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Graphical Abstract
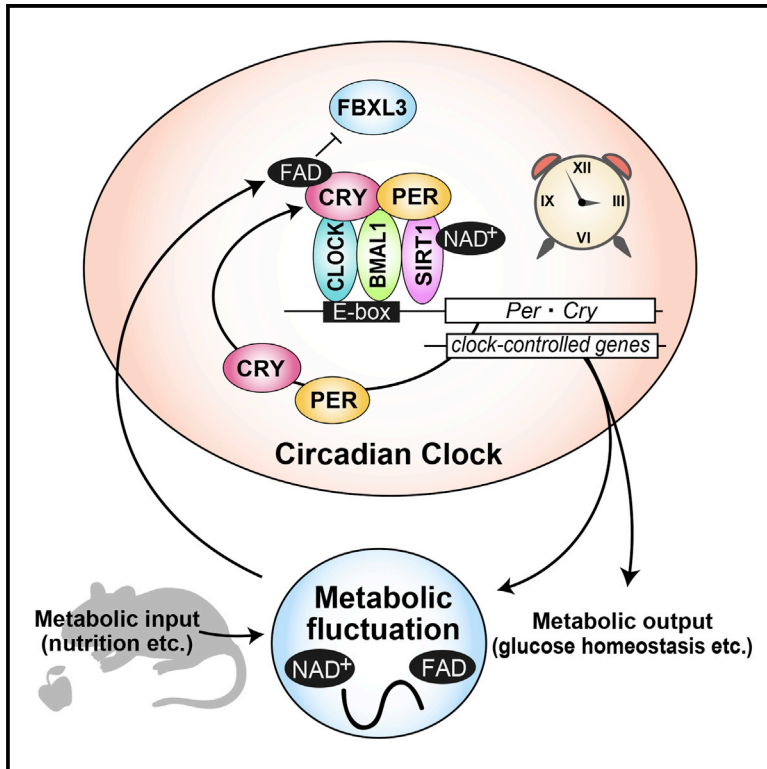
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In Brief

Hirano et al. examine a mechanism in which a co-factor, FAD, regulates CRYPTOCROME proteins and circadian regulation of metabolism. FAD stabilizes CRY proteins by competing with the CRY ubiquitin E3 ligase. Knockdown of *Rfk*, a biosynthetic enzyme of FAD, alters the expression profiles of CRY and clock-controlled metabolic genes.

Highlights

- FAD stabilizes CRYPTOCHROME (CRY) proteins by competing with FBXL3

- FAD concentration in the nucleus has a daily rhythm

- FAD lengthens the circadian period

- In vivo knockdown of *Riboflavin kinase* (*Rfk*) alters CRY and PER1 expression rhythms

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FAD Regulates CRYPTOCHROME Protein Stability and Circadian Clock in Mice

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SUMMARY

The circadian clock generates biological rhythms of metabolic and physiological processes, including the sleep-wake cycle. We previously identified a missense mutation in the flavin adenine dinucleotide (FAD) binding pocket of CRYPTOCHROME2 (CRY2), a clock protein that causes human advanced sleep phase. This prompted us to examine the role of FAD as a mediator of the clock and metabolism. FAD stabilized CRY proteins, leading to increased protein levels. In contrast, knockdown of *Riboflavin kinase* (*Rfk*), an FAD biosynthetic enzyme, enhanced CRY degradation. RFK protein levels and FAD concentrations oscillate in the nucleus, suggesting that they are subject to circadian control. Knockdown of *Rfk* combined with a riboflavin-deficient diet altered the CRY levels in mouse liver and the expression profiles of clock and clock-controlled genes (especially those related to metabolism including glucose homeostasis). We conclude that light-independent mechanisms of FAD regulate CRY and contribute to proper circadian oscillation of metabolic genes in mammals.

INTRODUCTION

Circadian rhythms are observed in organisms across the phylogenetic spectrum and are recognized in many biological functions from the molecular level through behavior (Schibler and Sassone-Corsi, 2002). Rhythms with approximately 24 hr periodicity are governed by the internal time-keeping system, the circadian clock. At its most basic level, the cell-autonomous circadian clock is constituted by a transcriptional/translational negative feedback loop, in which activators (CLOCK/BMAL1) and repressors (PERIOD1–3 [PER1–3]/CRYPTOCHROME1–2 [CRY1–2]) are coordinately regulated (Hardin and Panda, 2013; Reppert and Weaver, 2002). We have shown that mutations in some of the core clock genes (*PER2*, *PER3*, *Casein kinase Iδ*, *CRY2*, *DEC2*) affect the timing of sleep-wake behavior or sleep

duration in humans (He et al., 2009; Hirano et al., 2016b; Jones et al., 1999; Toh et al., 2001; Xu et al., 2005, 2007; Zhang et al., 2016). Gene targeting or mutations of the clock genes in rodent models cause circadian phenotypes but also, in some cases, lead to metabolic dysfunction (Doi et al., 2010; Turek et al., 2005; Marcheva et al., 2010; Rudic et al., 2004; Shimba et al., 2011).

The circadian clock is present in many peripheral tissues, playing important roles in local metabolism (Asher and Sassone-Corsi, 2015; Hirota and Fukada, 2004; Panda and Hogenesch, 2004; Schibler and Sassone-Corsi, 2002). It has been widely accepted that cellular metabolism is under the control of the circadian clock. Indeed, a majority of metabolites display daily oscillation of tissue and serum concentrations (Dallmann et al., 2012; Eckel-Mahan et al., 2012; Fustin et al., 2012; Hatori et al., 2012). These metabolites then feed back to the circadian clock by modulating enzymatic activity. Recent studies demonstrate that redox homeostasis is interconnected with the circadian clock. Cellular redox state represented by the relative ratio of flavin adenine dinucleotide (FAD) (oxidized)/NADPH (reduced) oscillates in the rodent suprachiasmatic nucleus (SCN) where the master clock resides (Wang et al., 2012). In mouse liver, circadian rhythmicity of oxidative function of mitochondria is observed (Kil et al., 2015; Peek et al., 2013). Furthermore, it was demonstrated that even without a functional transcriptional/translational negative feedback loop, the circadian oscillation of the redox state persists in peripheral clocks of various organisms (Edgar et al., 2012; O'Neill and Reddy, 2011; O'Neill et al., 2011). The involvement of NAD^+ in circadian regulation has been well characterized (Berger and Sassone-Corsi, 2016; Rutter et al., 2001). The concentration of NAD^+ and a rate-limiting enzyme of the biosynthetic pathway of NAD^+, nicotinamide phosphoribosyltransferase (NAMPT), cycle in a circadian manner (Nakahata et al., 2009; Ramsey et al., 2009). NAD^+ is a classic cofactor critical for enzymatic activity of SIRT1 (NAD^+-dependent histone deacetylase) in various species (Blander and Guarente, 2004; Imai et al., 2000; Landry et al., 2000; Rosenberg and Parkhurst, 2002; Tanny et al., 1999). Thus, SIRT1 activity is directed rhythmically by NAD^+, resulting in modulation of circadian transcription (Nakahata et al., 2008). In parallel, another group reported that SIRT1-mediated deacetylation of PER2 protein leads to its stabilization (Asher et al., 2008).

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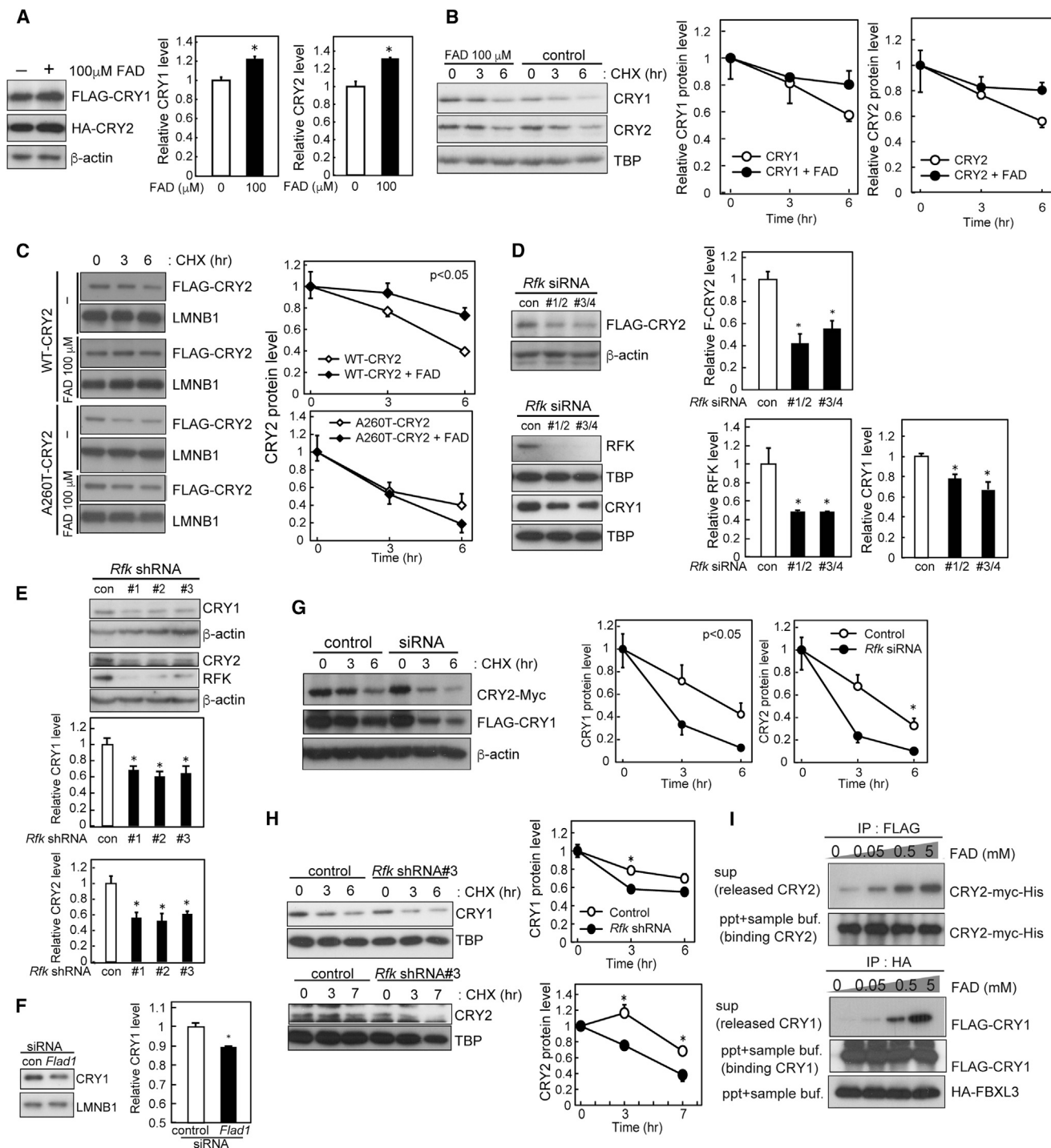
Figure 1. FAD Stabilizes CRY Protein

(A) Effect of FAD on CRY1 and CRY2 protein levels in HEK293 total cell lysates. Forty-two hours after transfection, HEK293 cells were treated with 100 μM FAD for 6 hr. Data are shown as means with SEM (n = 3, *p < 0.05 by Student's t test).

(B) Degradation assay of endogenous CRY1 and CRY2 protein in NIH 3T3 cells. The cells were pre-treated with 100 μM FAD (or PBS as a control) for 6 hr and then treated with 100 μg/mL cycloheximide (CHX) and/or 100 μM FAD. Cells were fractionated, and nuclear extracts were used for western blotting analysis. CRY protein levels at the starting point (t = 0 hr) were normalized to 1. Data are shown as means with SEM.

(C) Degradation assay of WT-CRY2 and A260T-CRY2 protein in HEK293 cells. Forty-two hours after transfection, HEK293 cells were pre-treated with FAD for 6 hr and then treated with 100 μg/mL CHX and/or 100 μM FAD. Cells were fractionated. CRY protein levels in the nuclear fraction at t = 0 hr were normalized to 1. Data are shown as means with SEM (n = 3). Significance (p < 0.05) was determined by two-way ANOVA.

Compared to NAD$^+$, the physiological importance of FAD has been ignored especially in the mammalian circadian clock. The major function of FAD is as a co-factor of flavoprotein catalyzing metabolic reactions (Powers, 2003). By switching its redox state (FAD $\rightleftharpoons$ FADH$_2$), an electron is transferred to or from the flavoprotein, thus modifying its activity. FAD also acts as a chromophore of photosensing proteins including CRY in plants and *Drosophila* (Oztürk et al., 2007). In plants and flies, FAD bound to CRY protein detects blue light and contributes to signal transduction pathways generating responses to light signals. In the mammalian molecular clock, CRY1 and CRY2 possess transcriptional repressor activity (Kume et al., 1999). Knockout of *Cry1* and *Cry2* in mice leads to behavioral rhythms being completely abolished (van der Horst et al., 1999; Vitaterna et al., 1999). Thus, the light-independent function of CRYs has been highlighted. We recently showed that a missense mutation in CRY2 causing amino acid conversion from Ala260 to Thr is responsible for familial advanced sleep phase (FASP) (Hirano et al., 2016b), which is a sleep trait characterized by early sleep and wake times (Jones et al., 1999). The Ala260 residue resides in the FAD binding pocket of CRY2 (Hitomi et al., 2009). FBXL3 (a CRY2 E3 ubiquitin ligase) binds competitively to the same pocket and promotes ubiquitylation of CRY proteins (Xing et al., 2013). The mouse model harboring the human CRY2 FASP mutation (A260T) recapitulated the human phenotype of advanced activity onset and offset. Although we found that these mutant mice exhibit altered light entrainment, whether this is a result of direct light sensing remains unclear (Hirano et al., 2016b). Thus, our study of the human mutation suggested that FAD may be a key molecule in the circadian clock through CRY regulation in vivo.

Here, we demonstrate that FAD stabilizes CRY proteins. A FAD synthetic enzyme (riboflavin kinase) oscillates in the nucleus, resulting in rhythmic FAD levels. In vivo knockdown of *Rfk*, Riboflavin (vitamin B2) kinase essential for FAD synthesis, altered the expression rhythms of CRY1, CRY2, and PER1. Expression levels of some metabolic genes were also affected by *Rfk* knockdown. These data demonstrate that cycling of cellular FAD ensures the proper oscillation of CRY expression and contributes to the overall regulation of cellular metabolism.

RESULTS

FAD Stabilizes CRY1 and CRY2

Previously, we demonstrated that FAD increased CRY2 protein levels in cell culture (Hirano et al., 2016b). We predicted that the effect of FAD on protein levels is conserved in CRY1 and 2 since the amino acid sequence of the FAD binding domain is well conserved. Similar to CRY2, treatment of cultured cells with FAD increased CRY1 (Figure 1A). A cycloheximide chase experiment suggested that the protein stability of endogenous CRY1 and CRY2 is also increased by FAD treatment in the nucleus, although they did not reach statistical significance (Figure 1B). CRY2 carrying the A260T mutation in the FAD binding pocket was less stable versus wild-type (WT) CRY2 in the nucleus (Figure 1C), as previously shown (Hirano et al., 2016b). Of note, mutant CRY2 was not stabilized by FAD treatment, while WT-CRY2 was (Figure 1C). This indicated that proper binding of FAD to CRY is needed for the stabilization.

FAD synthesis is a two-step reaction. Riboflavin (vitamin B2), a precursor of FAD, is first phosphorylated by Riboflavin kinase (RFK) and converted to flavin mononucleotide (FMN). FMN is then adenylated by FAD synthase (FADS, encoded by *Flad1* gene) to form FAD. RFK is thought to be the rate-limiting enzyme of the FAD biosynthetic pathway (Kohlmeier, 2003). We first knocked down the *Rfk* gene using small interfering RNAs (siRNAs) in NIH 3T3 cells to examine the effect of reduced FAD levels on CRY proteins. *Rfk* knockdown decreased RFK protein levels as expected. In addition, CRY2 (Figure 1D, upper) and CRY1 (Figure 1D, bottom) protein levels were also reduced by *Rfk* knockdown. Cells stably expressing small hairpin RNA (shRNA) targeting *Rfk* showed decreased endogenous CRY1 and CRY2 levels (Figure 1E). Similarly, reduction of CRY1 was observed by knockdown of the *Flad1* gene, supporting the effect of FAD deficiency (Figure 1F). Moreover, the degradation of CRY1 and CRY2 was enhanced in NIH 3T3 cells either transiently transfected with siRNAs (Figure 1G) or stably expressing shRNA for *Rfk* (Figure 1H). These results revealed that reduced FAD can promote the degradation of CRY1/2, leading to reduced protein levels.

Structural studies suggested that FAD interferes with the function of FBXL3 by competing with FBXL3 for binding to CRY2 (via the FAD binding pocket) (Nangle et al., 2013; Xing et al., 2013). We and another group previously showed that FAD competitively released CRY2 from CRY2-FBXL3 complex in vitro (Hirano et al., 2016b; Xing et al., 2013). We also found that this competitive function of FAD is significantly reduced by the CRY2 A260T mutation (Hirano et al., 2016b). Similarly, CRY1 is also released from CRY1-FBXL3 complexes by the presence of FAD in vitro in a dose-dependent manner (Figure 1I). Together, these results indicated that FAD competes with FBXL3 and increases stability of both CRY1 and CRY2 in cultured cells.

(D) CRY and RFK expression in NIH 3T3 cells transfected with *Rfk* siRNAs. Overexpressed FLAG-CRY2, endogenous CRY1 and RFK protein levels were analyzed by western blotting (n = 3, *p < 0.05 by Dunnett's test).

(E) CRY1 and CRY2 expression in NIH 3T3 cells stably expressing *Rfk* shRNAs (n = 3, *p < 0.05 by Dunnett's test).

(F) CRY1 expression in NIH 3T3 cells transfected with *Flad1* siRNAs (n = 3, *p < 0.05 by Student's t test).

(G) Degradation assay of CRY1 and CRY2 protein in *Rfk* knockdown cells. NIH 3T3 cells were transfected with *Rfk* siRNA and CRY expression vectors. Forty-two hours after transfection, the cells were treated with 100 μg/mL CHX. Statistical significance (*p < 0.05) was determined by two-way ANOVA followed by post hoc test).

(H) Degradation assay of endogenous CRY1 and CRY2 protein in NIH 3T3 cells stably expressing *Rfk* shRNAs. Statistical significance (*p < 0.05) was determined by two-way ANOVA followed by post hoc test).

(I) FBXL3 competition assay. FLAG-CRY1-HA-FBXL3 or Myc-CRY2-FLAG-FBXL3 complexes expressed in HEK293 cells were purified using HA or FLAG antibody. FAD was added to CRY-FBXL3 complexes and incubated at 4°C for 2 hr in vitro.

A

B

C
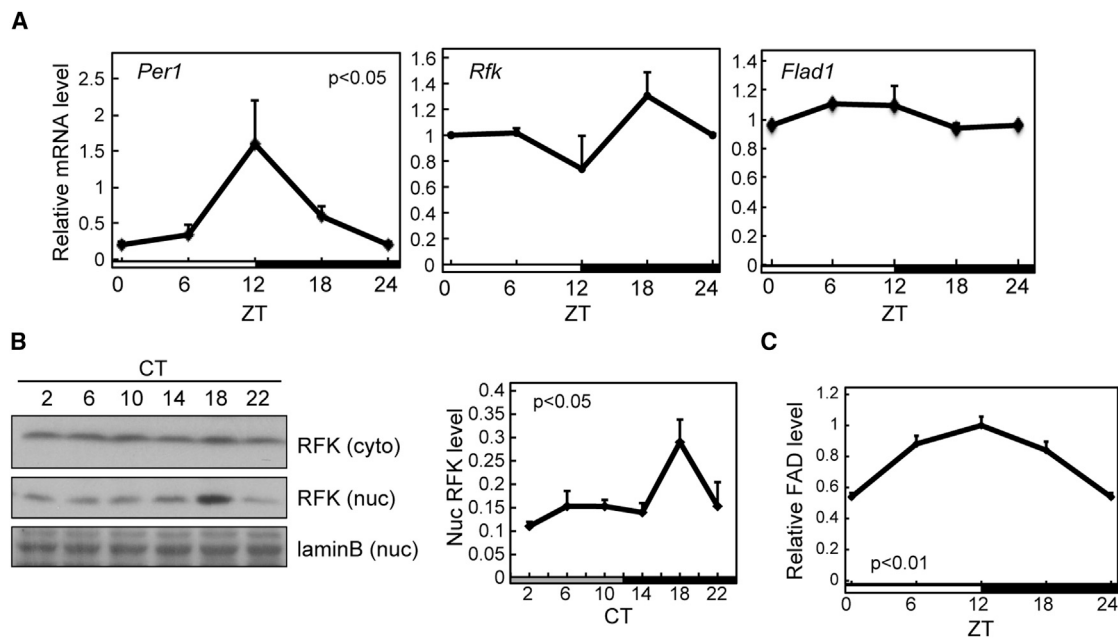
Figure 2. Rhythmic Expression of Components of the FAD Synthetic Pathway

(A) Temporal expression profiles of *Per1* (positive control), *Rfk*, and *Flad1* in mouse liver. C57BL/6J mice entrained to LD 12:12 were sacrificed every 6 hr. mRNA levels were normalized to *G3pdh*.

(B) Temporal expression profiles of RFK in mouse liver in DD. For DD, mice were sacrificed every 4 hr on the second day in DD. Liver was fractionated into nuclear and cytosolic fractions. Data are shown as means ± SEM (n = 3). Statistical significance (p < 0.05) was determined by one-way ANOVA.

(C) Relative FAD amounts in the mouse liver nucleus. Data are shown as means ± SEM (n = 3).

FAD Oscillates in the Nucleus

We then examined the expression rhythms of genes encoding enzymes critical for FAD biosynthesis. *Rfk* and *Flad1* mRNAs did not show significant expression rhythms (Figure 2A). On the other hand, we observed that nuclear RFK protein levels peaked in the middle of the night (CT18) in constant darkness (Figure 2B). Consistent with this, the nuclear concentration of FAD shows clear daily rhythms (Figure 2C) with the peak time at ZT12. These results suggested that FAD plays a time- and location-dependent role to regulate CRY stability by competing with FBXL3, which is supported by the fact that FBXL3 is predominantly localized in the nucleus (Godinho et al., 2007; Hirano et al., 2013; Yoo et al., 2013). Indeed, the peak time of FAD oscillation (around ZT12) coincides with the time when CRY1 and CRY2 protein start accumulating in liver, implying that FAD contributes to this accumulation.

FAD Affects the Circadian Period in Cell Culture

The stability of CRY1 and CRY2 significantly affects the circadian period at molecular and behavioral levels (Godinho et al., 2007; Hirano et al., 2014, 2016a; Ode et al., 2016; Siepka et al., 2007). Additionally, the FASP mouse model expressing A260T-CRY2 protein exhibits shorter circadian period of both behavioral rhythms and cellular rhythms in peripheral tissues (Hirano et al., 2016b). To examine the effect of FAD on the cellular clock, we analyzed PER2::LUC bioluminescence rhythms in the presence of FAD. FAD treatment lengthened the circadian period in a dose-dependent manner (Figure 3A). This is consistent with

the previous finding that inhibition of FBXL3 increased CRY protein stability, thus lengthening the circadian period (Godinho et al., 2007; Hirota et al., 2008; Siepka et al., 2007). In agreement with these observations, we found that CRY1 and CRY2 protein accumulated to higher levels when NIH 3T3 cells were treated with FAD after being synchronized by dexamethasone (DEX) (Figure 3B).

Riboflavin Deficiency and In Vivo Knockdown of *Rfk* Led to Lower CRY Levels

To investigate the in vivo role of FAD, we analyzed the protein expression rhythms in liver of *Rfk* knockdown mice. We prepared mice by entraining them to light-dark (LD) 12:12 with free access to normal chow or riboflavin (vitamin B2)-deficient chow. Subsequently, mice underwent tail vein injection with an *Rfk* siRNA to knock down *Rfk* in liver (Figure 4A). We found that 2 weeks of access to a riboflavin-deficient diet did not cause reduction of CRY levels, at least at ZT18 (Figure 4B, control). However, when subjected to a riboflavin-deficient diet, knockdown of *Rfk* decreased CRY1 and CRY2 at ZT18 (Figure 4B). Knockdown by *Rfk* siRNA was successful in mice whether they were maintained on a diet of normal or riboflavin-deficient chow. Next, we asked how FAD deficiency affects other clock proteins in constant darkness. Similar to the LD cycle, CRY protein levels were decreased at CT18. The effect of *Rfk* knockdown seemed time dependent, and accumulation of nuclear CRY proteins (from CT6 to CT18) was slow in the knockdown mice. Of note, chronic FAD treatment also disrupted the expression rhythms of CRY1 and
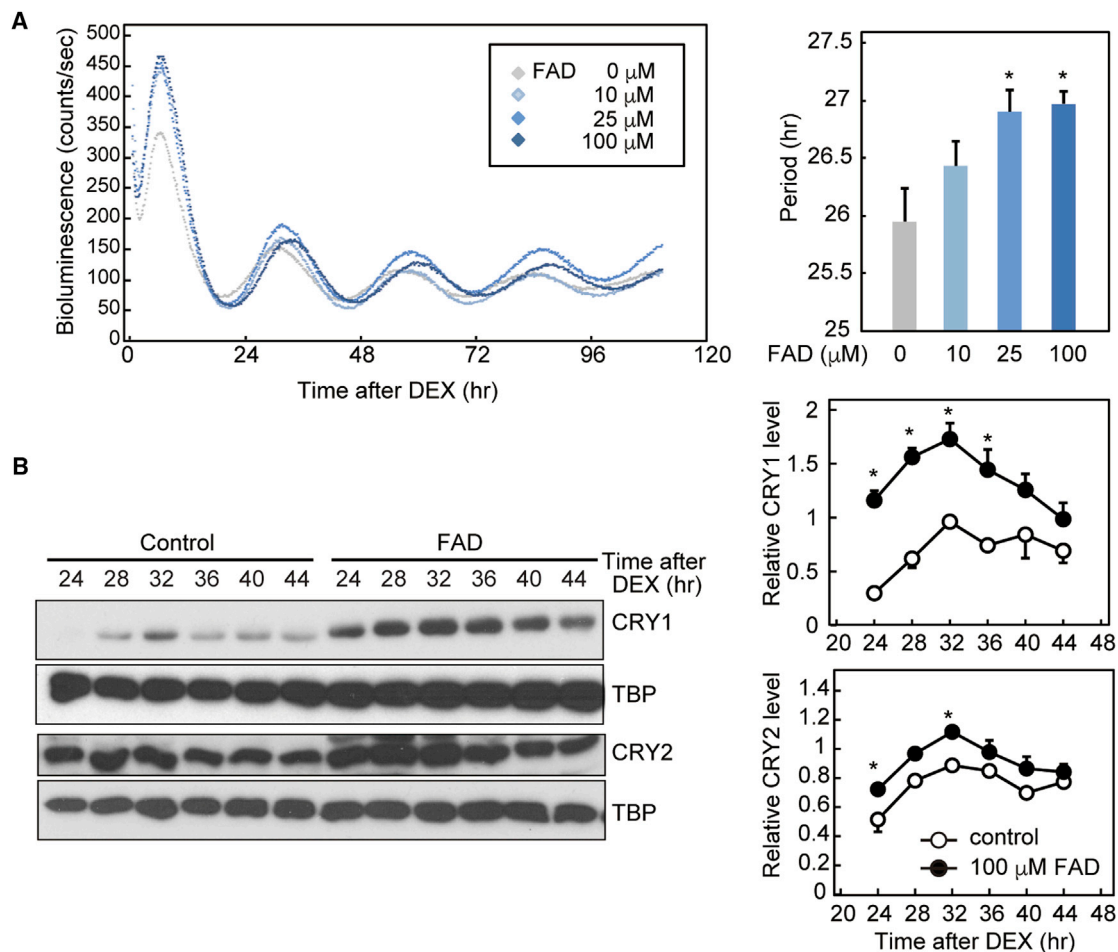
Figure 3. Cellular Rhythms of PER2::LUC MEFs Treated with FAD

(A) Representative rhythms of PER2::LUC bioluminescence in MEFs from $Per2^{Luc}$ knockin mice. Cellular rhythms of MEFs were synchronized by 100 nM DEX. Media was replaced by a recording media containing 10−100 μM FAD (or PBS) and 100 μM luciferin for recording of bioluminescence. Period lengths of the bioluminescence rhythms are shown as means ± SEM (n = 4, *p < 0.05 by Tukey's test).

(B) NIH 3T3 cells were synchronized by 100 nM DEX. Media was replaced by fresh media containing 100 μM FAD (or PBS). Total cell lysates were used for western blotting analysis. Data are shown as means ± SEM (n = 3, *p < 0.05 by two-way ANOVA followed by post hoc test).

CRY2 in NIH 3T3 cells (Figure 3B). Together, these results suggest a role for FAD in regulating expression rhythms of CRY. Interestingly, PER1 protein was also decreased by *Rfk* knockdown at CT12 and 18 (Figures 4C and 4D). Similar alterations were detected in cell-culture experiments, where knockdown of *Rfk* and *Flad1* decreased PER1 and CRY1 protein levels (Figure S1A).

**Expression of Clock Genes Is Altered by *Rfk* Knockdown**

In addition to the effects of CRY1 and CRY2 in regulating expression of clock genes, they are also thought to regulate expression of many metabolism-related genes (Oishi et al., 2003). A recent chromatin immunoprecipitation sequencing (ChIP-seq) study indicated that CRY rhythmically occupies not only E-boxes, but also nuclear receptor response elements to control gene transcription (Koike et al., 2012). In *Rfk* knockdown mice maintained on a riboflavin-deficient diet, gene expression was significantly decreased for *Per1* (consistent with reduced PER1 protein levels

seen in liver extracts) and other clock genes (Figures 4D and 4E). Decreased accumulation of CRY1/2 proteins around CT12–18 and reduction of clock gene expression were also observed by another siRNA targeting *Rfk* (Figures S1B and 1C). Among clock components, we found that *Per1* mRNA and protein levels were significantly affected by FAD depletion. As *Per1* is a target gene of CRY1, we performed a ChIP assay using CRY1 antibody in the knockdown mice. As shown in the previous ChIP-seq study (Koike et al., 2012), the CRY1 ChIP signal at the *Per1* promoter E-box is highest at CT0 (Figure S1D). The DNA binding of CRY1 was significantly decreased by *Rfk* knockdown, suggesting that FAD depletion altered CRY transcriptional activity.

The Effect of FAD Deficiency on Metabolic Pathways

We next examined the transcript levels of a series of genes related to metabolism in liver (Figures 5A and S2A). Glucagon promotes gluconeogenesis, and the expression of glucagon-inducible genes is inhibited by CRY protein through suppression
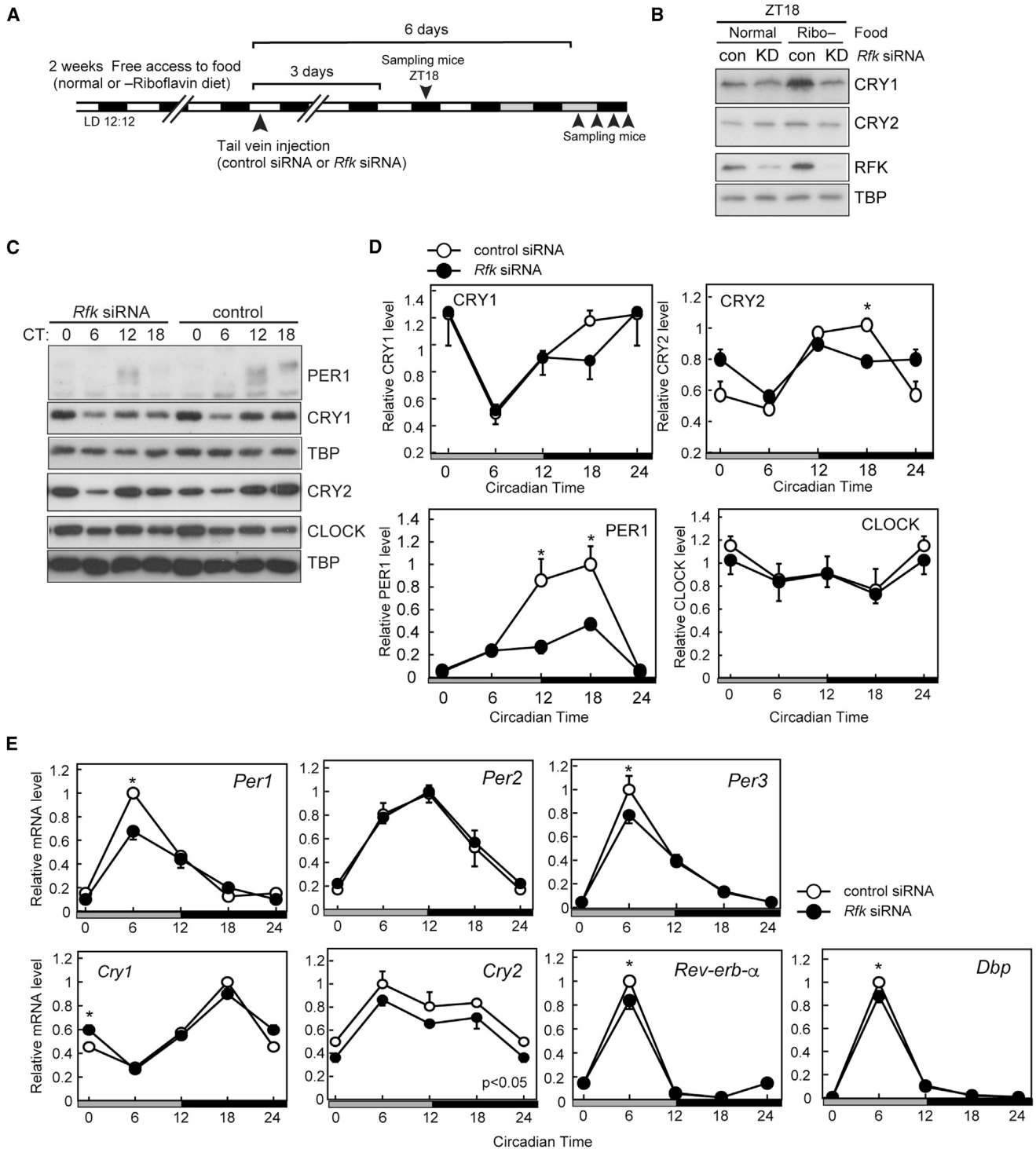
Figure 4. Effect of *Rfk* Knockdown on Clock Proteins in Mouse Liver

(A) Experimental scheme for in vivo knockdown of the *Rfk* gene. Mice were fed normal or riboflavin-deficient chow for 2 weeks. *Rfk* siRNA or control siRNA was injected into mice via the tail vein at ZT10–12. Three days or 6 days after the injection, mice were sacrificed to collect liver tissues.

(B) Nuclear protein levels of CRY1, CRY2, and RFK at ZT18. TATA-binding protein (TBP) was used as a nuclear marker.

(C) Nuclear protein expression of PER1, CLOCK, CRY1, and CRY2 at indicated CT in DD.

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of cyclic AMP (cAMP) signaling (Zhang et al., 2010). A small synthetic compound (KL001) that stabilizes CRY was shown to reduce the response to glucagon. Thus, KL001 lowered the induction of major glucagon targets such as *glucose-6-phosphatase* (*G6pc*) and *phosphoenolpryvate carboxykinase 1, cytosolic* (*Pck1*) genes in mouse hepatocytes (Hirota et al., 2008). We found that *G6pc* expression was increased by *Rfk* knockdown at CT6 and 12 in liver extracts of mice on a riboflavin-free diet (Figure 5A), while there was no effect on *Pck1*. These data suggest that gluconeogenesis is disturbed by depletion of cellular FAD. In addition, we found that blood glucose concentrations showed a tendency to increase and that glucose levels in liver were significantly increased by *Rfk* knockdown (Figure 5B). In HepG2 cells (derived from a liver hepatocellular carcinoma), FAD treatment decreased while *Rfk* knockdown increased the oxygen consumption rate (OCR) (Figure 5C), an indicator of cellular metabolism. Enhanced OCR generally represents reduction of glycolysis. Consistent with this, FAD treatment increased the extracellular acidification rate (ECAR), an indicator of glycolysis (Figure 5D). On the other hand, *Rfk* knockdown reduced the ECAR, indicating that FAD deficiency decreased glycolysis and increased gluconeogenesis, which is promoted by *G6pc*. Of note, these effects were cancelled by knockdown of *CRY1* and *CRY2*. As shown in Figure 5C, knockdown of *Rfk* increased OCR in HepG2 cells (Figure 5E, left). Knockdown of both *CRY1/2* and *Rfk* decreased OCR rather than further elevating it (Figure 5E, right), suggesting an additional regulatory pathway(s) is involved. On the other hand, *CRY1/2* knockdown attenuated the effect of *Rfk* knockdown on ECAR (Figure 5F). A previous study also showed that knockout of *Cry1* and *Cry2* decreased glycolysis in liver (Peek et al., 2013), supporting the critical role of CRY in glycolysis. Besides glucose homeostasis, some metabolic genes such as *Pparg* (regulator of hepatic steatosis), *Acs1*, and *Scd1* (regulators of sterol metabolism) were also modulated by the knockdown, suggesting a more global effect of FAD loss on cellular metabolism (Figures 5G and S2B). Taken together, our study emphasizes that cycling FAD feeds back to the circadian clock through its regulation of CRY stability, thereby controlling metabolic pathways (Figure 5H).

DISCUSSION

In the present study, we demonstrate that FAD levels are controlled in a circadian manner (Figure 2C). The peak timing of FAD rhythms was around ZT12 when CRY1 and CRY2 proteins start to accumulate in mouse liver (Lee et al., 2001). FAD deficiency affected CRY stability and accumulation in the circadian cycle (Figures 1 and 4C), suggesting that FAD-mediated stabilization contributes to the nuclear accumulation of CRY. We also found that RFK protein levels (the rate-limiting FAD biosynthetic enzyme) in the nucleus show circadian changes (Figure 2B). However, the mechanisms for rhythmic expression

of the riboflavin transporters or enzymatic activity of RFK and FADS remain elusive. Moreover, redox homeostasis (ratio of reduced to oxidative forms of FAD) plays a critical role in the proper maintenance of cellular physiology, and recent findings revealed that daily rhythms of redox state is an integral part of circadian regulation (Edgar et al., 2012; O'Neill et al., 2011; Peek et al., 2013; Wang et al., 2012). Thus, in addition to biosynthesis, the cellular redox state can contribute to the oscillation of FAD.

The expression of clock genes and clock-controlled genes was disturbed by *Rfk* knockdown (Figures 4D, 4E, 5A, and 5G). Interestingly, genes displaying significantly altered expression also have high ChIP scores in a comprehensive CRY1 and CRY2 ChIP-seq study (Koike et al., 2012), suggesting that they are direct targets of CRY. For example, ChIP scores in the *G6pc* promoter were ranked 111th (for CRY1) and 20th (for CRY2) among all genes (>10,000) with specific ChIP signals. On the other hand, *Pck1*, another target of glucagon, was ranked 852nd (CRY1) and 1,658[th] (CRY2) by ChIP score (Koike et al., 2012) and showed no change with *Rfk* knockdown (Figure 5A). Furthermore, we found that *CRY1/2* knockdown reversed or attenuated the effect of *Rfk* silencing in OCR and ECAR assays, suggesting the involvement of CRY-FAD regulation in glucose metabolism (Figures 5E and 5F). Congruent with these data, metabolomics analysis revealed altered glucose metabolism under FAD treatment in HepG2 cells (Figure S3). However, to ensure that this is relevant in vivo, further investigation using *Cry1/2* knockout mice or mice harboring mutations in the FAD binding domain of CRY1 and CRY2 will be needed. *Per1* also scored the highest in the previous ChIP study of CRYs and, in our studies, showed significant alterations in both mRNA expression and protein levels. We observed that *Rfk* knockdown reduced DNA binding of CRY1 to a target sequence of the *Per1* promoter (Figure S1D). However the reduction of *Per1* expression by *Rfk* knockdown argues that its regulation is even more complex. Previously, we and another group reported that mutation at the FAD binding site of CRY altered CRY repressor activity (Hirano et al., 2016b; Hitomi et al., 2009). Therefore, FAD deficiency may affect CRY repressor activity, which cannot be detected by the ChIP assay.

FAD is a co-factor of CRY in various species (Lin and Todo, 2005). In plants and flies, CRY harboring FAD detects blue light and contributes to the biological response to light by changing protein conformation. In plants, *Arabidopsis* CRY regulates development and growth in response to light. *Drosophila* CRY is a circadian photosensor contributing to clock resetting in response to light. Furthermore, dCRY acts as a light-dependent magnetosensor and knockout results in loss of magnetosensation (Gegear et al., 2008, 2010). Interestingly, human CRY2 can rescue the light-dependent magnetosensation phenotype of d*Cry*-deficient flies (Foley et al., 2011). Whether CRY2 is actually involved in magnetoreception in mammalian physiology is

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(D) Quantification of PER1, CLOCK, CRY1, and CRY2 protein levels shown in (C).

Data in (C) and (D) are shown as means ± SEM (n = 3, *p < 0.05 by two-way ANOVA followed by post hoc test).

(E) mRNA levels of indicated clock genes in mouse liver at specified CT in DD. mRNA levels were normalized to *G3pdh*. Data are shown as means ± SEM (n = 3, *p < 0.05 by two-way ANOVA followed by post hoc test).
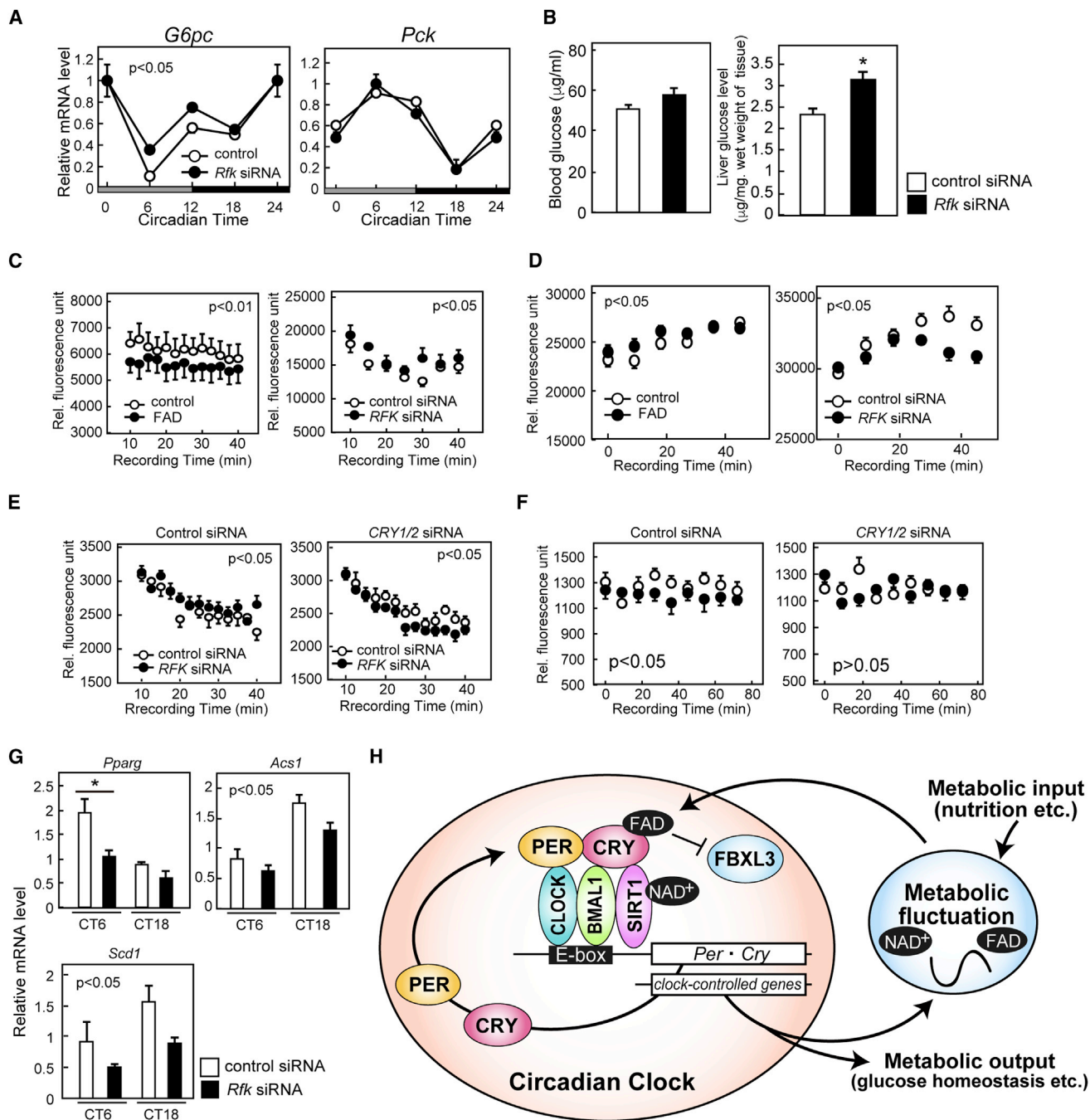
Figure 5. *Rfk* Knockdown Results in Altered Expression of Genes in Metabolic Pathways

(A) mRNA levels of *G6pc* and *Pck* in mouse liver. mRNA levels were normalized to *Tbp*. Data are shown as means ± SEM (n = 3, *p < 0.05 by two-way ANOVA).

(B) Glucose levels in blood serum (left) and liver (right) at CT12. Data are shown as means ± SEM (n = 3, *p < 0.05 by Student's t test).

(C) OCR assay in HepG2 cells. Cells were transfected with siRNA of *Rfk* or treated with 100 μM FAD for 24 hr. Data are shown as means ± SEM (n = 6). Statistical significance was determined by two-way ANOVA.

(D) ECAR assay in HepG2 cells. Cells were transfected with *Rfk* siRNA or treated with 100 μM FAD for 24 hr before recording fluorescence of a glycolysis probe. Data are shown as means ± SEM (n = 5 for FAD treatment, n = 9 for *Rfk* knockdown). Statistical significance was determined by two-way ANOVA.

(E) OCR assay in HepG2 cells transfected with siRNA of Rfk and/or *CRY1/2*. Data are shown as means ± SEM (n = 9).

(F) ECAR assay in HepG2 cells transfected with siRNA of *Rfk* and/or *CRY1/2*. Data are shown as means ± SEM (n = 9).

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unknown. On the other hand, mammalian CRYs have acquired repressor activity of transcription and are considered core components in the circadian clock. CRY repressor activity is light independent (Griffin et al., 1999), and the central clock (SCN) does not detect light directly. Thus, the link between FAD and the mammalian circadian clock in the physiological context was unclear. A recent report revealed that LSD1, a lysine-specific FAD-dependent histone demethylase, regulates the light resetting of the circadian clock through protein kinase C (PKC)-α-mediated phosphorylation of LSD1 (Nam et al., 2014). Phosphorylated LSD1 (at Ser112) interacts with CLOCK/BMAL1 complexes and modulates E-box-mediated gene expression. Knockout of *Lsd1* or knockin of a mutation at the phosphorylation site of LSD1 (Ser112 to Ala) led to decreased amplitude of gene expression in mouse embryonic fibroblasts and behavioral rhythms in mice. However, histone demethylation was intact in these *Lsd1* mutant mice. Thus, it is likely that the role of LSD1 in circadian phenotypes is independent of FAD. Furthermore, the circadian periods of these *Lsd1* mutants remain indistinguishable from normal mice. Here, we found that FAD treatment indeed modulates the circadian period (Figure 3A). The data presented in this study therefore demonstrate that FAD contributes to circadian rhythmicity by binding to CRYs and that a deficiency of FAD causes dysregulations of metabolism.

Riboflavin (vitamin B2) is an essential nutrient for mammals. Because mammals are unable to synthesize it, we depend on dietary intake of riboflavin (Powers, 2003). Deficiency of riboflavin causes abnormal development, neurodegeneration, cardiovascular disease, alterations of DNA damage repair, and cancer (Powers, 2003). Although the effect of riboflavin deficiency may act through different pathways, some of these effects may be mediated through disturbance of CRY protein levels and altered circadian regulation. Indeed, CRY proteins are highly associated with cancer development and DNA damage (Lee et al., 2013; Ozturk et al., 2009; Papp et al., 2015). In the present study, we found that administration or deletion of FAD altered glucose metabolism through CRYs (Figure 5), indicating a significant role for the FAD-CRY pathway in normal physiology.

EXPERIMENTAL PROCEDURES

Mice
All experimental protocols were conducted according to US National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. Mice were kept in cages with free access to food and water. Riboflavin-deficient chow was purchased from Research Diets.

Cell Culture and Constructs
HEK293 cells, NIH 3T3 cells, and HepG2 cells were purchased from ATTC. Authentication of the cell lines was performed by ATCC according to their protocol. Cultures were tested for mycoplasma contamination, and all cell lines used for experiments were mycoplasma free. HEK293 cells and NIH 3T3 cells were cultured in DMEM (Corning) containing 10% fetal bovine serum (FBS) and

100 U/mL penicillin-streptomycin (Thermo Fisher Scientific) and maintained by standard methods. HepG2 cells were maintained in MEM containing 10% FBS, non-essential amino acids, and 1 mM sodium pyruvate. Mouse embryonic fibroblasts (MEFs) were prepared from E12.5 embryos of $Per2^{luc}$ knockin mice (Yoo et al., 2004). After removing the head, paws, and internal organs, embryos were chopped and incubated in 0.25% trypsin in PBS for 24 hr at 4°C. After incubation for 30 min at 37°C in 0.25% trypsin in PBS, cells were dissociated by pipetting in DMEM. The supernatant was cultured in a cell-culture dish with DMEM and maintained by standard methods. Cells were transfected with Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) according to manufacturer's protocol. DNA constructs used for transfections are listed in Supplemental Experimental Procedures (Hirano et al., 2016b).

Bioluminescence Rhythms in Cell Culture
Cellular rhythms were synchronized by treatment with 100 nM DEX for 2 hr. Media was changed to the recording media: phenol-red free DMEM (Sigma-Aldrich) containing 10 mM HEPES (pH 7.0), 3.5 g/L D-glucose, and 0.1 mM luciferin potassium salt (Thermo Fisher Scientific). Bioluminescence was continuously recorded in a LumiCycle 32 instrument (Actimetrics). Bioluminescence was detrended by subtracting 24 hr average of bioluminescence using the LumiCycle analysis software to calculated periods.

Expression Profiles of Proteins and Genes
C57BL/6J male mice (~10 weeks old) were entrained to LD 12:12 for at least 10 days. For expression rhythms of RFK in DD, mice were sacrificed every 4 hr in dim red light on the second day of DD. For LD, mice were sacrificed every 6 hr. Liver tissues were collected, followed by nuclear extraction (Yoshitane et al., 2009) and mRNA extraction.

Nuclear and Cytosolic Fractionation of Cells
Cells were resuspended in the lysis buffer (10 mM HEPES-potassium hydroxide [KOH] [pH 7.8 at 4°C], 10 mM KCl, 0.1 mM EDTA, and 0.2% NP-40) containing 1 mM DTT and protease inhibitor (Thermo Fisher Scientific) and incubated for 10 min on ice. Cell membranes were disrupted by vortexing twice for 15 s, followed by centrifugation for 10 min at 20,000 × g. The pellet (nuclear fraction) was washed with the lysis buffer without NP-40 and dissolved in SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 50 mM DTT, 2% SDS, 10% glycerol).

Western Blotting
For whole-cell extracts, HEK293 cells were lysed in SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 50 mM DTT, 2% SDS, and 10% glycerol). Preparation of the cytosolic and the nuclear fractions of mouse liver was performed as previously described (Yoshitane et al., 2009). Protein samples were separated by SDS-PAGE. Tissues were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) with blocking in T-TBS (50 mM Tris-HCl [pH 7.4], 137 mM NaCl, and 0.1% Tween 20) containing 1% skim milk. Primary antibodies were reacted in the blocking solution at 4°C overnight. Then, secondary antibodies were reacted in the blocking solution at room temperature (RT) for 2 hr. Proteins were detected with the Western Lightning Plus ECL (PerkinElmer). Band intensities were determined using ImageJ software. β-actin was used as a loading control for total cell lysates, and TATA-binding protein (TBP) was used as the nuclear marker. Proteins were detected with the following antibodies: anti-cMyc 9E10 (Santa Cruz Biotechnology, sc-40), anti-FLAG M2 (Sigma-Aldrich, F1804), anti-HA Y11 (Santa Cruz Biotechnology, sc-805-G), anti-β-actin (Abcam, AC-15), anti-LMNB1 (Abcam, ab16048), anti-TBP (Santa Cruz), anti-hPER1 (Thermo Fisher Scientific, PA1-524), anti-mPER2 (Alpha Diagnostic International, PER-21A), anti-CLOCK (MBL International, D333-3), anti-CRY1 (MBL International, PM081), and anti-CRY2 (MBL International, PM082). Secondary antibodies used were goat anti-mouse immunoglobulin G horseradish peroxidase (IgG-HRP) (Santa Cruz

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(G) mRNA levels of indicated metabolism-relevant genes in mouse liver. mRNA levels were normalized to *Tbp*. Data are shown as means ± SEM (n = 3, *p < 0.05 by two-way ANOVA).

(H) Proposed model of CRY regulation by FAD. The classical clock generates gene expression rhythms including *Rfk* and *Nampt*, which rhythmically synthesize FAD and NAD+. FAD binding to CRY prevents FBXL3-mediated CRY degradation, leading to stabilization. Time-dependent FAD synthesis modulates CRY expression rhythms and amplitudes, which regulate the gene expression rhythms of important genes for metabolism.

Biotechnology, sc-2005), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2006), and goat anti-guinea pig IgG-HRP (Santa Cruz Biotechnology, sc2438).

Real-Time qPCR

Total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific) from liver samples and purified by RNeasy Mini kit (QIAGEN). cDNA was synthesized from 1.5 μg RNA by GoScript (Promega) using dT_{15} primer. Quantification of mRNA was performed with GoTaq Real-Time qPCR Kits (Promega) using gene-specific primers (Table S1). mRNA levels were normalized by mouse *Gapdh* or *Tbp* levels.

FAD Concentration

FAD was measured with an FAD assay kit (Sigma-Aldrich) following the manufacturer's protocol. Briefly, the nuclear extract was added to 2 vol of 8% (w/w) perchloric acid (PCA), followed by vortexing the mixture for 30 s. The lysate was centrifuged for 10 min at $1,500 \times g$, and the supernatant was used for FAD measurements.

FAD Competition Assay

Flavin adenine dinucleotide disodium salt hydrate (FAD, Sigma-Aldrich) was diluted in PBS to make a 100 mM stock solution. HEK293 cells were transfected with plasmid vectors for CRY (FLAG-CRY1 or CRY2-Myc-His) and FBXL3 (HA-FBXL3 or FLAG-FBXL3). Forty-two hours after transfection, the cells were treated with 10 μM MG132 (Calbiochem) for 6 hr. CRY-FBXL3 complex was purified with anti-FLAG M2 affinity gel (Sigma-Aldrich) or HA antibody. FAD was incubated with CRY1-FBXL3 complex binding to anti-FLAG M2 affinity gel or protein G Sepharose (GE Healthcare) in 40 μL PBS for 2 hr on ice. After centrifugation, the supernatant was collected as the "released CRY" sample. CRY still binding to FBXL3 was eluted by adding SDS sample buffer.

Tail Vein Injection of siRNA

siRNA for *Rfk* was purchased from Thermo Fisher Scientific (siRNA#1: catalog # S79629; siRNA#2: catalog # S79628). siRNA solution for the injection was prepared according to the manual of Invivofectamine 3.0 (Thermo Fisher Scientific). BALB/c male mice (8 weeks old) were fed a normal diet or riboflavin-free diet for 2 weeks. Mice (10 weeks old at the injection) were anesthetized with isoflurane during the procedure, and siRNA targeting *Rfk* or equivalent amounts of control siRNA (3 mol/mice each siRNA) were injected through the tail vein using a 28G syringe. Injections were performed at ZT10–12.

Glucose Concentration

Four days after the injection of siRNAs, blood was collected from mouse tail veins. Blood was incubated for 60 min at 37°C and centrifuged at 3,000 rpm for 20 min. The supernatant was collected and used for a glucose oxidation assay (Sigma-Aldrich). For glucose concentration in liver lysate, proteins were removed by PCA precipitation. One-third volume of 4 M PCA was added to total liver lysate, followed by incubation on ice for 5 min. After centrifugation at $13,000 \times g$ for 2 min, the supernatant was collected. Ice-cold 2 M KOH was added to titrate pH to ~7.0. Samples were centrifuged at $13,000 \times g$ for 15 min at 4°C, and the supernatant was used for the glucose oxidation assay (Sigma-Aldrich).

OCR and ECAR

HepG2 cells were cultured in 96-well plates and transfected with indicated siRNAs. Twenty-four hours after the transfection, cell-culture media were replaced by media containing the OCR fluorescence probe (Abcam, OCR kit) and cultured for 24 hr. Media was changed to recording media: phenol-red-free DMEM (Sigma-Aldrich) containing 10 mM HEPES (pH 7.0), 3.5 g/L D-glucose, and 10% FBS. Florescence was continuously measured every 2.5 or 5 min with a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek). For ECAR, transfected cells were cultured for 48 hr, and culture media was changed to glycolysis buffer containing probe (Abcam, ECAR kit). Florescence was measured every 1.5 min using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek).

Statistical Analysis

All error bars in the figures represent SEM. No statistical analysis was used to predetermine the sample sizes. Experiments were not randomized and were not analyzed blindly. Data were statistically analyzed using R software and Prism7. To assess statistical significance, data were obtained from three or more independent experiments. Two-tailed paired Student's t test, Tukey's test, one-way ANOVA, or two-way ANOVA followed by Sidak test (as post hoc test) was used. p values <0.05 were considered to represent a statistically significant difference.

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