SCF<sub>FBXL3</sub> ubiquitin ligase targets cryptochromes at their cofactor pocket

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The cryptochrome (CRY) flavoproteins act as blue–light receptors in plants and insects, but perform light–independent functions at the core of the mammalian circadian clock. To drive clock oscillations, mammalian CRYs associate with the Period proteins (PERs) and together inhibit the transcription of their own genes. The SCF<sub>FBXL3</sub> ubiquitin ligase complex controls this negative feedback loop by promoting CRY ubiquitination and degradation. However, the molecular mechanisms of their interactions and the functional role of flavin adenine dinucleotide (FAD) binding in CRYs remain poorly understood. Here we report crystal structures of mammalian CRY2 in its apo, FAD–bound and FBXL3–SKP1–complexed forms. Distinct from other cryptochromes of known structures, mammalian CRY2 binds FAD dynamically with an open cofactor pocket. Notably, the F–box protein FBXL3 captures CRY2 by simultaneously occupying its FAD–binding pocket with a conserved carboxy–terminal tail and burying its PER–binding interface. This novel F–box–protein–substrate bipartite interaction is susceptible to disruption by both FAD and PERs, suggesting a new avenue for pharmacological targeting of the complex and a multifaceted regulatory mechanism of CRY ubiquitination.

Cryptochromes (CRYs) are evolutionarily conserved FAD-binding proteins that share close sequence homology with DNA photolyases and are widespread in both plant and animal kingdoms<sup>1,2</sup>. Using FAD as a chromophore, plant cryptochromes function as blue-light photoreceptors and regulate a broad range of light responses during plant growth and development<sup>1,3</sup>. In animals, cryptochromes are classified into two types<sup>4,5</sup>. Type I cryptochromes, exemplified by Drosophila melanogaster CRY, maintain blue-light sensitivity and have a key role in photic entrainment of the insect circadian clock<sup>6</sup>, whereas type II cryptochromes are photo-insensitive and evolved as central components of the molecular clock<sup>7,8</sup>.

Mammalian CRYs, the prototypic type II cryptochromes, act together with PERs, CLOCK and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1) to constitute a transcription negative feedback loop that oscillates with a circadian periodicity of approximately 24 h (ref. 9). In this molecular clockwork, CLOCK and BMAL1 heterodimerize and activate the transcription of CRY and PER genes, whose protein products in turn form complexes in the cytoplasm and translocate into the nucleus to suppress their own gene expression by binding and inhibiting CRY<sup>10</sup>–BMAL1 (refs 10, 11). In mammals, this basic clock circuitry drives the circadian oscillations of both the master clock in the brain and the peripheral clocks throughout the body<sup>9,12</sup>. The master clock is synchronized to light–dark cycles perceived by the retina and coordinates clocks in the peripheral tissues through a variety of mechanisms, such as feeding behaviour, body temperature and hormonal secretion<sup>13–15</sup>.

Despite their functional divergence, many plant and animal cryptochromes are functionally linked to protein ubiquitination and degradation. For example, light-activated Arabidopsis thaliana CRY1 mediates seedling photomorphogenesis by inhibiting COP1 ubiquitin ligase<sup>16,17</sup>, whereas Drosophila CRY modulates the insect circadian rhythm by promoting light-dependent degradation of the clock protein Timeless (TIM) and itself via the Jetlag ubiquitin ligase<sup>18,19</sup>. In both cases, photo-excitation of FAD is thought to induce conformational changes within the cryptochrome proteins and alter their interactions with the target proteins<sup>20,21</sup>. Recent biochemical and genetic studies have shown that mammalian CRYs are also functionally connected to the ubiquitin–proteasome system<sup>22–24</sup>. The SCF<sub>FBXL3</sub> ubiquitin ligase complex (SKP1–CUL1–F-box protein complex containing the F-box protein FBXL3) has been identified to regulate the clock by promoting the degradation of CRYs.

As an integral part of the clock circuitry, SCF<sub>FBXL3</sub>-catalysed CRY degradation needs to be regulated tightly. Indeed, the AMP-activated protein kinase (AMPK), a cellular metabolic sensor, has been shown to phosphorylate mouse CRY1 and accelerate its degradation by enhancing CRY1 binding to FBXL3 (ref. 25). Moreover, a recent circadian chemical screen has identified a period-lengthening small molecule that can compete with FAD for CRY binding and stabilize CRYs by inhibiting their ubiquitination<sup>26</sup>. These activities of the compound suggest that SCF<sub>FBXL3</sub>-mediated CRY ubiquitination may be tunable by the FAD cofactor, whose function in mammalian CRYs remains elusive<sup>26</sup>. To provide the missing structural framework for understanding the functions of mammalian CRYs and the regulatory mechanisms of CRY–FBXL3 interaction, we report here the crystal structures of mammalian CRY2 in three different functional forms.

**Structure of murine apo CRY2 PHR**

Cryptochromes in general contain a conserved photolyase-homology region (PHR) and a unique cryptochrome C-terminal (CCT) extension (CCE) (Supplementary Fig. 1a). We first determined the 2.7 Å crystal structure of the proteolytically stable mouse CRY2 PHR domain (amino acids 1–512) (Supplementary Fig. 2 and Supplementary Table 1). As expected, murine CRY PHR adopts a canonical photolyase
fold, consisting of an amino-terminal α/β domain (N-terminal domain containing both α-helices and β-sheets), a C-terminal α-helical domain, and a connecting linker sequence (Fig. 1a). At the sequence level, vertebrate CRYs are more homologous to metazoan (6–4) photolyases than plant or type I animal cryptochromes (Supplementary Table 2). After superposition, murine CRY2 PHR can be aligned with the most homologous structure of Drosophila (6–4) photolyase with a root mean squared deviation (r.m.s.d.) of 1.1 Å over 431 equivalent Cα positions (Supplementary Fig. 3). However, unlike all known structures of (6–4) photolyases and cryptochromes27, the murine CRY2 PHR structure represents the apo form of the flavoprotein (Fig. 1a). We found no electron density of FAD at its expected cofactor-binding pocket, despite the well-defined and conserved FAD-binding cavity. This feature is in agreement with previous reports showing that only a small fraction of mammalian CRYs contains FAD when isolated from mammalian cells or various heterologous systems. Similar to Drosophila (6–4) photolyase, murine CRY2 PHR does not co-purify with a second cofactor that acts as a light-harvesting antenna in some bacterial photolyases28–31.

**Structure of FAD-bound murine CRY2 PHR**

Oxidized FAD is naturally fluorescent with an emission maximum at approximately 525 nm. In the presence of excess murine CRY2 PHR, FAD fluorescence is strongly quenched, suggesting that murine CRY2 PHR still retains specific FAD-binding activity (Fig. 1b). By titrating the amount of protein added, we estimated that FAD binds to isolated PHR still retains specific FAD-binding activity (Fig. 1b). By titrating approximately 525 nm. In the presence of excess murine CRY2 PHR, FAD at its expected cofactor-binding pocket, despite the well-defined and conserved FAD-binding cavity. This feature is in agreement with previous reports showing that only a small fraction of mammalian CRYs contains FAD when isolated from mammalian cells or various heterologous systems. Similar to Drosophila (6–4) photolyase, murine CRY2 PHR does not co-purify with a second cofactor that acts as a light-harvesting antenna in some bacterial photolyases28–31.

**Graphs and Figures**

**Figure 1** | Structure of murine CRY2 PHR in apo- and FAD-bound forms. 

- **a**, Overall structure of the apo-bound form of the mouse CRY2 PHR (mCRY2 PHR) domain. 
- **b**, mCRY2 PHR induced quenching of FAD fluorescence. The inset shows FAD-fluorescence quenching with mCRY2 PHR concentration titrated. Error bars, s.d. 
- **c**, A close-up view of FAD bound to mCRY2 PHR with positive Fc – F0, electron density contoured at 1.5 σ and calculated before the cofactor was built (green mesh). 
- **d**, mCRY2 PHR and the Drosophila (6–4) photolyase (dm(6–4)-PL) are superimposed, with their FAD cofactors shown as sticks and their FAD carbon atoms in yellow and green, respectively.
the missense mutations in the alleles known as after hours and overtime, Cys358Ser and Ile364Thr substitutions, are mapped to the LRR10 and LRR11, respectively (Fig. 4c and Supplementary Fig. 6). Because both residues are located in the hydrophobic core of the LRR domain, their substitution by polar amino acids probably perturbs the local conformation of LRR-C.

The C-terminal tail of FBXL3

The C-terminal tail of FBXL3 represents the hallmark of the FBXL3-CRY2 interface by sticking out from the flat concave surface of LRR-C and penetrating into CRY2 (Fig. 5a). The 12-amino-acid C-terminal region of FBXL3 is mostly invariant in vertebrates and terminates with a tryptophan residue (Supplementary Fig. 6). This sequence caps the LRR10 and LRR11, respectively (Fig. 4c and Supplementary Fig. 6). Notably, the last residue of the tail, Trp 428, reaches the core of the FAD-binding pocket and physically occupies the cofactor site.

Superposition analysis shows that the FBXL3 C-terminal tail approaches the FAD-binding pocket from the same angle as the (6-4) DNA lesion approaches the FAD-binding pocket in the Drosophila (6-4) photolyase-DNA complex structure (Fig. 5b). To expand the entrance, Met 425 of FBXL3 pushes aside an important CRY2 loop, which we name the interface loop. To enter the pocket, the FBXL3 tail stacks its Pro 426 residue against Trp 310 of CRY2 and flips the side chain of CRY2 His 373 with Thr 427 (Fig. 5c, d). In the FAD-bound form, these CRY2 residues directly contact the cofactor. Inside the pocket, the side chain of FBXL3 Trp 428 occupies the central space of the cavity, which corresponds to the narrow gap between the isoalloxazine and adenine rings of FAD. While donating a hydrogen bond to the backbone carbonyl group of Gln 307 in CRY2, the indole ring of Trp 428 makes multiple hydrophobic contacts with CRY2. The carboxyl group of Trp 428 forms additional hydrogen bonds with CRY2 Ser 414 and a signature arginine residue, Arg 376 (Fig. 5c, d). Overall, this network of interactions anchors the FBXL3 tail at the very centre of the FAD-binding pocket, predicting that FAD and the F-box protein will compete to bind the cryptochrome protein.

Interface between FBXL3 and CRY2 PHR

In addition to the C-terminal tail, the FBXL3 LRR domain also forms extensive interfaces with CRY2 through its solenoid fold. At the apical ridge of LRR-N, four FBXL3 loops from LRR1–LRR4 interact with the back of the α-helical domain of CRY2 opposite to the FAD-binding pocket (Figs 3b and 4c). This part of the interface is mediated largely by polar interactions (Supplementary Fig. 7).

In contrast to LRR-N, the FBXL3 LRR-C subdomain binds CRY2 through a predominantly hydrophobic interface. On its concave wall, LRR-C presents a cluster of more than ten hydrophobic residues strictly
conserved in vertebrate CRYs (Fig. 6a and Supplementary Fig. 6). These residues, plus a circle of peripheral polar amino acids, recognize a large complementary surface on CRY2 adjacent to the cofactor pocket. The interface on the CRY2 side is constructed by three key structural motifs; the interface loop, the C-terminal helix and the CCS region (Fig. 6b). With a string of strictly conserved aromatic residues (Supplementary Fig. 8), the CRY2 interface loop is buttressed by Met425 from the FBXL3 tail on one side and the CRY2 C-terminal helix on the other. The CRY2 CCS region adopts a well-ordered loop structure that wraps back onto the interface loop and the C-terminal helix. Together, these three motifs contribute a panel of hydrophobic residues grouped at the centre of the LRR-C binding interface. By making direct contacts with both FBXL3 and CRY2 PHR, the conserved CCS region of CRY2 probably takes up its structural role at the interface through an ‘induced fit’ mechanism (Supplementary Fig. 9).

To map regions that are critical for FBXL3–CRY2 interaction, we tested the binding activity of a series of FBXL3 and CRY2 mutants. Disruption of interactions at the apical ridge of LRR-N did not affect the complex formation (Asp339Ala and Tyr490Ala (Fig. 6c), Asp339Arg and Arg496Ala (Fig. 6d and Supplementary Fig. 7)), indicating a supportive role of LRR-N in recruiting CRY2. By contrast, CRY2 binding was impaired severely by either single amino acid substitution or truncation of the FBXL3 tail (Pro426Ala, Trp428Ala, Asp423A (Fig. 6c)). Similarly, alteration of a CRY2 residue in the cofactor pocket also compromised complex association (Trp310Ala (Fig. 5d, 6d)). These results pinpoint the FBXL3 C-terminal tail as a critical region34, was able to dislodge CRY2 from FBXL3 (Fig. 6e). We were able to confirm that free FAD, but not flavin mononucleotide (FMN), FAD in regulating the FBXL3–CRY2 interaction. In a purified system, A close-up view of the mCRY2 FAD-binding pocket showing key FAD- and FBXL3-interacting residues. FAD and the FBXL3 tail are shown as sticks. Dashed yellow lines represent hydrogen bonds.

hydrophobic-to-charged mutation was introduced to each of the three LRR-C-interacting structural motifs in CRY2; that is, the interface loop (Phe428Asp), the C-terminal helix (Ile499Asp) and the CCS region (Leu517Asp) (Fig. 6b, d). The functional importance of this part of the interface and the FBXL3 tail-gripping pocket was underscored further by the stabilization of the CRY2 mutants with hot-spot residues altered at these sites (Fig. 6f and Supplementary Fig. 9).

The C-terminal helix of CRY2 and its two basic residues, Arg501 and Lys503, are thought to be essential for PER binding32,33. In complex with FBXL3, the entire CRY2 C-terminal helix is masked by the LRR domain of the F-box protein (Fig. 3b and Supplementary Fig. 9). We found by chance that mutation of Asp339, which is in close proximity to Arg501, also showed defective interaction with PER1 (Fig. 6d and Supplementary Fig. 7). These lines of evidence strongly suggest that PERs and FBXL3 share an overlapping CRY2-binding interface, which should prevent them from binding CRY2 simultaneously. Indeed, PER2-bound CRY2 is completely devoid of FBXL3 (Supplementary Fig. 10). Importantly, just like FAD, a purified C-terminal fragment of PER2, which corresponds to a previously mapped minimal CRY-binding region34, was able to dislodge CRY2 from FBXL3 (Fig. 6e). We were able to verify this with an immuno blot analysis.

Figure 5 | Interaction between the FBXL3 C-terminal tail and the CRY2 FAD-binding pocket. 
(a) Surface representation of the FBXL3 LRR-C subdomain with its C-terminal tail inserted into the FAD-binding pocket of mCRY2 (blue ribbons). (b) The structure of Drosophila (6–4) photolyase–DNA complex shown from the same orientation as mCRY2 in a. FAD is shown as spheres. The damaged DNA substrate is shown as a stick diagram. (c, d) A close-up view of the mCRY2 FAD-binding pocket showing key FAD- and FBXL3-interacting residues. FAD and the FBXL3 tail are shown as sticks. Dashed yellow lines represent hydrogen bonds.

Figure 6 | Structural and functional analyses of the FBXL3–murine CRY2 interface. 
(a) Surface and ribbon diagrams of the FBXL3 LRR-C subdomain showing hydrophobic and polar residues that are involved in mCRY2 binding. Amino acids selected for mutational analyses are labelled. The C-terminal tail and some N-terminal repeats are shown as ribbon diagrams. (b) Ribbon diagrams of the mCRY2 structural elements involved in FBXL3 LRR-C interaction. Select interface residues are shown as sticks. A part of the FBXL3 tail (magenta) is shown for reference. (c) Interactions of retrovirus-expressed FBXL3 mutants with endogenous mCRY2 in mouse embryonic fibroblasts (MEFs) assessed by co-immunoprecipitation (IP) and western blot analysis. (d) Flag-mCRY2 complexes were immunoprecipitated with an anti-Flag resin from transfected HEK293T cells and assessed for binding to FBXL3, PER1, BMAL1 and SKP1. Arg376Ala served as a negative control as it is probably detrimental to mCRY2 folding (see Supplementary Discussion for Cys430Ala and FBXL3–Cys340Ser mutants). (e) Two in vitro competition assays showing the ability of FAD and PER2, but not FMN, to disrupt a preformed FBXL3--mCRY2 complex. (f) MEFs were infected with retroviruses expressing either mCRY2 or mCRY2 mutants and treated with cycloheximide (CHX) for the indicated time in hours. Total extracts were examined by immunoblot analysis.
to confirm that the abundance of PER2 had a direct effect on the stability of both CRY1 and CRY2 (Supplementary Fig. 1)24,35, probably by competing with the SCF<sub>FBXL3</sub> E3 ligase.

**Discussion**

The structure of the mammalian cryptochrome reveals an unexpected open FAD-binding pocket that probably evolved in vertebrates to enable dynamic FAD binding. This unique property is built on the plasticity of the phosphate loop guiding the entrance of the pocket. Intriguingly, this loop is highly conserved among vertebrates (Supplementary Fig. 8), raising the possibility that its alteration by phosphorylation25,26 or CRY-binding proteins might regulate the affinity of FAD (Supplementary Fig. 11).

Importantly, our structural results have unravelled the functional requirement for the dynamic FAD-binding pocket in CRYs and a novel mechanism of substrate-recognition by the SCF ubiquitin ligase. In contrast to the canonical degron-based substrate-engagement scheme27,28, FBXL3 takes advantage of the deep but accessible cofactor pocket of CRYs to capture the substrate (Supplementary Fig. 12). This interaction mode establishes FAD and its analogues as potential tor pocket of CRYs to capture the substrate (Supplementary Fig. 12).

The unexpected interface between CRY2 and FBXL3 suggests that their interactions can be regulated by multiple mechanisms. As a common metabolic redox cofactor in the cell, it is possible that FAD directly controls the stability of CRYs by competing with the ubiquitin ligase. Although oxidized FAD binds murine CRY2 with a modest affinity (see Supplementary Discussion), its interaction with FAD directly controls the stability of CRYs by competing with the ubiquitin ligase. This function is reminiscent of the stabilization of PERs by CRYs against SCF<sup>FBXL3</sup>-mediated degradation1,14. Such interplay between the two obligate functional partners could serve as a mechanism for synchronizing their stability to ensure robust clock oscillation and phase shift. In fact, the CRY1-desaturating effect of Ser71 phosphorylation by AMPK may be partially explained by its reported activity in blocking PER2 binding29.

**METHODS SUMMARY**

The full Methods provides detailed information about all experimental procedures, including description of protein purification; protein crystallization, data collection and structure determination; FAD fluorescence assay; in vitro competition assay; and mutagenesis, cell culture, binding and stability analyses.

**Full Methods** and any associated references are available in the online version of the paper.

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**Author Contributions** The protein purification and crystallization experiments were conceived by W.X., T.R.H. and N.Z., and conducted by W.X. and N.Z. and N.Z. determined and analysed the structures. FAD fluorescence and in vitro competition experiments were conceived by W.X. and T.R.H. and N.Z., and conducted by W.X. and T.R.H. Mutational and binding studies, and stability analyses were conceived by L.B., M.P., W.X. and N.Z., and conducted by L.B. and T.M.F.B. and M.F.B. conducted native mass spectrometric experiments.

**Author Information** Structural coordinates and structural factors for FBXL3–CRY2–SKP1, CRY2–FAD and CRY2 are deposited in the Protein Data Bank under accession numbers 3H6G and 4IF6 respectively. Supplementary Information is available at http://www.nature.com/reprints. The authors declare no competing financial interests.

Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to N.Z. (nzheng@uwashington.edu).
METHODS
Recombinant protein purification. The mouse CRY2 (amino acids 1–544) was expressed as a glutathione S-transferase (GST) fusion protein in High Five (Invitrogen) suspension insect cells and isolated by glutathione affinity chromatography using buffer containing 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10% Glycerol, 10 mM DTT (dithiothreitol) and 0.5 mM FAD. The protein was purified further by cation exchange and gel filtration chromatography after on-column cleavage by tobacco etch virus (TEV) protease. During purification, the polyepitope produced a series of C-terminal degradation products that were not affected by the presence or absence of FAD and diithiothreitol. Two shorter versions of the protein, murine CRY2 with amino acids 1–527 and murine CRY2 with amino acids 1–512, were subsequently purified. Native mass spectrometry analysis confirmed that murine CRY2 with amino acids 1–512 was stable, but murine CRY2 with amino acids 1–527 was not (Supplementary Figs 1 and 2). The mouse CRY2 (amino acids 1–544) protein was co-expressed with full-length human FBXL3 and SKP1 in High Five suspension insect cells (human FXBL3 is 97% identical to mouse FBXL3, and the F-box and LRR regions of human and mouse FBXL3 differ by only 3 amino acids; Supplementary Fig. 6). FBXL3 was fused with an N-terminal GST tag, whereas the other two proteins were tag-free. The CRY2–FBXL3–SKP1 complex was isolated from the soluble cell lysate by glutathione affinity chromatography. After on-column tag cleavage by TEV, the complex was purified further by anion exchange and gel filtration chromatography and then concentrated by ultrafiltration to 10 mg mL\(^{-1}\) in a buffer of 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM DTT. Mouse PER2 (amino acids 1095–1235) was overexpressed as a GST-fusion protein in Escherichia coli and purified by glutathione affinity, anion exchange and gel filtration chromatography.

Crystallization, data collection and structure determination. The crystals of the CRY2–FBXL3–SKP1 complex were grown at 4 °C by the hanging-drop vapour diffusion method, using 1.5 l protein complex sample mixed with an equal volume of reservoir solution containing 0.2 M ammonium citrate, 13–14% PEG3350 and 7% acetonitrile. Diffraction-quality crystals that were obtained were subjected to a post-crystallization dehydration procedure by gradually increasing the concentration of PEG3350 to 30%, and then directly frozen in liquid nitrogen. This procedure consistently improved the resolution of the crystals by approximately 4 Å. The CRY2–FBXL3–SKP1 derivative crystals were prepared by soaking the native crystals in the buffer containing 15% PEG3350 and 7% acetonitrile supplemented with 0.2 mM KAu(CN)\(_4\) for 12 h, followed by soaking in 0.5 mM KAu(CN)\(_4\) for 12 h and then 1 mM KAu(CN)\(_4\) for a further 12 h. After soaking, the crystals were dehydrated by the same method as native crystals. The crystals of murine CRY2 were also grown at 4 °C by the hanging-drop vapour diffusion method with 1.5 l protein mixture with an equal volume of reservoir solution containing 0.1 M MES- imidazole, pH 6.5, 7.2% PEG8K, 20% ethylene glycol (v/v), 0.03 M NaF, NaBr and NaCl. The crystals were directly frozen in liquid nitrogen. The CRY2–FAD crystals were obtained by soaking the murine CRY2 crystals with 1 mM FAD in the reservoir buffer with extra 7.5% Glycerol for 2 h before freezing in liquid nitrogen. All data sets were collected at the BL8.2.1 and BL8.2.2 beamlines at the Advanced Light Source of the Lawrence Berkeley National Laboratory. Reflection data were indexed, integrated and scaled with the HKL2000 package. The murine CRY2 PHR domain structure was determined by molecular replacement using the Dreosiphila (6-4) photolyase structure (PDB:3CVU) as the search model. Soaking with FAD altered the unit cell and substantially improved the murine CRY2 PHR domain crystals. For the apo structure, translation liberation screw-motion (TLS) refinement was necessary to reduce the R-free value below 0.30. The CRY2–FBXL3–SKP1 structure was solved by molecular replacement combined with single-wavelength anomalous dispersion phases (MR-SAD) using the software PHENIX. The structural models were manually built, refined and rebuilt with the programs COOT and PHENIX.

FAD fluorescence assay. FAD fluorescence and binding studies were carried out in a PBS buffer, pH 7.4, with 10% glycerol at room temperature in a half-plate with a total volume of 100 l. Interaction was quantified by measuring the decrease in FAD fluorescence (excitation at 450 nm and emission at 520 nm) after binding to mouse CRY2. FAD concentration was held constant at 10 lM in all wells, and murine CRY2 PHR was serially diluted three times from 300 nM to 292 nM. Non-specific FAD binding (background) was measured in parallel with equal molar concentrations of ovalbumin or lysozyme, which do not bind FAD. All protein concentrations were re-confirmed by in-gel replicate. The binding data was fit using a standard nonlinear regression curve fitting method with a log(inhibitor) versus response model in Prism 5 (GraphPad Software).

In vitro competition assay. Strep-tagged full-length FBXL3, GST-tagged murine CRY2 (amino acids 1–544) and His-tagged SKP1 were co-expressed in monolayer High Five insect cells. The complex was purified without elution by Strep-Tactin affinity resin in a buffer containing 100 mM Tris-HCl, pH 8.0, 300 mM NaCl and 20 mM DTT. The Strep-Tactin resin beads with the immobilized complex were subsequently aliquoted in a volume of 100 l with 1 mg ml\(^{-1}\) total protein and placed in a panel of 7.5 mm gravity columns. The beads were incubated with 300 l of oxidized FAD, FMN or purified murine CRY2 (amino acids 1095–1235) in a serial dilution (FAD: 0, 5, 50, 500, 5000 M; FMN: 50 and 500 M; murine PER2: 0, 2.5, 5, 10, 20 and 40 pM) for 3 h at 4 °C. Flow-through and subsequent two-column-volume wash fractions were collected from each column and the GST–CRY2 protein released from the column was examined with SDS–PAGE and detected by western blot analysis using anti-GST antibody.

Cell culture. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine serum. Immortalized mouse embryonic fibroblasts (MEFs) were maintained in DMEM containing 10% fetal bovine serum (FBS). HEK293T cells were transfected with PEI (Polysciences) according to the manufacturer’s instructions. Cycloheximide (Sigma) was used at a final concentration of 100 lM. For retrovirus production, GP-293 packaging cells (Clontech) were used. Forty-eight hours after transfection, the virus-containing medium was collected and supplemented with 8 lM polybrene (Sigma). Cells were then removed by infecting the replacement cell culture medium with the viral supernatant for six hours. Forty-eight hours after infection, cells were selected with 1 mg ml\(^{-1}\) puromycin.

Co-immunoprecipitation binding studies. Cells were collected and subsequently lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% NP40, plus protease and phosphatase inhibitors). Immunoprecipitation was carried out with anti-Flag agarose beads (Sigma). Immunocomplexes were washed five times with lysis buffer and eluted in a serial dilution (FAD: 0, 5, 50, 500, 5000 M; FMN: 50 and 500 M; murine PER2: 0, 2.5, 5, 10, 20 and 40 pM) in Laemmli buffer containing 1% SDS. Cell extracts or Flag-immunocomplexes were analysed by SDS–PAGE and then by immunoblot analysis with the following antibodies: anti-Flag (Sigma), anti-CRY1 and CRY2 (Bethyl), anti-PER1 (Alpha Diagnostic), anti-PER2 (Bethyl), anti-BMAL1 (Bethyl), anti-Skp1 (Invitrogen) and anti-FBXL3 (ref. 22).

Plasmids. Wild-type and mutant mouse CRY2 complementary DNAs were subcloned into pcDNA3.1–Flag. Mouse PER2 was cloned into pMT-Myc. For retrovirus production, cDNAs encoding Flag or HA-tagged FBXL3 or CRY2 wild-type and mutants, were subcloned into the retroviral vector pBABE-Puro (Cell Bioslabs). Point mutations were generated using the QuickChange Site-directed Mutagenesis kit (Stratagene).

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