Supplementary Information

Cryptochrome Mediates Circadian Regulation of cAMP Signalling and Hepatic Gluconeogenesis

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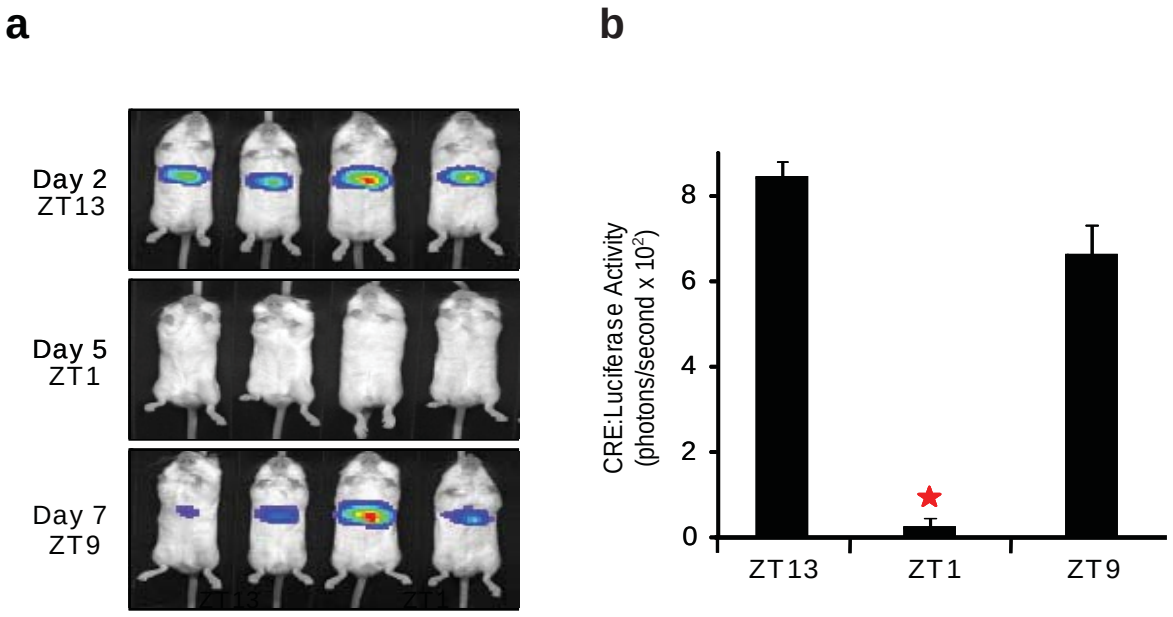
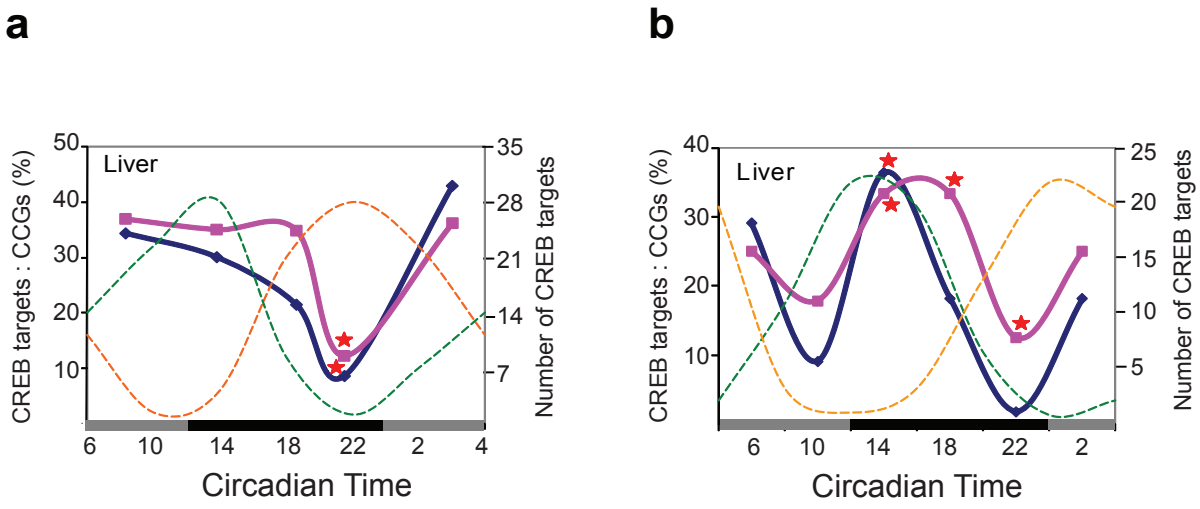
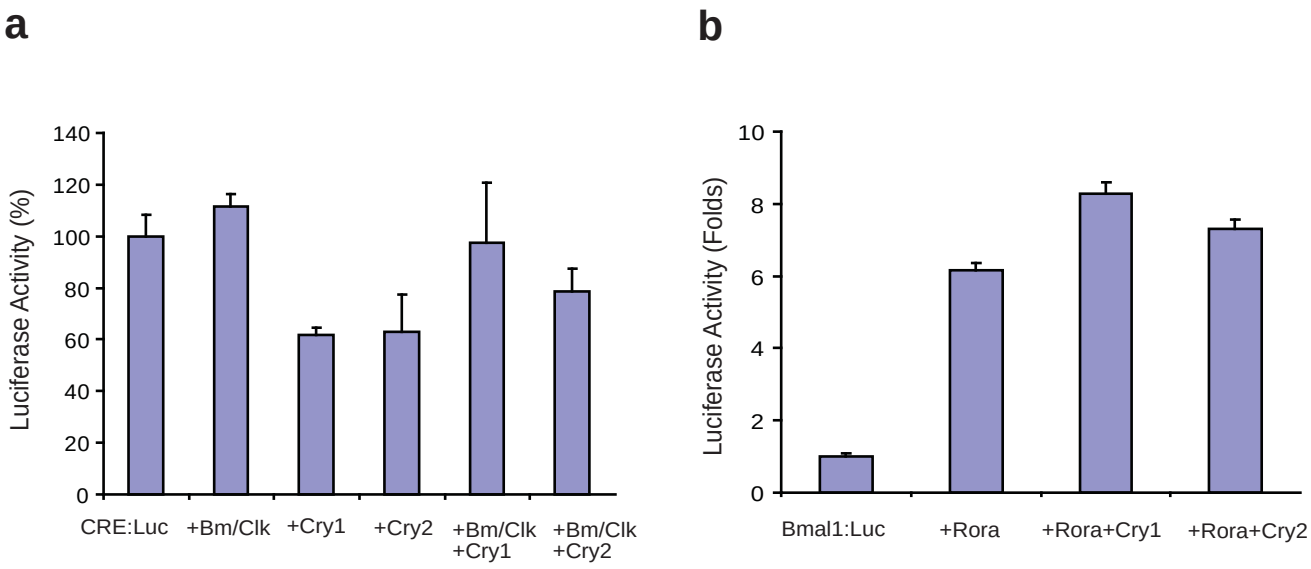


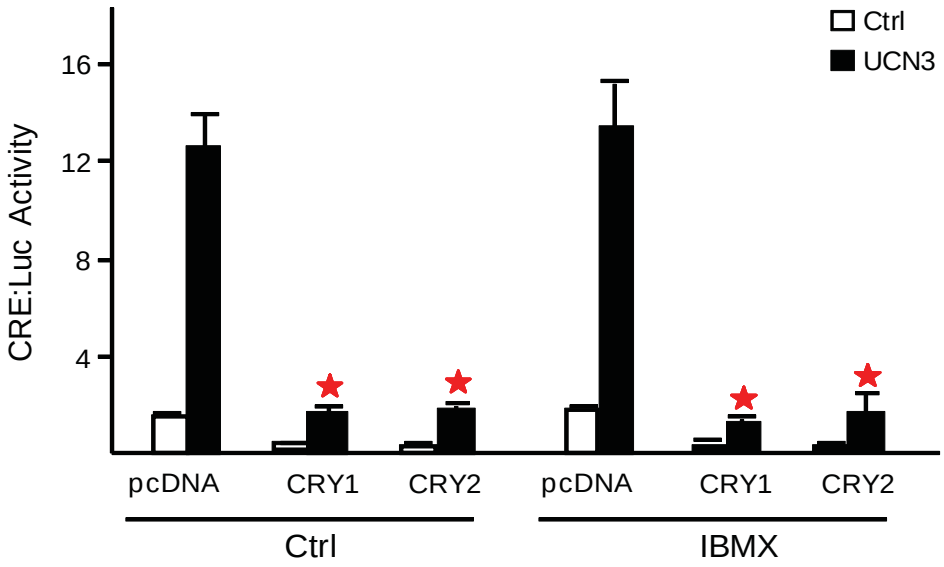
Supplementary Figure 1: Circadian gating of CRE:Luc induction by glucagon stimulation. Adenoviruses carrying a CRE:Luc reporter were injected into mice via tail vein at ZT3-8 on day 0. Mice were then fasted for three hours prior to glucagon injection, followed by injection of luciferin 1 hr later. Luciferase activity was monitored through bioluminescence imaging at the indicated time points (ZT13, ZT1 and ZT9; Left panel) and normalized with b-galactosidase activity (right panel). To minimize variation among individual animals, imaging was conducted in the same mouse at different time-points, i.e., the same mice were placed at the same order in each panel. Note that the CRE:Luc activity was repressed at ZT1.



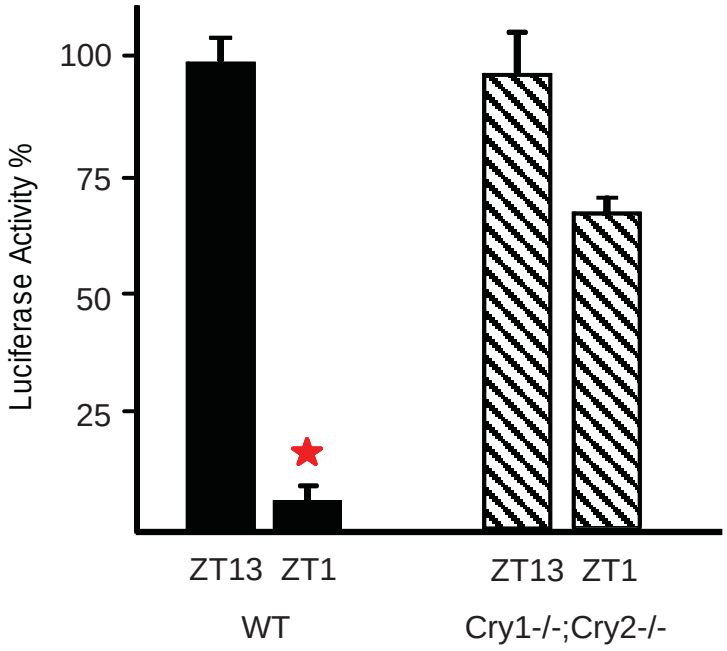
Supplementary Figure 2: Bioinformatics analysis of CREB targets among CCGs. The sum (blue solid) and ratio (pink solid) of CREB target genes are plotted against the circadian time. Analyses were based on the CCG data sets reported by Ueda et al (**a**) and by Panda et al (**b**) (see ref. S1 and S2). The standard used to define a CREB target was described before (ref. S3). For comparison of gene expression, Per2 (green dot) and Bmal1 (red dot) expression patterns versus circadian time were also shown. Note that both CREB targets numbers and the ratios are significantly less at CT22, compared to average CREB targets vs non-CREB target CCGs. Asterisks indicate $P < 0.05$ as analyzed by contingency tables, two-tailed Fisher's exact test.



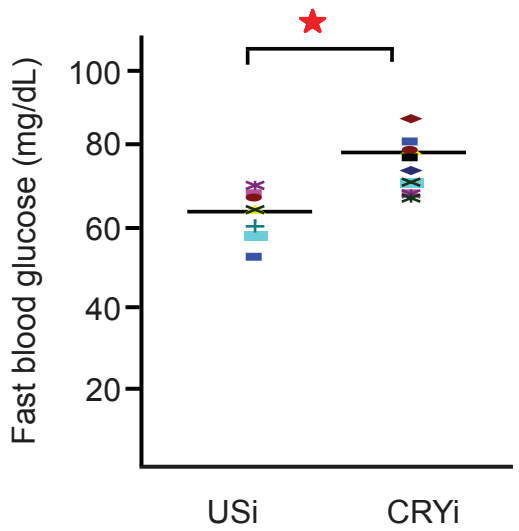
Supplementary Figure 3: Specificity of CRY repression on CREB-dependent signaling. **a**, CRE:Luc reporter activity is not modulated by BMAL1/CLOCK, suggesting that inhibition by CRY is not E-box mediated. **b**, CRY did not inhibit Bmal1:Luc expression activated by RORa.



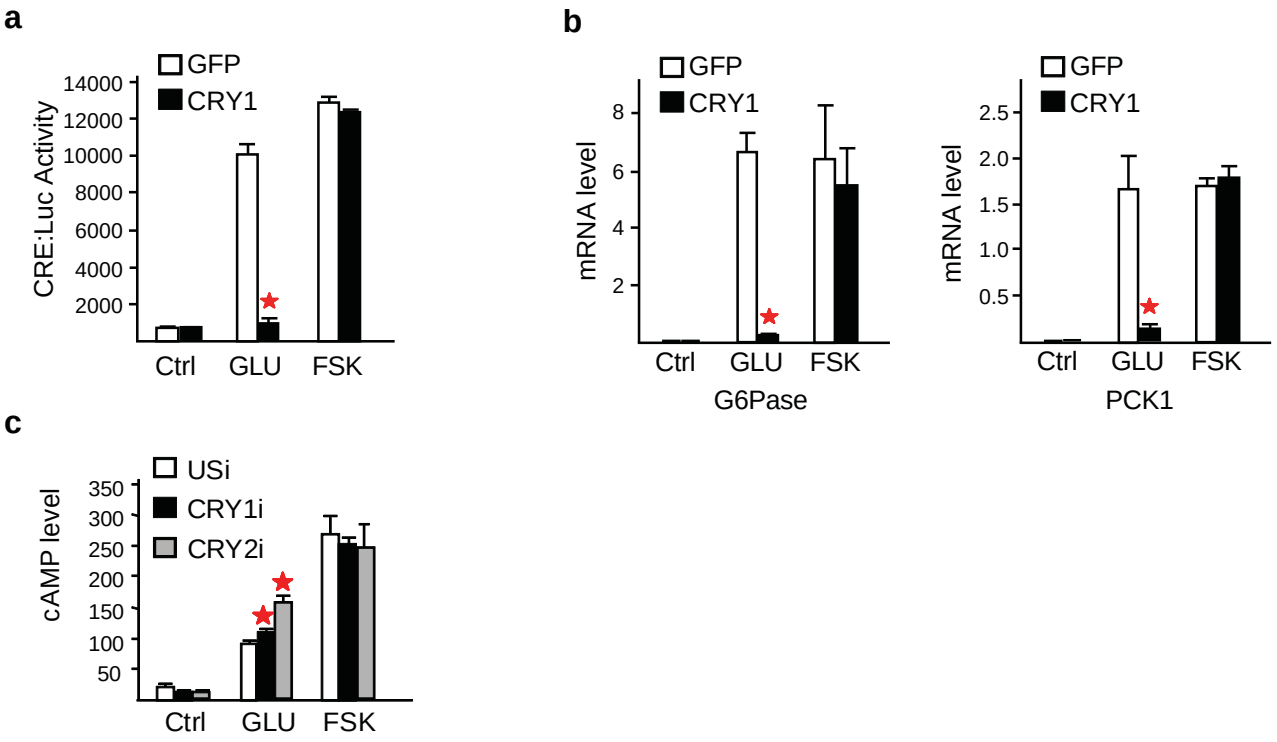
Supplementary Figure 4: CRE:Luc reporter activity in HEK293T cells transfected with CRFR2B and exposed to UCN3. Co-treatment with phosphodiesterase inhibitor IBMX indicated. Asterisks indicate $P < 0.05$, n=3.



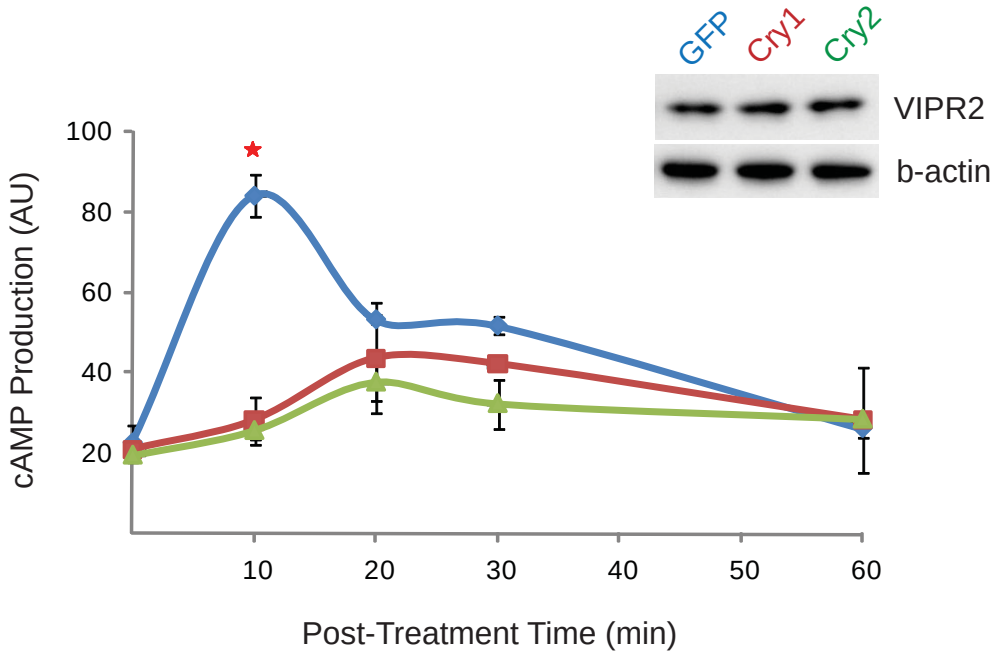
Supplementary Figure 5: Loss of circadian gating in the liver of Cry1/2 knockouts. In the absence of CRY function in Cry1-/-;Cry2-/- mice, CRE:Luc expression at ZT1 was significantly higher than those in WT ($p < 0.001$ for WT and $p = 0.291$ for Cry1-/-;Cry2-/- mice; $n = 3$).



Supplementary Figure 6: Circulating blood glucose concentrations is higher in fasted CRY-deficient mice. Mice were fasted for overnight and blood glucose was measured at ZT1. Asterisk indicates $P < 0.02$; $n = 8$ for each genotype. All data are shown in individual dots.



Supplementary Figure 7: CRY1 inhibits CRE:Luc reporter activity and gluconeogenic gene expression in cultured primary hepatocytes. a. Primary hepatocytes from CRE:Luc transgenic mice were infected with Ad-GFP or Ad-Cry1 for 24 hr, then stimulated with glucagon or adenyl cyclase activator FSK indicated. **b.** Gluconeogenic gene expression in primary hepatocytes stimulated with glucagon or FSK. **c**. RNAi-mediated knockdown of CRY1 and CRY2 increases cAMP accumulation. Intracellular cAMP levels were measured in primary hepatocytes exposed to glucagon (100nM) or FSK (10uM). All data are shown in mean and s.e.m representing at least three independent experiments. Asterisks indicate $P < 0.05$, $n = 3$.



Supplementary Figure 8: Inhibition of cAMP production by CRY overexpression in fibroblasts. VIPR2/CRE:Luc fibroblast cells infected with GFP control (blue), Cry1 (red), or Cry2 (green) were used for cAMP production assay after 2 nM VIP treatment. $P < 0.001$ at time point of 10 min post-treatment. All data are shown in mean and s.e.m representing three independent experiments. Insert shows equal amount of VIPR2 (V5-tagged) gene expression among these cell lines.

Supplementary detailed methods:

Cell Culture

HEK293T cells were cultured in DMEM with 10% FBS and transfected with plasmids by using lipofectamine2000. Mouse primary hepatocytes were isolated and cultured as previously described. For reporter studies, Ad-CRE:Luc and RSVβ-gal infected hepatocytes (1 pfu virus^{-1} cell^{-1}) were exposed to GLU (100 nM) for 4–6 h. For cAMP studies, Lv or Ad-CRY1, CRY2 or GFP infected hepatocytes (1 pfu virus^{-1} cell^{-1}) were exposed to GLU (100 nM) or FSK (10 μM, Sigma) for 5 min without 3-isobutyl-1-methylxanthine (IBMX) pretreatment or 30 min with half hour IBMX (500 μM) pre-treatment, intracellular cAMP levels were determined by cAMP Elisa kit (Roche) and normalized to protein concentration.

Glucose production assay

Primary hepatocytes were prepared and cultured as described previously (ref. S4). Briefly, liver from fed mice (8–12 week-old C57Bl/6, male) was perfused with Hank's balanced salt solution (HBSS), followed by treatment with 0.02% collagenase. Cell viability was higher than 70% as assessed by the trypan blue exclusion test. Cells were seeded as 5×10^5 per well in a 6-well dish in M199 Medium (Invitrogen) supplemented with 10% fetal bovine serum. After a 6 h attachment, adenoviruses were applied in 1ml fresh M199 medium at multiplicity of infection (M.O.I) of 5 for each CRYi and 10 for control USi for 120 min; replaced with 2ml fresh M199 medium supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), and glutamine (0.29 mM). After incubation for 42–45 h, cells were treated with glucagon (10 μg ml^{-1}, Sigma) for 5 h, gently washed 3 times with PBS, followed by incubation with glucose production

buffer (PBS supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate) for additional 2 h. Supernatant was collected for measuring glucose concentration using a fluorometric assay (BioVision). The readings were normalized with total protein in whole-cell lysates.

Cell fractionation

HEK293T cells were collected, washed twice with PBS, resuspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 1mM EDTA, 100 μM PMSF and proteinase inhibitors), passed through 27-G needles 6 times, and then centrifuged at 500 × *g* for 5 min. The pellets were re-extracted with 1 ml lysis buffer three more times and the combined supernatants were ultracentrifuged at 40,000 × *g* for 30 min. The resulting supernatant was allocated as cytosol fraction and the pellet was resuspended in extraction buffer (75 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol and 12.5 mM $MgCl_2$, 100 μM PMSF and proteinase inhibitors) as membrane fraction. All the above procedures were carried either on ice or under 4 °C. Protein concentrations of both fractions were measured by Bio-Rad protein assay kit, adjusted to 1 μg μl^{-1} and kept in –80 °C freezer.

In vitro adenyl cyclase activity assay

Cytosol fraction with GFP or HA-CRY1 protein was prepared from HEK293T cells infected with Ad-GFP or Ad-CRY1 adenovirus, respectively by following the above protocol. For AC activity assay, equal volume of membrane and cytosol fractions (25 μl + 25 μl) were mixed, incubated at 37 °C for 5 min, and then mixed with 25 μl AC reaction buffer (5 mM Tris-HCl, pH 7.4, 17.5 mM MgCl2, 120 μM ATP, 3 μM GTP, 1.5 μM FAD, freshly adding 60 mM phosphocreatine and 0.375 Unit Creatine phosphokinase) in the presence or absence of

isoproterenol (100 μM final) or FSK (1 μM final). Reactions were carried at 37°C for 30 min, stopped by mixing with 20 μl 2.2M HCl, and then neutralized by 20 μl 2.2M NaOH. Levels of cAMP were measured by ELISA kit (R&D) by following its manual. To exclude the possibility that isoproterenol or FSK interrupts cAMP ELISA assay, isoproterenol (100 μM final) or FSK (1 μM final) were added in the mixture of lysis, extraction, and reaction buffers alone for cAMP assay. For the pre-clearance of HA-CRY1, cytosol fractions from Ad-HA-CRY1 infected HEK293T cells were immuneprecipitated by anti-HA or mouse IgG beads at 4 °C for 1 hour.

GST pull-down

GST-CRY1 and control GST-GFP proteins were prepared as described (ref S5). Briefly, bacteria BL21 AI (Invitrogen) harbouring either construct DNA were grown till $O.D._{600}$ = 0.7–1.0, subsequently induced by Isopropyl β-D-1-thiogalacto-pyranoside (IPTG, 1mM) and Arabinose (0.2% w:v) for 5 h at room temperature with 250 rpm shaking. After sonication, cell lysates were incubated with Glutathione-Sepharose beads (GE Healthcare) for purification. For the pull-down assay, 1×10^7 293T cells were prepared for membrane fractionation as described above. Membrane pellets were dissolved in pull-down buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 2mM Dithiothreitol (DTT), 100 μM PMSF and proteinase inhibitors), and mixed with 10 μg GST proteins as determined by Coomassie Brilliant Blue staining. After 2 h incubation at 4 °C and washing 5 times with pull-down buffer, pellets were suspended in SDS loading buffer. For each loading, 1% input samples were applied for quantification.

Statistical analyses

All studies were performed on at least three independent occasions. Results are reported as mean and s.e.m. Except for described, the comparison of different groups was carried out using a two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Supplementary References:

1. Ueda, H.R. A transcription factor response element for gene expression during circadian night. *Nature* **418**, 534-539 (2002).
2. Panda, S., *et al.* Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109**, 307-320 (2002).
3. Zhang, X., *et al.* Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proceedings of the National Academy of Sciences* **102**, 4459-4464 (2005).
4. Dentin, R., *et al.* Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. *J. Biol. Chem.* **279**, 20314-20326 (2004).
5. Sanada, K., Harada, Y., Sakai, M., Todo, T. & Fukada, Y. Serine phosphorylation of mCRY1 and mCRY2 by mitogen-activated protein kinase. *Genes to Cells* **9**, 697-708 (2004).