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# Supplemental Data



# Cycling of CRYPTOCHROME Proteins Is Not Necessary for Circadian-Clock Function in Mammalian Fibroblasts

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## Supplemental Experimental Procedures

### Purification Procedure for CP Proteins

Polyhistidine-tagged CP-CRY1, CP-CRY2, CP-mutCRY1, and CP-CRE proteins were expressed in *E. coli* strain BL21 (DE3) and purified by TALON metal affinity chromatography (Clontech). Bacterial cultures were grown to an $OD_{600}$ of 0.6–1.0, and protein expression was induced with 0.5 mM IPTG. After harvesting, the cells were lysed in equilibration/wash buffer (20 mM HEPES [pH 8.0] and 200 mM NaCl). After centrifugation, the supernatant was incubated with TALON resin for 30 min. Then, the resin was washed with equilibration/wash buffer for 20 min and then washed with the same equilibration/wash buffer with the addition of 20 mM imidazole. The proteins were eluted with equilibration/wash buffer that included 100 mM imidazole. After the TALON affinity chromatography, eluted proteins were dialyzed against PBS buffer supplemented with 5% glycerol and concentrated with a Microcon centrifugal filter (Millipore).

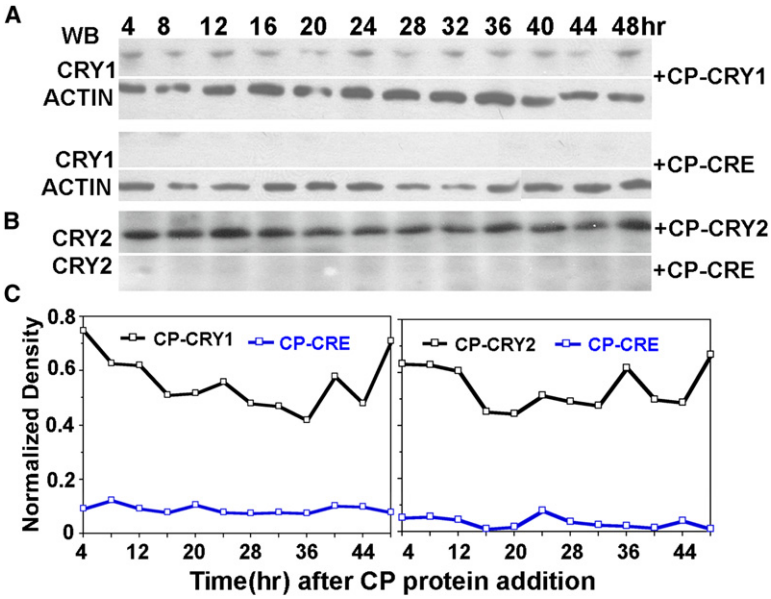


Figure S1. Immunoblot Confirmation of Uptake of CP-CRY1 and CP-CRY2 into Rat-1 Fibroblasts

The immunoblots used antibodies directed against CRY1 (A) or CRY2 (B). A total of 50 nM CP-CRY1, 50 nM CP-CRY2, or 50 nM CP-CRE were added to cultured Rat-1 fibroblasts in DMEM + 10% FBS. At various times over the following 48 hr, the cells were washed with PBS and treated with proteinase K (5 μg/μl) for 10 min at 37°C to eliminate nonspecific adherence of proteins to the cell surface. (C) shows the densitometry of these blots as a function of time and normalized to the actin controls. In this paper, we used antibodies directed against mouse clock proteins to probe for both mouse and rat clock proteins. The sequence comparisons of these proteins are as follows: rCRY1 is 95% identical with mCRY1; rCRY2 is 97% identical with mCRY2; rBMAL1 is 99% identical with mBMAL1; and rCLOCK is 95% identical with mCLOCK. In the case of the BMAL1 antibody, the immunogen was a peptide that is conserved among mouse, rat, and human BMAL1, and the antibody is known to react with the BMAL1 of all three species. Similarly, the CLOCK antibody is known to recognize CLOCK from hamster, human, mouse, and rat.

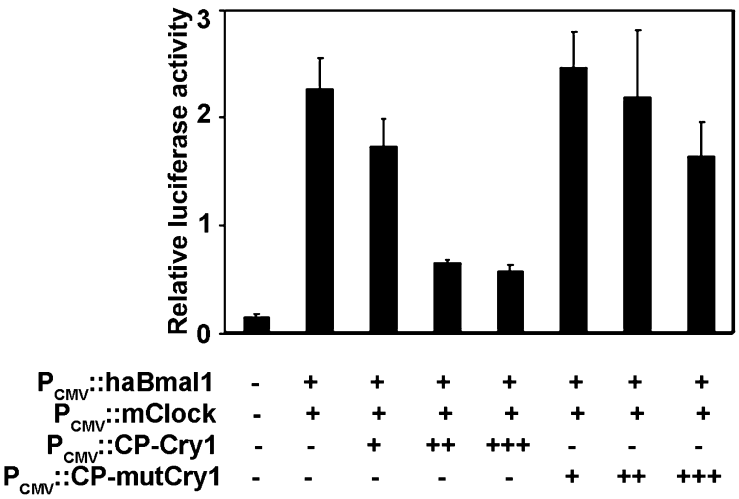


Figure S2. Suppression of BMAL1/CLOCK-Mediated Transcription by $P_{CMV}$::Cry1 and $P_{CMV}$::mutCry1

HEK293 cells were transfected with *haBmal1*, mouse *Clock*, and an E box-containing promoter $P_{PK2}$-driven luciferase reporter ($P_{PK2}$::luc). Transfection of either mutant CRY1 or wild-type CRY1 can suppress $P_{PK2}$ activity in a dose-dependent manner, but mutant CRY1 is less effective than wild-type CRY1. The values are mean ± SD of three replicates. Concentrations of transfected plasmids were as follows: +, 5 ng/ml; ++, 50 ng/ml; and +++, 250 ng/ml.

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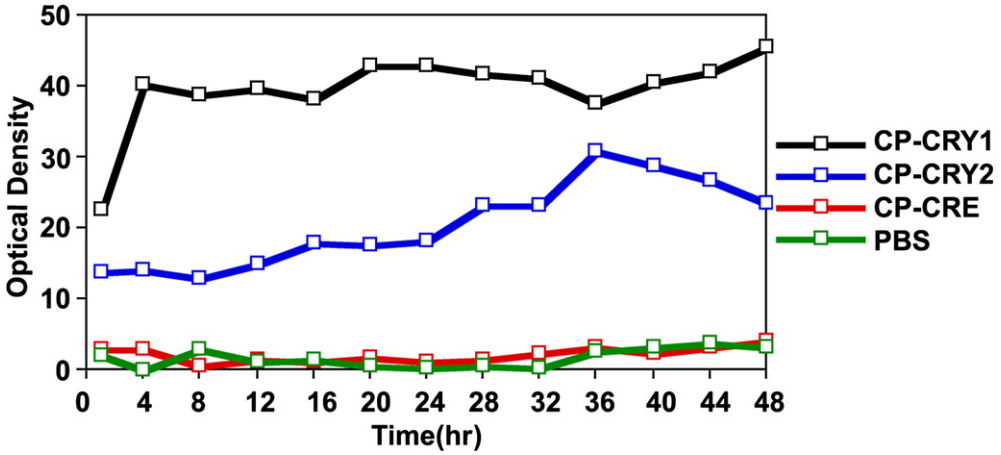


Figure S3. Densitometry of Data Shown in Figure 4A

These data show that CP-CRY1 induces BMAL1 levels at least 20-fold and CP-CRY2 induces BMAL1 levels at least 10-fold above basal levels. These levels of BMAL1 do not show circadian fluctuations.

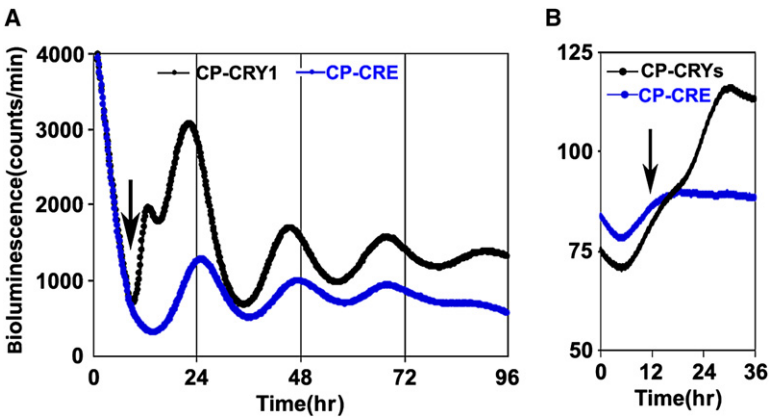


Figure S4. Induction of $P_{Bmal1}$ by CP-CRY Proteins in Rat-1 Fibroblasts and in *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ Mouse Fibroblasts

(A) A Rat-1 line that was stably transfected with the $P_{Bmal1}$::Fluc reporter construct and that showed a robust circadian rhythm of luciferase activity was used to assay the induction of $P_{Bmal1}$ activity by CP-CRY1. The cells were cultured, treated with forskolin for 2 hr, and put into the Lumicycle as described in the Experimental Procedures. An amount of 50 nM CP-CRY1 or 50 nM of the control protein CP-CRE was added to the cell samples at the time marked with the arrow and the luminescence was recorded for 5 days.

(B) *mCry1*$^{-/-}$*mCry2*$^{-/-}$ mouse fibroblasts were transfected with the $P_{Bmal1}$::Fluc reporter plasmid by nucleofection (see Experimental Procedures). Twelve hours later, the cells were treated with forskolin for 2 hr and then 50 nM CP-CRY1 + 50 nM CP-CRY2 were added to the cells. In both (A) and (B), the treatment with CP-CRY elicits an increase in the luminescence of the $P_{Bmal1}$::Fluc reporter, indicating CP-CRY stimulates $P_{Bmal1}$ activity.

### CP Proteins Do Not Evoke a $Ca^{2+}$ Flux

Other researchers using the positively charged Tat peptide as a cell-permeation tag found that the Tat sequence could mediate a $Ca^{2+}$ flux across cell membranes and elicit phase shifting by itself in SCN brain slices [S1]. We explicitly tested whether our MTS-tagged CP proteins might have the same side effect. By using the $Ca^{2+}$ indicator dye fura-2, we found that not CP-CRY1, CP-CRY2, or CP-CRE can activate a rapid $Ca^{2+}$ flux in Rat-1 fibroblasts (Figure S6). Our PTD is based on hydrophobicity, whereas the TAT peptide is based on positively charged residues, so it is reasonable that the mechanism by which MTS mediates transduction of proteins across membranes differs from that of the TAT peptide. This result, coupled with the fact that CP-CRE does not activate BMAL1 (Figures 4A and 4B) or cause phase resetting (Figures 5A and 5D), indicates that the phase resetting and BMAL1 activation we observe with CP-CRY1 and CP-CRY2 is likely to be due to a CRY-specific action and not due to a nonspecific increase of membrane permeability.

### Supplemental References

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S2. Lin, K., Sadée, W., and Quillan, J.M. (1999). Rapid measurements of intracellular calcium using a fluorescence plate reader. Biotechniques *26*, 318–326.

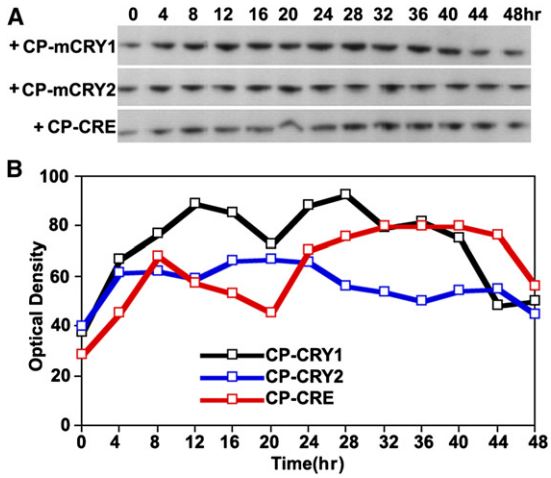


Figure S5. CLOCK-Protein Abundance Is Not Significantly Induced by CP-CRYs in Rat-1 Fibroblasts

(A) CLOCK protein expression in response to continuous treatment of CP-CRY1, CP-CRY2, or CP-CRE. CLOCK protein abundance was assessed by immunoassay.

(B) Densitometry of data depicted in (A).

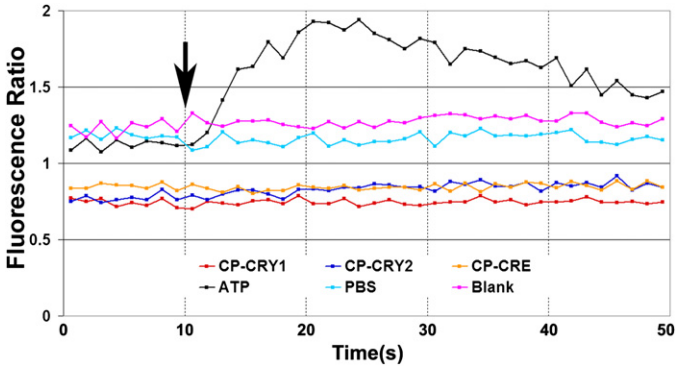


Figure S6. CP Proteins Do Not Evoke a $Ca^{2+}$ Flux

Rat-1 fibroblasts were loaded with the $Ca^{2+}$ indicator dye fura-2 with the membrane-permeable ester form, fura-2/AM, in accordance with standard methods [S2, S3]. ATP (100 μM) provokes a rapid increase of cytosolic $Ca^{2+}$, but not CP-CRY1 (100 nM), CP-CRY2 (100 nM), CP-CRE (100 nM), or the PBS control elicit an increase of cytosolic $Ca^{2+}$.

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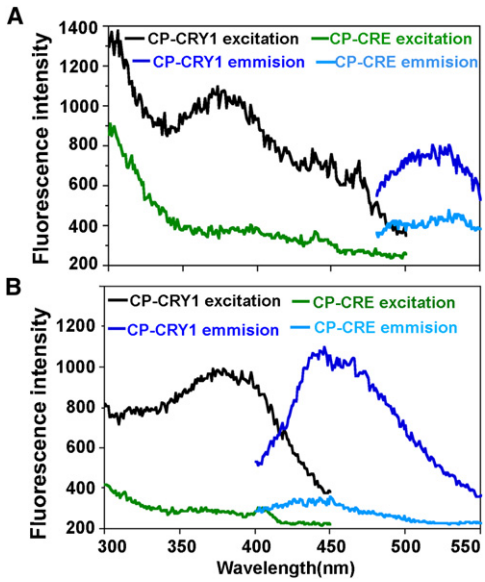


Figure S7. CP-CRY Proteins Have Incorporated Cryptochrome's Cofactors FAD and Pterin

(A) Fluorescence-excitation and -emission spectra of CP-CRY1 at pH 2. We recorded the fluorescence-excitation spectrum of CP-CRY1 (black) and CP-CRE (green) by monitoring emission at 520 nm and recorded the fluorescence-emission spectrum of CP-CRY1 (blue) and CP-CRE (cyan) by monitoring excitation at 450 nm. (B) Fluorescence-excitation and -emission spectra of CP-CRY1 at pH 10. We recorded fluorescence-excitation spectra of CP-CRY1 (black) and CP-CRE (green) by monitoring emission at 460 nm and recorded fluorescence-emission spectra of CP-CRY1 (blue) and CP-CRE (cyan) by monitoring excitation at 380 nm. The spectrum taken at pH 2 (A) shows that there is flavin, and the spectrum taken at pH 10 (B) shows that the flavin fluorescence is quenched at high pH, and this indicates that the flavin is FAD. The spectrum at pH 10 (B) also shows that high pH converts MTHF to the highly fluorescent oxidized pterin, consistent with the presence of both chromophores in CP-CRY1 [S4]. We conclude that CP-CRY1 contains FAD and a pterin as the two chromophore/cofactors, like the majority of the members of the photolyase/cryptochrome family.

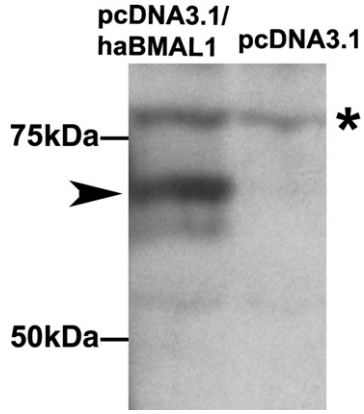


Figure S8. Characterization of the Antibody Directed against BMAL1

An anti-BMAL1 polyclonal IgG (Oncogene catalog #PC539, lot #D13920-1) was used for immunoblotting of BMAL1. For characterization of the anti-BMAL1 polyclonal IgG (#PC539, Oncogene), a plasmid containing hamster Bmal1 driven by the CMV promoter (pcDNA3.1/haBMAL1) was transfected in HEK 293 cells with LipofectAMINE 2000 reagent with pcDNA3.1 as a vector control. Twenty-four hours after transfection, cells were collected and separated on 7.5% gel for immunoblotting.