# Supporting Information

## Yin et al. 10.1073/pnas.1000438107

### SI Materials and Methods

**Reagents.** The Bmal1-luc (WT or RORE mutant) reporter constructs have been described previously (1). All plasmids were confirmed by automated sequencing analysis. All siRNA oligos were obtained from Dharmacon. Dexamethasone, forskolin, succinylacetone, and heme were purchased from Sigma-Aldrich. Anti-Arfbp1 was purchased from Usbio. Anti-M2 agarose beads were obtained from Sigma-Aldrich. MG132 and cycloheximide were purchased from Biomol. GSK-3β inhibitor IX was purchased from EMD Bioscience.

**Reporter Gene Assays.** Reporter gene assays were performed in 293T cells following a previously described protocol (1). In a typical experiment, 100 ng of luciferase plasmid was mixed with 200–400 ng of expression constructs of transcription factors. In the siRNA knockdown experiments, ~100 pmol of siRNA oligos was used to tranfected target cells along with reporter vectors. Equal amounts of DNA were used for all transfection combinations by adding appropriate amounts of vector DNA. Relative luciferase activities were determined at 48–72 h after transfection. Light units were normalized to the cotransfected β-galactosidase expression plasmid. Fold repression was calculated as the activity of a given reporter after transfection with control siRNA divided by the activity of the same reporter in the presence of gene-specific siRNA oligos. All transfection experiments were performed in triplicate.

**Q-PCR Primers.**

Arf-bp1 (human): forward, TTGGGCTAGATGGTGACACA; reverse, GCCTAATCTGGAGGAAGCTG
Pam (human): forward, TGAAGACAAGAAGCAAGTCTGAA; reverse, ACGCAATGGCGTACATTACA
Arf-bp1 (mouse): forward, CCCATGGAGACAGATGAACC; reverse, TCGGCCTAATCTGGAAGAAG
Pam (mouse): forward, ATGGTGAAAGCACAAAAATCG; reverse, GCGTCTCCAGAGATGAGCTT
Cry1 (mouse): forward, AGCGCAGGTGTCGGTTATGAGC; reverse, ATAGACGCAGCGGATGGTGTCG
Per2 (mouse): forward, CAGCTGCCCTCCCGGGATCT; reverse, TCGGAGCTCTCGCCTCTGGC
Bmal1 (mouse): forward, GCAGTGCCACTGACTACCAAGA; reverse, TCCTGGACATTGCATTGCAT

1. Yin L, Lazar MA (2005) The orphan nuclear receptor Rev-erbalpha recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian *Bmal1* gene. *Mol Endocrinol* 19:1452–1459.

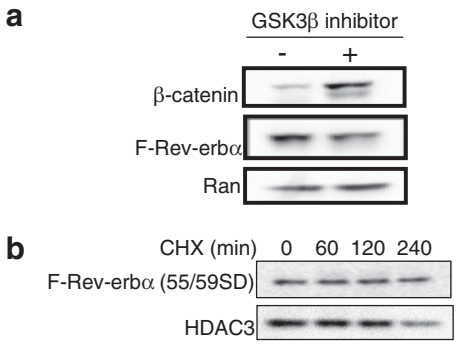


**Fig. S1.** Effect of GSK3β inhibitor and cycloheximide on Rev-erbα protein. (*A*) Effect of GSK3β inhibitor on f-Rev-erbα protein. HFR8 cells were treated with DMSO or 5 nM GSK3β inhibitor Ix for 12 h, and the levels of f-Rev-erbα were determined by immunoblot analysis. The β-catenin blot was shown as a positive response to inhibition of GSK3β. (*B*) The protein half-life of f-Rev-erbα 55/59SD mutant. The 293T cells were transfected with f-Rev-erbα 55/59SD expression vector and treated with cycloheximide (20 μg/mL) for 0, 60, 120, and 240 min. The levels of f-Rev-erbα were determined by immunoblot analysis.

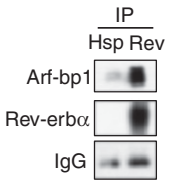


**Fig. S2.** Interaction of endogenous Rev-erbα and Arf-bp1. HepG2 cells treated with lithium and Mg132 were subjected to immunoprecipitation with anti–Rev-erbα.

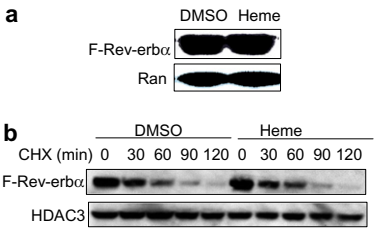


**Fig. S3.** Lack of effect of heme on Rev-erbα protein stability. (*A*) HFR cells were incubated in serum-free medium for 8 h before heme treatment (10 μM for 12 h). The cell lysates were collected for immunoblot analysis of the levels of f-Rev-erbα. (*B*) HFR cells were incubated in serum-free medium supplemented with succinylacetone (5 mM) for 16 h to deplete heme before being exposed to DMSO or 6 μM heme for 6 h. Cells were then treated with cycloheximide (20 μg/mL) for the indicated times. The levels of Rev-erbα and HDAC3 were determined by immunoblot analysis.

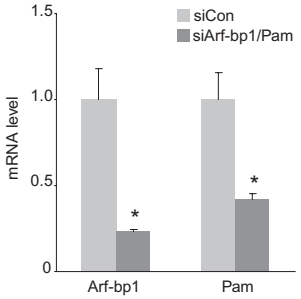


**Fig. S4.** Validation of Arf-bp1 and Pam knockdown. Shown are the mRNA levels for Arf-bp1 and Pam in HFR cells in which both Arf-bp1 and Pam were silenced using specific siRNA oligos. Plotted data are mean ± SD. **P* < 0.05 by Student's *t* test.

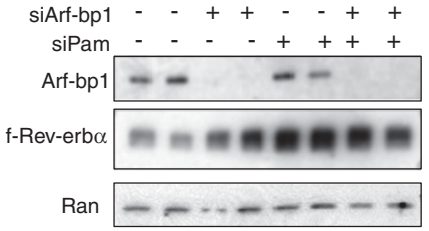


**Fig. S5.** Effects of Arf-bp1 or Pam knockdown on f-Rev-erbα protein. HFR cells were transfected with control siRNA, Arf-bp1 alone or Pam alone, or Arf-bp1 and Pam siRNA oligos together. Cell lysates were analyzed by immunoblotting for the levels of Arf-bp1, f-Rev-erbα, and Ran as a loading control.

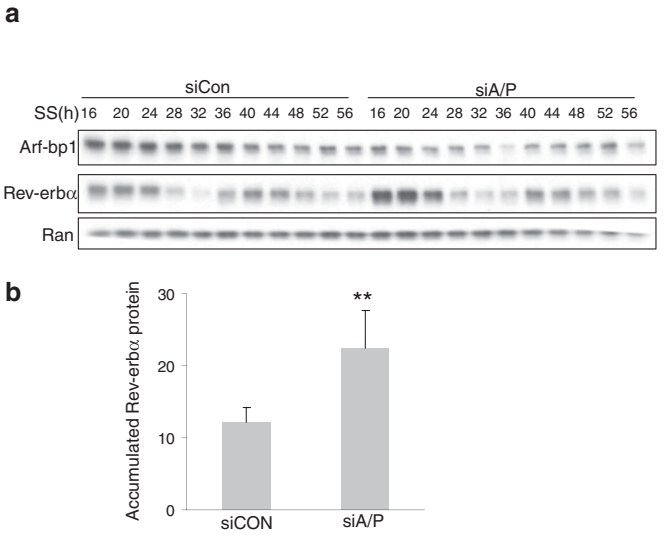


**Fig. S6.** Effect of Arf-bp1 and Pam knockdown on Rev-erbα protein oscillation. (*A*) Circadian oscillation in endogenous Rev-erbα protein in synchronized Hepa1c1c-7 cells with both Arf-bp1 and Pam knockdown. The representative immunoblot analysis results of three replicates are shown for the levels of Arf-bp1, Rev-erbα, and Ran (loading control) during two circadian cycles. (*B*) The circadian variation of Rev-erbα protein between control or Arf-bp/Pam knockdown was calculated by AUC analysis. Plotted data are mean area ± SD. ***P* < 0.01.

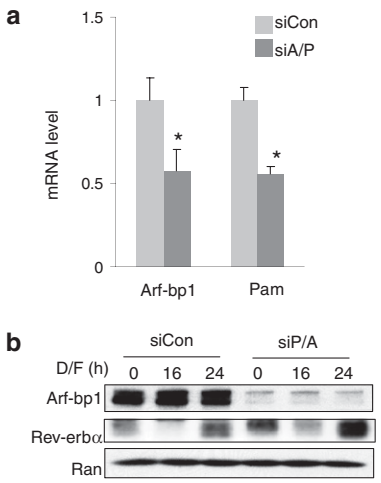


**Fig. S7.** Effect of Arf-bp1 and Pam knockdown on the molecular clock. (*A*) Hepa1c1c-7 cells were transfected with control or Arf-bp1 and Pam siRNA, and then synchronized by addition of dexmethasome and forskolin. The mRNA levels for both Arf-bp1 and Pam after knockdown transfection were measured by qPCR. Plotted data are mean ± SD. $*P < 0.05$ by Student's *t* test. (*B*) The levels of endogenous Rev-erbα protein were measured by immunoblot analysis at time 0, 16, and 24 h after synchronization.