRY isoforms in the clock mechanism. We constructed a simple mathematical model of the PER-CRY negative feedback loop (Figs. 4A and S15A) (14). The model successfully reproduced period shortening and lengthening by dose-dependent knockdown of *Cry1* and *Cry2*, respectively (22) (Fig. S15B), and also period shortening by stabilization of cytosolic CRY2 (23) (Fig. S15C). For period lengthening by KL001-

dependent CRY stabilization, the model predicted that the stabilization occurs in the nucleus (Figs. 4B left panel and S15D). Indeed, amounts of CRY1 and CRY2 proteins were increased and sustained, respectively, in a nuclear fraction of unsynchronized U2OS cells after KL001 treatment, although amounts of PER1 were reduced (Fig. S16). Furthermore, *in silico* stabilization of nuclear CRY2 in a *Cry1* knockout background and nuclear CRY1 in a *Cry2* knockout background both caused period lengthening (Fig. 4B, middle and right panels). Consistent with this prediction, continuous treatment with KL001 lengthened the period in both *Cry1* knockout and *Cry2* knockout fibroblasts in a dose-dependent manner (Figs. 4C, S17A, and S17B). Similarly, the compound caused period lengthening in CRY1 knockdown and CRY2 knockdown U2OS cells (Fig. S17C) and in SCN explants from *Cry1* knockout and *Cry2* knockout mice (Fig. S17D). Thus, both CRY isoforms share a similar functional role in the period regulation, despite different free-running periods in their knockouts (Fig. 4A). With both CRY1 and CRY2 feedback loops intact, the nuclear CRY1/CRY2 ratio controls the period in a bidirectional manner, *i.e.*, more CRY1 causes longer periods, and more CRY2 causes shorter periods (Figs. S15B and S15C).

In the liver, CRY proteins negatively regulate fasting hormone-induced transcription of the *Pck1* and *G6pc* genes, which encode rate-limiting enzymes of gluconeogenesis (4, 5). We therefore tested the effect of KL001 on expression of these genes in mouse primary hepatocytes. KL001 repressed glucagon-dependent induction of *Pck1* and *G6pc* genes in a dose-dependent manner without affecting their basal expression (Fig. 4D). Consistent with this result, KL001 treatment repressed glucagon-mediated activation of glucose production (Fig. 4E). This repression was specific, because basal glucose production (Fig. 4E) and cellular lactate dehydrogenase activity (Fig. S18) were unaffected. Altogether our results demonstrate the potential of KL001 to control fasting hormone-induced gluconeogenesis. Given that human genome-wide association studies identified an association of the *CRY2* gene locus with fasting blood glucose concentrations and presentation of type 2 diabetes (24, 25), KL001 may provide the basis for a therapeutic approach for diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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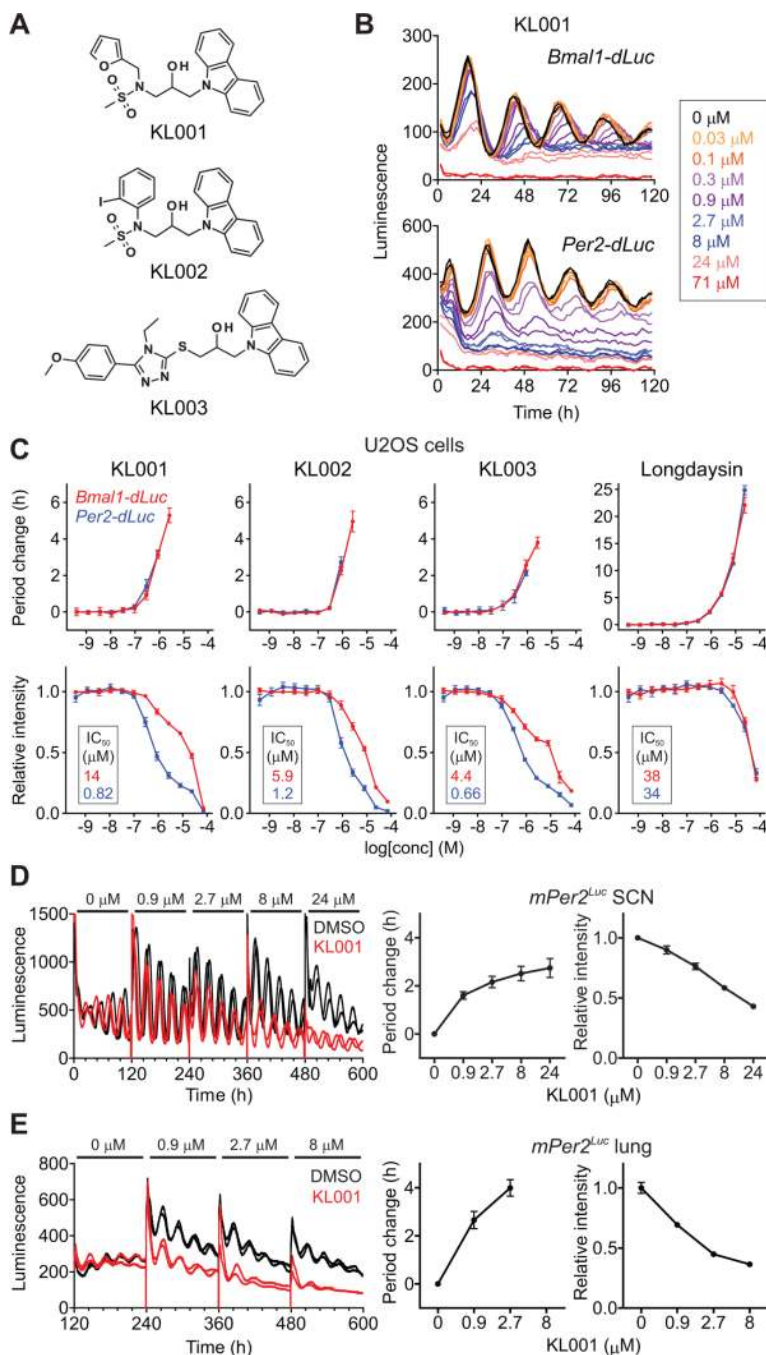


Fig. 1. Carbazole derivatives lengthen the circadian period. **(A)** The chemical structure of carbazole derivatives. **(B and C)** Luminescence rhythms of *Bmal1-dLuc* and *Per2-dLuc* reporter U2OS cells were monitored in the presence of various concentrations of compound. Representative traces ($n = 2$) are shown in (B). The changes of period and luminescence intensity (an average of 24–120 h; IC₅₀ values are indicated in insets of bottom panels) relative to DMSO control are shown in (C) as mean \pm SEM ($n = 4$). When arrhythmic, the period was not plotted. **(D and E)** Luminescence rhythms of *mPer2^{Luc}* knock-in reporter in mouse SCN (D)

and lung (E) explants were monitored in the presence of increasing concentration of KL001 (each for 120 h). Data are mean \pm SEM ($n = 6$ for SCN and 5 for lung).

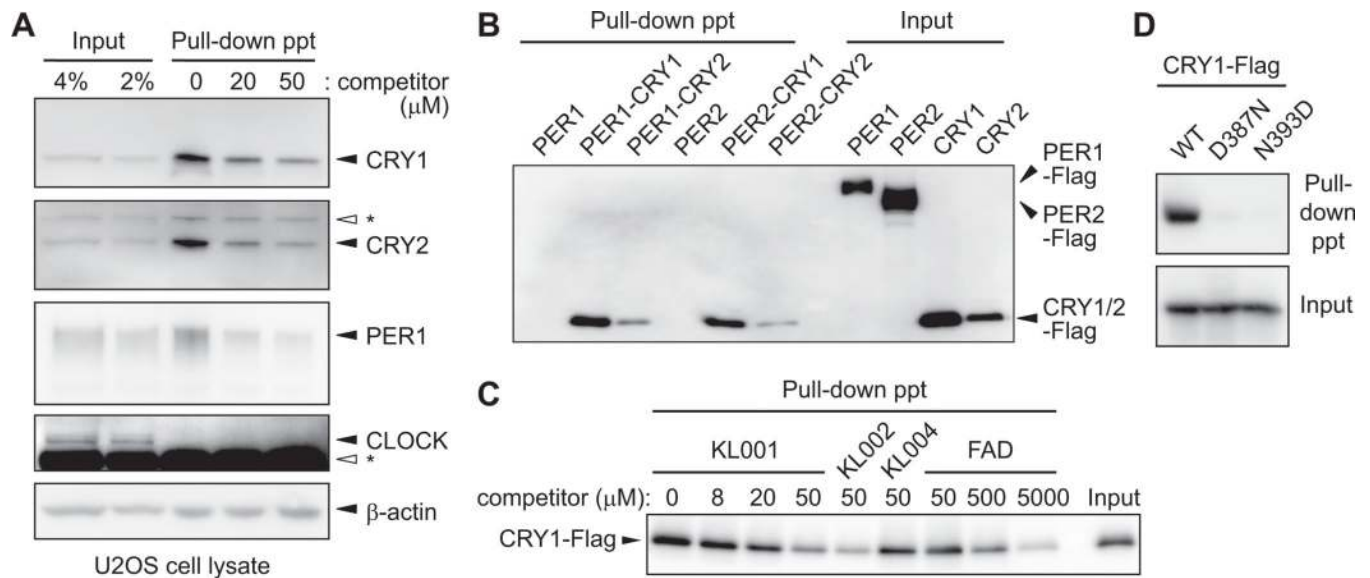


Fig. 2. KL001 interacts with CRY1 and CRY2. **(A)** Agarose-conjugated KL001-linker compound was incubated with lysate of unsynchronized U2OS cells in the presence of various concentrations of free KL001 as a competitor. Bound proteins were identified by protein immunoblotting. Asterisk indicates nonspecific band. ppt, pellet. **(B)** Flag-tagged PER or CRY was transiently overexpressed in HEK293T cells, and lysates containing PER (PER) or a mixture of lysates containing PER and CRY (PER-CRY) were subjected to the pull-down assay. Bound proteins were detected with anti-Flag antibody. **(C)** Effects of KL001, KL002, KL004 and FAD on interaction of CRY1-Flag with KL001-agarose conjugate. **(D)** Wild type (WT) or FAD binding site mutant (D387N or N393D) of CRY1-Flag was subjected to the pull-down assay.

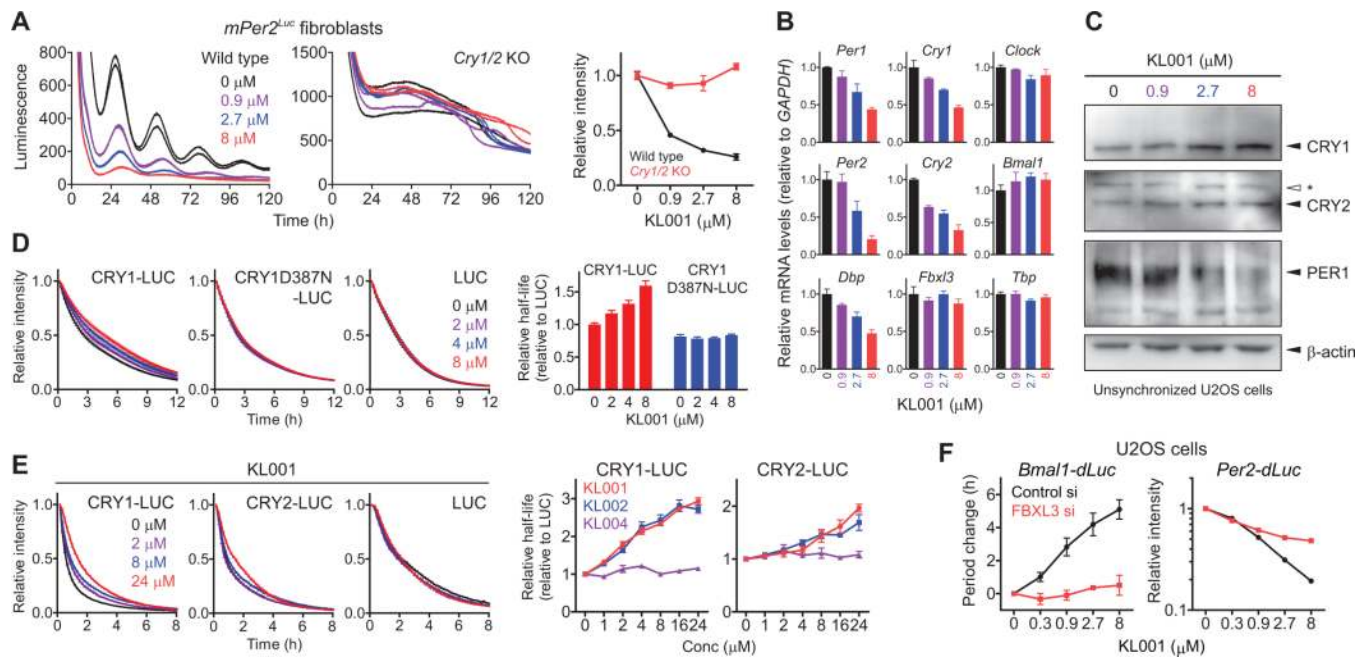


Fig. 3. KL001 stabilizes CRY proteins. **(A)** Effect of KL001 on *mPer2^{Luc}* knock-in reporter in *Cry1/2* double knockout mouse fibroblasts. Data are mean \pm SEM ($n = 4$). **(B and C)** Confluent unsynchronized U2OS cells were treated with KL001 for 48 h, and then subjected to RT-qPCR (B; mean \pm SEM, $n = 3$) or protein immunoblot (C) analysis. **(D)** Luciferase-fused CRY1 (CRY1-LUC), its D387N mutant (CRY1D387N-LUC) or luciferase (LUC) was transiently overexpressed in HEK293T cells. The cells were treated with KL001 for 24 h, and then cycloheximide was added for luminescence recording. Profiles are shown by setting peak luminescence as 1 (left panels). Half-life of CRY1-LUC or CRY1D387N-LUC relative to LUC is shown by setting CRY1-LUC 0 μ M condition as 1 (right panel). Data are mean \pm SEM ($n = 8$). **(E)** Effects of KL001, KL002 and KL004 on CRY1 and CRY2 stability in HEK293 stable cell lines expressing CRY1-LUC, CRY2-LUC or LUC. Data are mean \pm SEM ($n = 4$). **(F)** Effect of FBXL3 knockdown on the action of KL001 in *Bmal1-dLuc* or *Per2-dLuc* reporter U2OS cells. Data are mean \pm SEM ($n = 4$).

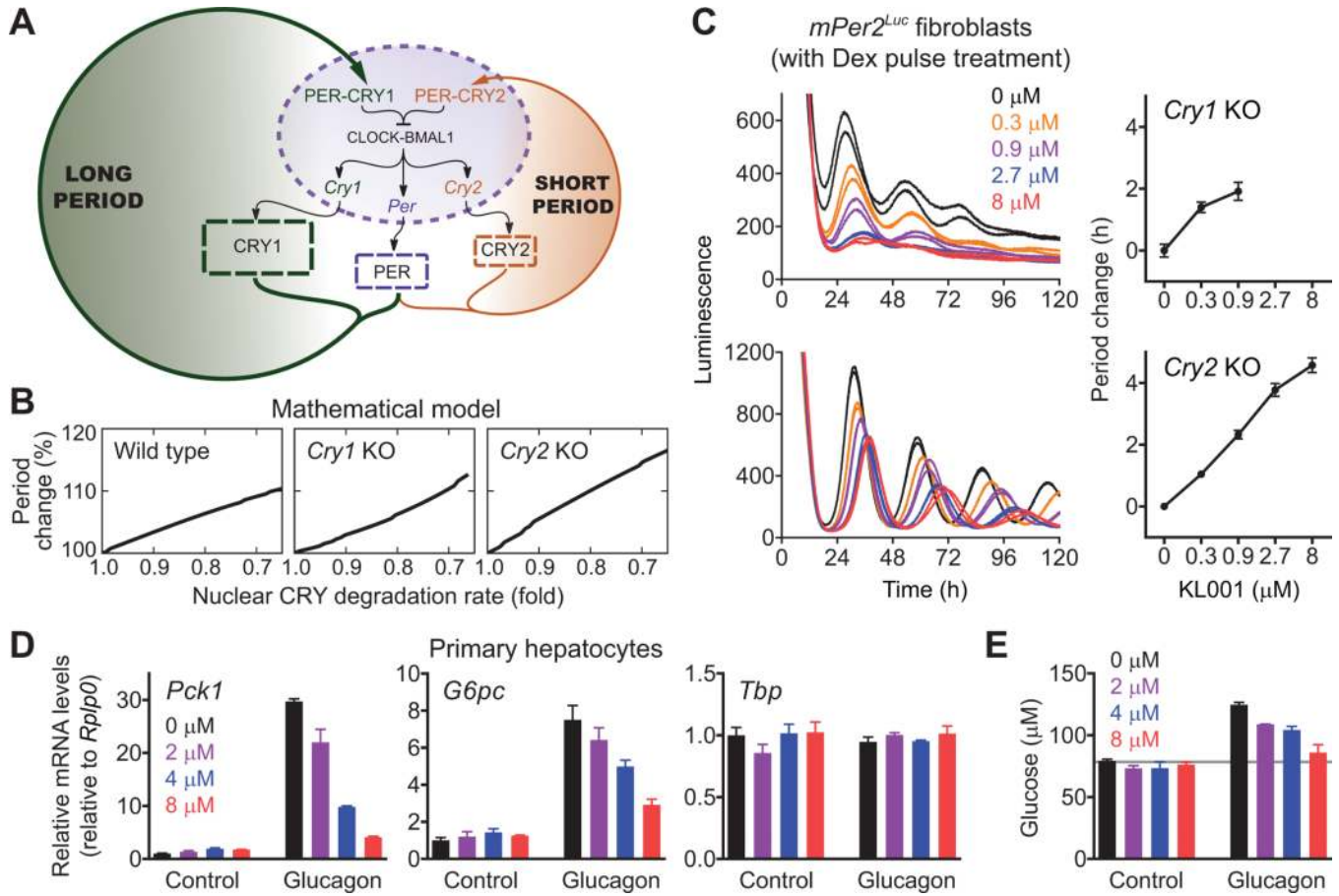


Fig. 4. Application of KL001 to define roles of CRY isoforms (A to C) and to control hepatic gluconeogenesis (D and E). (A) Scheme of the mathematical model consisting of the two parallel CRY feedback loops. (B) Effect of nuclear CRY stabilization on the period in wild type, *Cry1* knockout and *Cry2* knockout cells *in silico*. (C) *Cry1* or *Cry2* knockout *mPer2^{Luc}* knock-in mouse fibroblasts were stimulated with dexamethasone (Dex) for 2 h, and luminescence rhythms were monitored in the presence of KL001. Data are mean \pm SEM ($n = 4$). (D and E) Mouse primary hepatocytes were treated with KL001 for 18 h and then stimulated with 10 nM glucagon for 2 h (D, for RT-qPCR analysis) or 3 h (E, for glucose assay). To measure glucose production, the cells were further incubated with glucose-free buffer containing 20 mM sodium lactate and 2 mM sodium pyruvate for 4 h (E). Data are mean \pm SEM ($n = 3$).