Identification of Small Molecule Activators of Cryptochrome

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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 phenotypic 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, our studies using 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 timekeeping 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 *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, and cardiovascular and metabolic diseases (*1*–*3*). Thus, identification of clock-modulating small molecules may prove useful for the treatment of circadian-related disorders. Through cell-based 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

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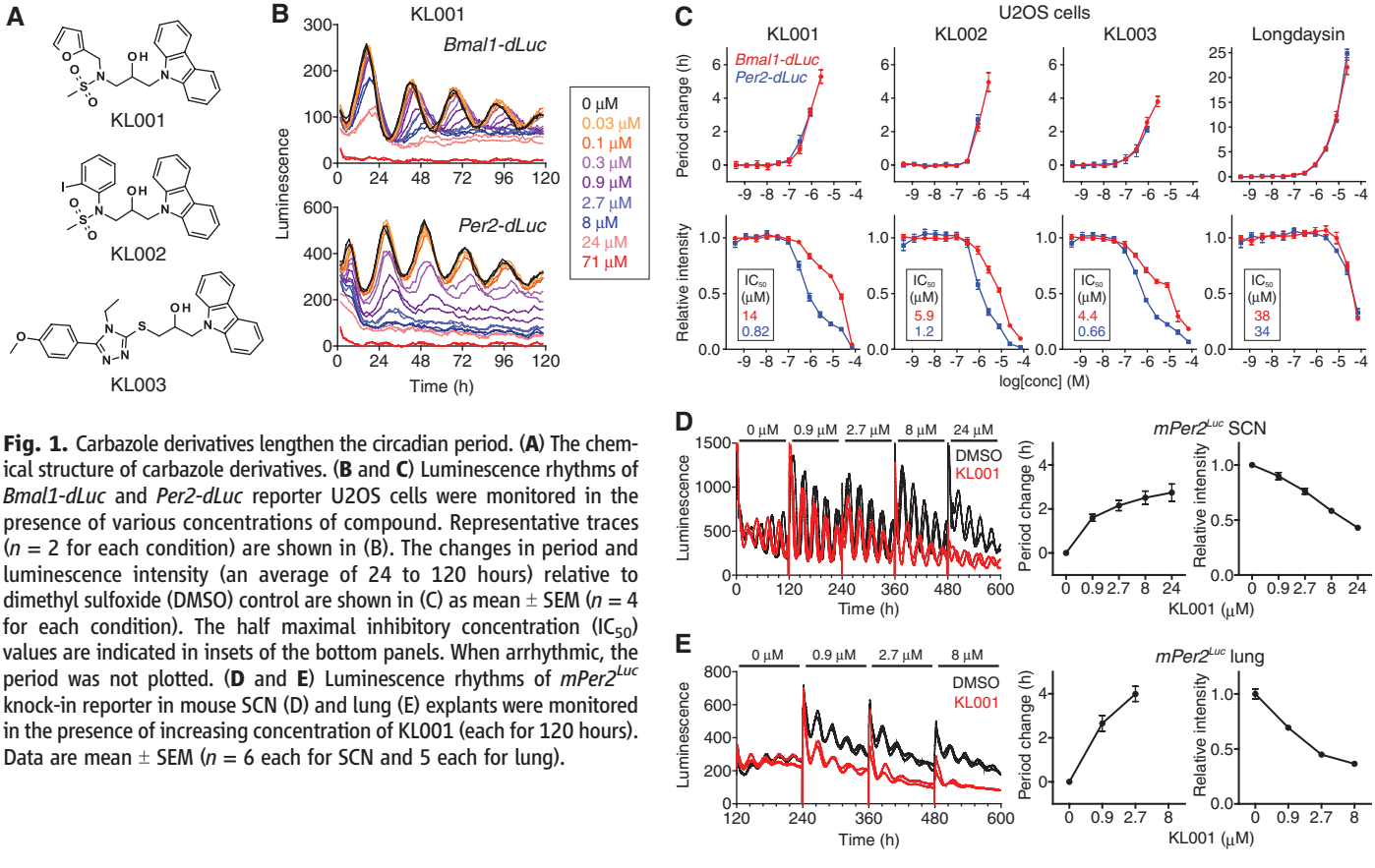

Fig. 1. Carbazole derivatives lengthen the circadian period. (**A**) The chemical structure of carbazole derivatives. (**B** and **C**) Luminescence rhythms of *Bmal1-dLuc* and *Per2-dLuc* reporter U2OS cells were monitored in the presence of various concentrations of compound. Representative traces (n = 2 for each condition) are shown in (B). The changes in period and luminescence intensity (an average of 24 to 120 hours) relative to dimethyl sulfoxide (DMSO) control are shown in (C) as mean ± SEM (n = 4 for each condition). The half maximal inhibitory concentration (IC_{50}) values are indicated in insets of the bottom panels. 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 hours). Data are mean ± SEM (n = 6 each for SCN and 5 each for lung).

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 to KL003) (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* (Fig. 1, B and C, and fig. 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 (Fig. 1, B and C, and fig. S1). High concentrations of KL001 (>50 μM) 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 explants of mouse suprachiasmatic nucleus (SCN) and lung (Fig. 1, D and E). 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

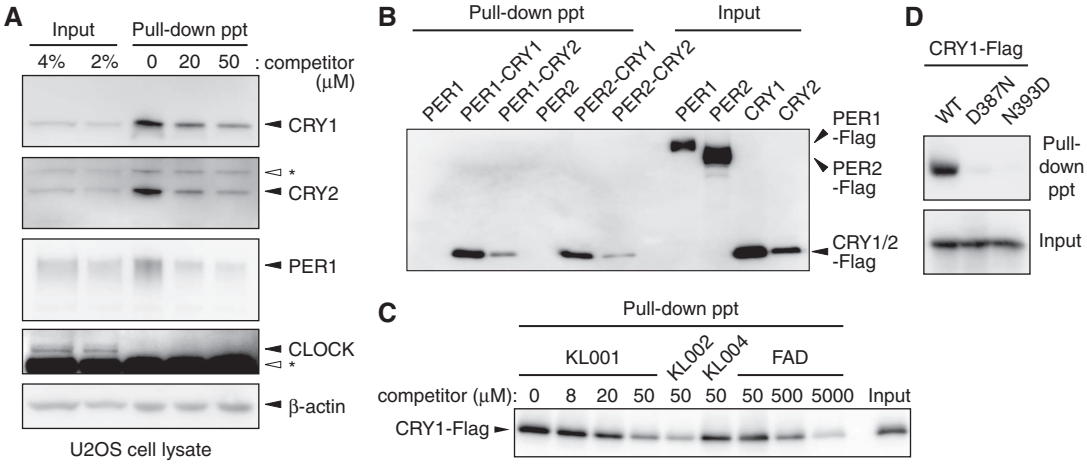

Fig. 2. KL001 interacts with CRY1 and CRY2. (**A**) Agarose-conjugated KL001-linker compound was incubated with lysate of unsynchronized U2OS cells in the presence of various concentrations of free KL001 as a competitor. Bound proteins were identified by protein immunoblotting. Asterisk indicates nonspecific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

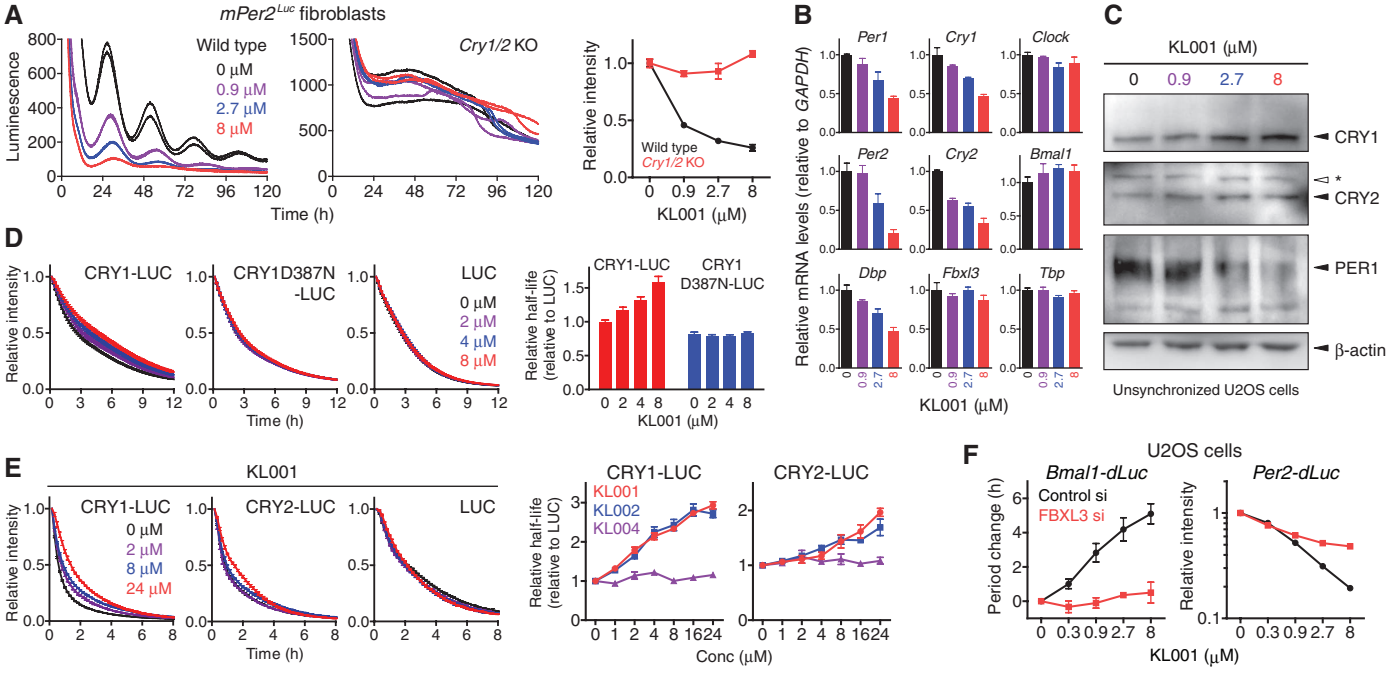

Fig. 3. KL001 stabilizes CRY proteins. (**A**) Effect of KL001 on *mPer2*^{Luc} knock-in reporter in *Cry1/2* double-knockout mouse fibroblasts. Data are mean ± SEM (n = 4 for each condition). (**B** and **C**) Confluent unsynchronized U2OS cells were treated with KL001 for 48 hours and then subjected to reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) (B; mean ± SEM, n = 3 for each condition) 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 hours, and then cycloheximide was added before 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 μM condition as 1 (right panel). Data are mean ± SEM (n = 8 for each condition). (**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 ± SEM (n = 4 for each condition). (**F**) Effect of FBXL3 knockdown on the action of KL001 in *Bmal1-dLuc* or *Per2-dLuc* reporter U2OS cells. Data are mean ± SEM (n = 4 for each condition).

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) Iδ, CKIα, 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 μM 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 μM of 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 (HEK) 293T cells transiently overexpressing Flag-tagged core clock proteins, the KL001-agarose conjugate interacted with CRY1 and CRY2, but not PER1, PER2, CLOCK, or BMAL1 (Fig. 2B and fig. 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 family proteins (*17*), inhibited CRY1 interaction with the affinity resin when added in excess (500 to 5000 μM) (Fig. 2C and fig. 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 (WT) cells was abolished in the *Cry1/2* double-knockout cells (Fig. 3A). Similarly, small interfering RNA (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 (Fig. 3E and fig. S12). The effect of each compound on CRY stability is consistent with their effects on the circadian period (Fig. 1C and fig. S5B) and CRY1 interaction (Fig. 2C),

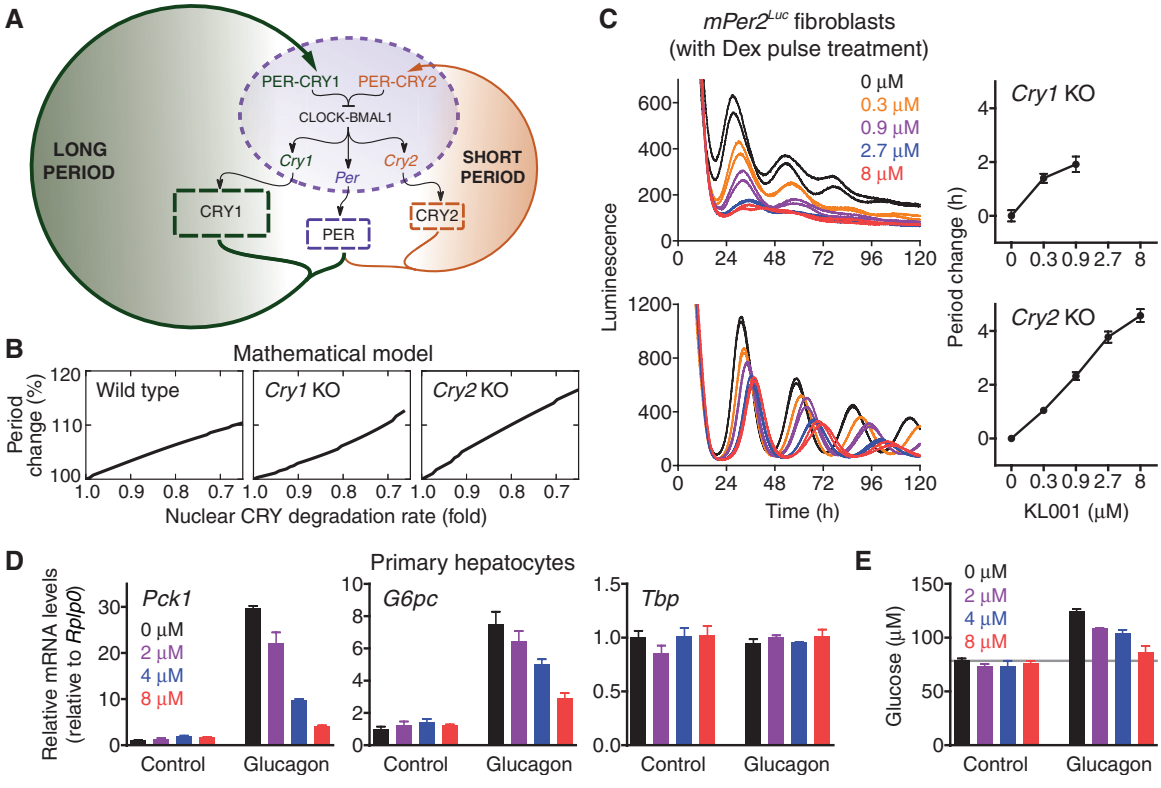

Fig. 4. Application of KL001 to define roles of CRY isoforms (A to C) and to control hepatic gluconeogenesis (D and E). (**A**) Scheme of the mathematical model consisting of the two parallel CRY feedback loops. (**B**) Effect of nuclear CRY stabilization on the period in WT, *Cry1* knockout and *Cry2* knockout cells in silico. (**C**) *Cry1* or *Cry2* knockout *mPer2*^{Luc} knock-in mouse fibroblasts were stimulated with dexamethasone (Dex) for 2 hours, and luminescence rhythms were monitored in the presence of KL001. Data are mean ± SEM (n = 4 for each condition). (**D** and **E**) Mouse primary hepatocytes were treated with KL001 for 18 hours and then stimulated with 10 nM glucagon for 2 hours (D, for RT-qPCR analysis) or 3 hours (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 hours (E). Data are mean ± SEM (n = 3 for each condition).

connecting the compound-dependent CRY stabilization with period lengthening. Because CRY proteins are targets of an E3 ubiquitin ligase complex SCF^{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 μM) 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 (Fig. 3F and fig. 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 used 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 (Fig. 4A and fig. 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 (Fig. 4B, left panel, and fig. 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 (Fig. 4C and fig. S17, A and B). 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; that is, more CRY1 causes longer periods and more CRY2 causes shorter periods (fig. S15, B and C).

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.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S18
Tables S1 to S4
References (*26*–*43*)

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Extreme Bendability of DNA Less than 100 Base Pairs Long Revealed by Single-Molecule Cyclization

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The classical view of DNA posits that DNA must be stiff below the persistence length [<150 base pairs (bp)], but recent studies addressing this have yielded contradictory results. We developed a fluorescence-based, protein-free assay for studying the cyclization of single DNA molecules in real time. The assay samples the equilibrium population of a sharply bent, transient species that is entirely suppressed in single-molecule mechanical measurements and is biologically more relevant than the annealed species sampled in the traditional ligase-based assay. The looping rate has a weak length dependence between 67 and 106 bp that cannot be described by the worm-like chain model. Many biologically important protein-DNA interactions that involve looping and bending of DNA below 100 bp likely use this intrinsic bendability of DNA.

Bending and looping of lengths of DNA below 100 base pairs (bp) is ubiquitous in cellular processes such as regulated gene expression in bacteria and eukaryotes (*1*, *2*), packaging of DNA in viral capsids, and DNA storage complexes in eukaryotes (*3*). Quantifying the intrinsic bendability of DNA at these biologically important length scales is essential for understanding DNA-protein interactions. According to a widely used approximation, DNA duplex is modeled as an elastic rod and its mechanical properties are described by the worm-like chain (WLC) model. Persistence length (l_p) is a measure of the bending rigidity of DNA; for a DNA

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