

function; yet, each superfamily of those enzymes comprises different enzyme families, from which genes may have been recruited. Also, the pathway of acetyl-CoA assimilation and phosphoenolpyruvate formation may differ. At last, one may be able to trace back the roots of the individual enzymes and come to tentative conclusions regarding the evolution of this cycle. Carboxyphosphate, which is used in the biotin-dependent carboxylase reactions, is an attractive model for carbon fixation during chemoevolution.

Several questions remain. What is the function of two different copies of 4-hydroxybutyryl-CoA dehydratase genes in autotrophic Sulfolobales? What are the functions of acetyl-CoA carboxylase and the enzymes of part I of the previously unrecognized pathway in various heterotrophic Archaea? Does the second part of the pathway, when operated in the reverse direction, serve as an acetyl-CoA assimilation route in some Archaea that lack the first part of the cycle? Which of the potential autotrophic pathways is operating in *Archaeoglobus*, which harbors the genes for the reductive acetyl-CoA pathway, for the proposed new pathway, and for RuBisCO (31, 32)?

#### References and Notes

1. G. Wächtershäuser, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 200 (1990).

2. O. K. Kandler, K. O. Stetter, *Zentbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. C 2*, 111 (1981).
3. M. Ishii *et al.*, *Arch. Microbiol.* **166**, 368 (1997).
4. C. Menendez *et al.*, *J. Bacteriol.* **181**, 1088 (1999).
5. N. P. Burton, T. D. Williams, P. R. Norris, *Arch. Microbiol.* **172**, 349 (1999).
6. M. Hügler, H. Huber, K. O. Stetter, G. Fuchs, *Arch. Microbiol.* **179**, 160 (2003).
7. H. Holo, *Arch. Microbiol.* **151**, 252 (1989).
8. G. Strauss, G. Fuchs, *Eur. J. Biochem.* **215**, 633 (1993).
9. H. Huber, K. O. Stetter, in *Bergey's Manual of Systematic Bacteriology*, Vol. 1, D. R. Boone, R. W. Castenholz, Eds. (Springer-Verlag, New York, ed. 2, 2001), pp. 198–202.
10. K. O. Stetter, *Syst. Appl. Microbiol.* **10**, 172 (1988).
11. C. M. Preston, K. Y. Wu, T. F. Molinski, E. F. DeLong, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6241 (1996).
12. M. Könneke *et al.*, *Nature* **437**, 543 (2005).
13. B. M. Martins, H. Dobbek, I. Çinkaya, W. Buckel, A. Messerschmidt, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 15645 (2004).
14. U. Näser *et al.*, *Bioorg. Chem.* **33**, 53 (2005).
15. A. Gerhardt, I. Çinkaya, D. Linder, G. Huismann, W. Buckel, *Arch. Microbiol.* **174**, 189 (2000).
16. Materials and methods are available as supporting material on Science Online.
17. B. Alber *et al.*, *J. Bacteriol.* **188**, 8551 (2006).
18. S. Chuakrut, H. Arai, M. Ishii, Y. Igarashi, *J. Bacteriol.* **185**, 938 (2003).
19. M. Hügler, R. S. Krieger, M. Jahn, G. Fuchs, *Eur. J. Biochem.* **270**, 736 (2003).
20. S. J. Hallam *et al.*, *PLoS Biol.* **4**, e95 (2006).
21. G. G. B. Tcherkez, G. D. Farquhar, T. J. Andrews, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 7246 (2006).
22. R. J. Ellis, *Trends Biochem. Sci.* **4**, 241 (1979).

23. U. Jahn, H. Huber, W. Eisenreich, M. Hügler, G. Fuchs, *J. Bacteriol.* **189**, 4108 (2007).
24. Y. Hu, J. F. Holden, *J. Bacteriol.* **188**, 4350 (2006).
25. U. Scherf, B. Söhling, G. Gottschalk, D. Linder, W. Buckel, *Arch. Microbiol.* **161**, 239 (1994).
26. S. Yooshep *et al.*, *PLoS Biol.* **5**, e16 (2007).
27. D. B. Rusch *et al.*, *PLoS Biol.* **5**, e77 (2007).
28. M. B. Karner, E. F. DeLong, D. M. Karl, *Nature* **409**, 507 (2001).
29. A. E. Ingalls *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 6442 (2006).
30. A. Francis, J. M. Beman, M. M. M. Kuypers, *ISME J.* **1**, 19 (2007).
31. H.-P. Klenk *et al.*, *Nature* **390**, 364 (1997).
32. M. W. Finn, F. R. Tabita, *J. Bacteriol.* **185**, 3049 (2003).
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#### Supporting Online Material

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Materials and Methods

Figs. S1 to S7

Tables S1 and S2

References

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## Rev-erba, a Heme Sensor That Coordinates Metabolic and Circadian Pathways

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The circadian clock temporally coordinates metabolic homeostasis in mammals. Central to this is heme, an iron-containing porphyrin that serves as prosthetic group for enzymes involved in oxidative metabolism as well as transcription factors that regulate circadian rhythmicity. The circadian factor that integrates this dual function of heme is not known. We show that heme binds reversibly to the orphan nuclear receptor Rev-erba, a critical negative component of the circadian core clock, and regulates its interaction with a nuclear receptor corepressor complex. Furthermore, heme suppresses hepatic gluconeogenic gene expression and glucose output through Rev-erba-mediated gene repression. Thus, Rev-erba serves as a heme sensor that coordinates the cellular clock, glucose homeostasis, and energy metabolism.

Circadian rhythms are intrinsic time-keeping mechanisms conserved throughout the animal kingdom (1–3). Many aspects of mammalian behavior and physiology, including sleep-wake cycles, blood pressure, body temperature, and metabolic pathways are controlled by the circadian clock (3–5). At the molecular level, cellular rhythms are generated and maintained through interconnected transcriptional-translational feedback loops of clock genes, which are conserved in the central pacemaker and in peripheral tissues (2, 3). The nuclear receptor Rev-erba has been identified as a link between positive and

negative loops of the circadian clock by controlling rhythmic expression of the *Bmal1* gene (6–9). This repression function is dependent on recruitment of the nuclear receptor corepressor–histone deacetylase 3 (NCoR-HDAC3) complex directly to the *Bmal1* gene.

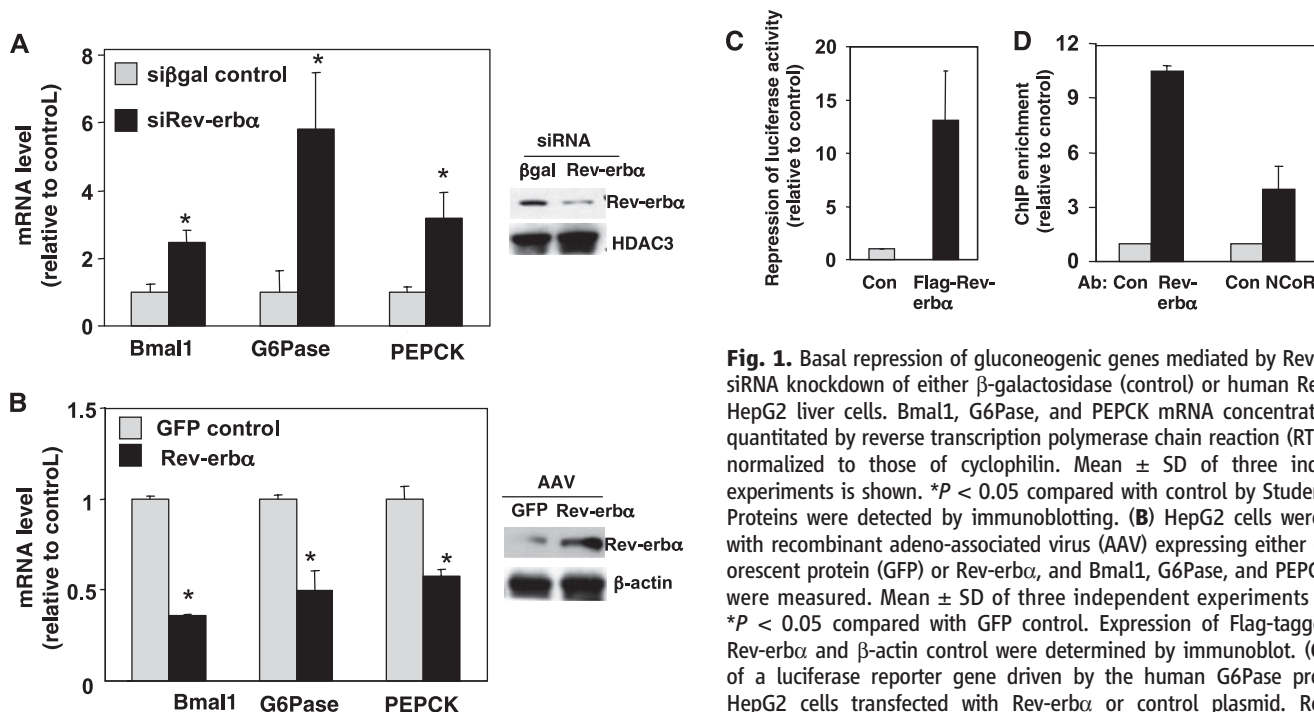
Several genomewide expression studies have highlighted that genes involved in glucose metabolism, lipid metabolism, heme biosynthesis, and mitochondrial adenosine triphosphate (ATP) synthesis all exhibit a circadian pattern of expression (1, 10–12). For example, gluconeogenic genes such as phosphoenol pyruvate carboxykinase

(PEPCK) and glucose 6-phosphatase (G6Pase) are typical clock-controlled metabolic-related genes (12). Furthermore, mice deficient in either *Bmal1* or *Clock* genes exhibit abnormal metabolic activities (13–15). Despite the evidence linking circadian clocks and metabolism, the mechanism by which the circadian clock is integrated into metabolic systems remains poorly understood.

To investigate the role of Rev-erba in metabolic gene regulation, we first depleted Rev-erba from HepG2 human hepatoma cells with small interfering RNA molecules (siRNAs). Expression of genes encoding gluconeogenic enzymes G6Pase and PEPCK was significantly increased when Rev-erba amounts were reduced (Fig. 1A). By contrast, loss of Rev-erba did not affect the expression of acetyl-coenzyme A (CoA) carboxylase or fatty acid synthase, which are involved in fatty acid metabolism (fig. S1). Overexpression of full-length Rev-erba in HepG2 cells decreased expression of G6Pase, PEPCK, and the known Rev-erba target *Bmal1* (Fig. 1B). Further, Rev-erba repressed expression of a luciferase

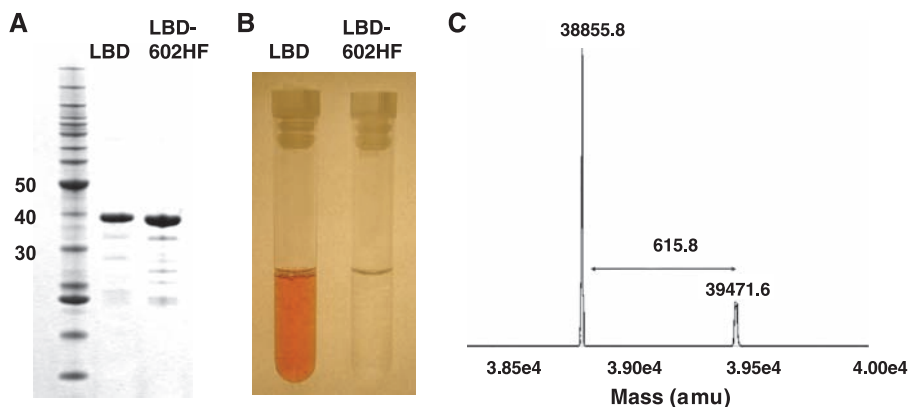
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**Fig. 1.** Basal repression of gluconeogenic genes mediated by Rev-erb $\alpha$ . **(A)** siRNA knockdown of either  $\beta$ -galactosidase (control) or human Rev-erb $\alpha$  in HepG2 liver cells. Bmal1, G6Pase, and PEPCK mRNA concentrations were quantitated by reverse transcription polymerase chain reaction (RT-PCR) and normalized to those of cyclophilin. Mean  $\pm$  SD of three independent experiments is shown. \* $P$  < 0.05 compared with control by Student's  $t$  test. Proteins were detected by immunoblotting. **(B)** HepG2 cells were infected with recombinant adeno-associated virus (AAV) expressing either green fluorescent protein (GFP) or Rev-erb $\alpha$ , and Bmal1, G6Pase, and PEPCK mRNAs were measured. Mean  $\pm$  SD of three independent experiments is shown. \* $P$  < 0.05 compared with GFP control. Expression of Flag-tagged mouse Rev-erb $\alpha$  and  $\beta$ -actin control were determined by immunoblot. **(C)** Activity of a luciferase reporter gene driven by the human G6Pase promoter in HepG2 cells transfected with Rev-erb $\alpha$  or control plasmid. Results are expressed as mean  $\pm$  SD of triplicate samples. **(D)** ChIP assays to detect

association of endogenous Rev-erb $\alpha$  and NCoR with the proximal region of the human G6Pase promoter. Anti-GFP was used as a negative control. Mean  $\pm$  range of duplicate samples is shown.



**Fig. 2.** Binding of heme to Rev-erb $\alpha$ . **(A)** Purified Rev-erb $\alpha$  LBD and 602HF mutant in *E. coli* in the presence of 75 mM hemin. 1  $\mu$ l of purified protein was separated by SDS-polyacrylamide gel electrophoresis and stained by Coomassie blue. Numbers to the left indicate molecular mass in kD. **(B)** Rev-erb $\alpha$  LBD, but not the 602HF mutant, is red. Both proteins are shown at concentrations of 3.7 mg/ml. **(C)** Mass spectrometric determination of Rev-erb $\alpha$  LBD-bound hemin. amu, atomic mass units.

reporter driven by the human G6Pase promoter (Fig. 1C). With chromatin immunoprecipitation (ChIP), we observed Rev-erb $\alpha$ , along with NCoR, in the vicinity of potential Rev-erb $\alpha$  binding sites (ROREs) (Fig. 1D). Deletion of the distal RORE from human G6Pase promoter abrogated Rev-erb $\alpha$ -dependent repression activity on this promoter (fig. S2). Taken together, these results suggest that Rev-erb $\alpha$  recruits the NCoR-HDAC3 corepressor complex to actively repress the expression of gluconeogenic genes and in turn may have a role in circadian oscillation of gluconeogenesis.

Heme serves as a prosthetic group for a large number of cellular proteins with diverse biological functions, including mitochondrial respiration,

hormone synthesis and metabolism, and nitric oxide synthesis (16–18). Heme has been identified as the prosthetic group of two clock proteins, NPAS2 (19) and PER2 (20), and implicated in the coregulation of heme biosynthesis and circadian rhythm (20, 21). The homolog of Rev-erb $\alpha$  in *Drosophila melanogaster*, E75, binds heme, which regulates E75 function by increasing protein stability (22, 23). When the Rev-erb $\alpha$  ligand binding domain (LBD, amino acids 281 to 614, optimized for production and purification) was purified from *Escherichia coli* grown in the presence of hemin (Fig. 2A), the protein was red (Fig. 2B). This red color was abolished when His<sup>602</sup> (H602) was mutated to phenylalanine

(602HF, Fig. 2B). Ultraviolet-visible spectrophotometric analysis revealed that the Rev-erb $\alpha$  LBD contained a chromophore with  $\lambda_{\max}$  of 414 nm that shifted to 428 nm in the presence of dithionite (fig. S3), suggesting that heme was bound. By contrast, little absorbance in the 400 to 430 nm range was observed for the 602HF mutant. Mass spectrometric analysis of the Rev-erb $\alpha$  LBD revealed the presence of heme (Fig. 2C), which was not identified in the 602HF mutant (fig. S4). These results demonstrate that Rev-erb $\alpha$  binds to heme and that H602 is critical for this function. Heme bound to the Rev-erb $\alpha$  LBD was displaced by molar excess of heme analogs (fig. S5), indicating that heme binding to Rev-erb $\alpha$  is exchangeable and reversible.

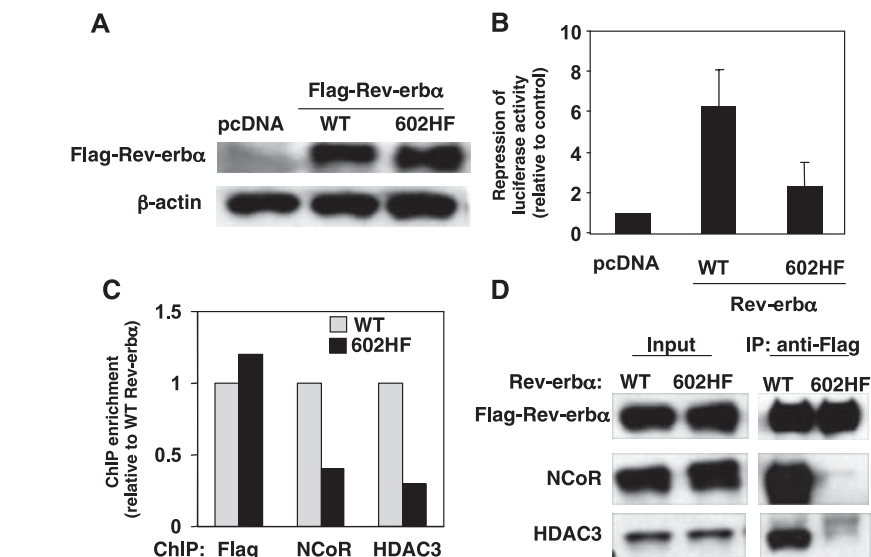
We next assessed the biological function of heme binding to Rev-erb $\alpha$  in mammalian cells. In human embryonic kidney 293T cells, the 602HF mutant that does not bind heme was at least as stable as wild-type (WT) Rev-erb $\alpha$  (Fig. 3A). However, the repression function of this mutant was attenuated on a Bmal1-luciferase vector (9) (Fig. 3B). A similar impairment of the 602HF mutant in repression was observed by using two additional reporter gene constructs that are repressed by WT Rev-erb $\alpha$  (fig. S6). ChIP demonstrated that the 602HF mutant bound at least as well as WT Rev-erb $\alpha$  to the Rev-erb $\alpha$ -responsive Bmal1. By contrast, recruitment of both NCoR and HDAC3 by the 602HF mutant was diminished relative to that of WT Rev-erb $\alpha$  (Fig. 3C). When Flag epitope-tagged Rev-erb $\alpha$  was stably expressed and immunoprecipitated from HeLa cells, WT Rev-erb $\alpha$  was associated with NCoR and HDAC3, whereas the 602HF mutant was not

(Fig. 3D). Thus, the 602HF mutation specifically impaired recruitment of the NCoR-HDAC3 complex to Rev-*erb* $\alpha$ .

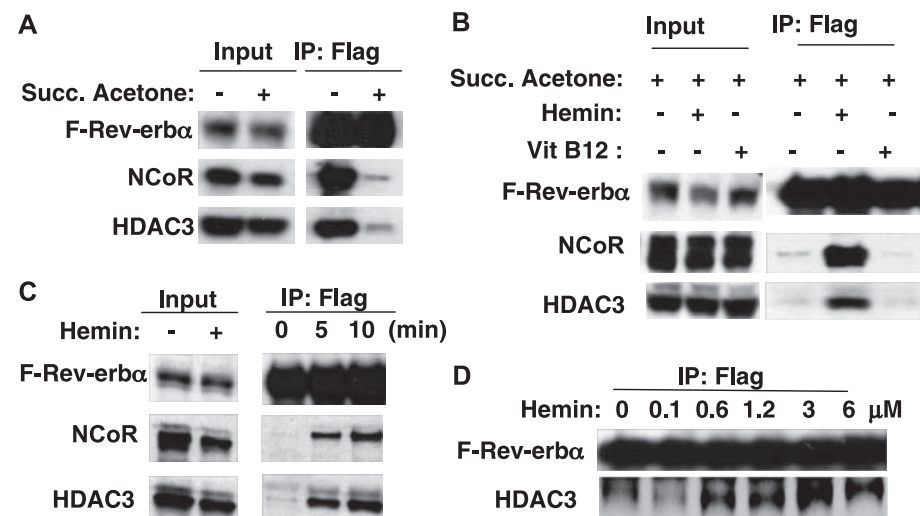
To test whether heme binding to Rev-*erb* $\alpha$  directly regulates its interaction with the NCoR-HDAC3 corepressor complex, we cultured HeLa cells that stably express Rev-*erb* $\alpha$  in serum-free medium supplemented with succinylacetone (SA) to deplete heme (24, 25). Heme depletion was confirmed by the characteristic increase in abundance of mRNA encoding  $\delta$ -aminolevulinic synthase 1 (ALAS1) (fig. S7). The interaction between Rev-*erb* $\alpha$  and NCoR-HDAC3 was nearly abolished in heme-depleted cells (Fig. 4A). This was reversed by incubation of the cells with hemin for 6 hours, whereas the porphyrin-like vitamin B12 did not stabilize the Rev-*erb* $\alpha$ -corepressor complex (Fig. 4B). In vitro, stable association of the NCoR-HDAC3 complex with Rev-*erb* $\alpha$  occurred within 5 min of hemin treatment of extracts from heme-depleted cells (Fig. 4C). Furthermore, the ability of heme to stabilize the association between Rev-*erb* $\alpha$  and NCoR-HDAC3 was dependent on heme concentration (Fig. 4D and fig. S8). Interaction of bacterially expressed Rev-*erb* $\alpha$  LBD with a short peptide derived from NCoR suggested that heme stabilization of the interaction between full-length Rev-*erb* $\alpha$  and endogenous NCoR depends on factors other than the LBD alone (fig. S9).

We tested whether the heme-dependent recruitment of the NCoR-HDAC3 corepressor complex affected expression of circadian and metabolic Rev-*erb* $\alpha$  target genes. Consistent with our biochemical findings, heme depletion significantly increased the expression of the core clock gene *Bmal1* (fig. S10A), whereas hemin treatment significantly suppressed *Bmal1* expression (fig. S10B), indicating that intracellular heme concentrations might regulate this Rev-*erb* $\alpha$  target. Hemin treatment also repressed transcription of the PEPCK and G6Pase genes in human HepG2 liver cells (Fig. 5A). Conversely, heme depletion by knockdown of ALAS1 (fig. S12) significantly induced G6Pase expression and in a manner that was reversed by addition of hemin (Fig. 5B), demonstrating the dependence of G6Pase transcription on heme concentrations. The repressive effect of heme was abrogated when the abundance of Rev-*erb* $\alpha$  was reduced by siRNA, indicating that the heme effect was Rev-*erb* $\alpha$ -dependent (Fig. 5C). Moreover, heme-dependent recruitment of NCoR and HDAC3, and a concomitant reduction in histone acetylation, was observed by ChIP at the endogenous *G6Pase* gene (Fig. 5D). Hemin treatment also repressed the expression of G6Pase and PEPCK in primary mouse hepatocytes (fig. S10) and blunted production of glucose (Fig. 5E), demonstrating the metabolic relevance of heme binding to Rev-*erb* $\alpha$ .

The circadian expression of Rev-*erb* $\alpha$  is regulated both transcriptionally, by BMAL1-CLOCK (26) and by Rev-*erb* $\alpha$  itself (27), as well as posttranslationally, by glycogen synthe-



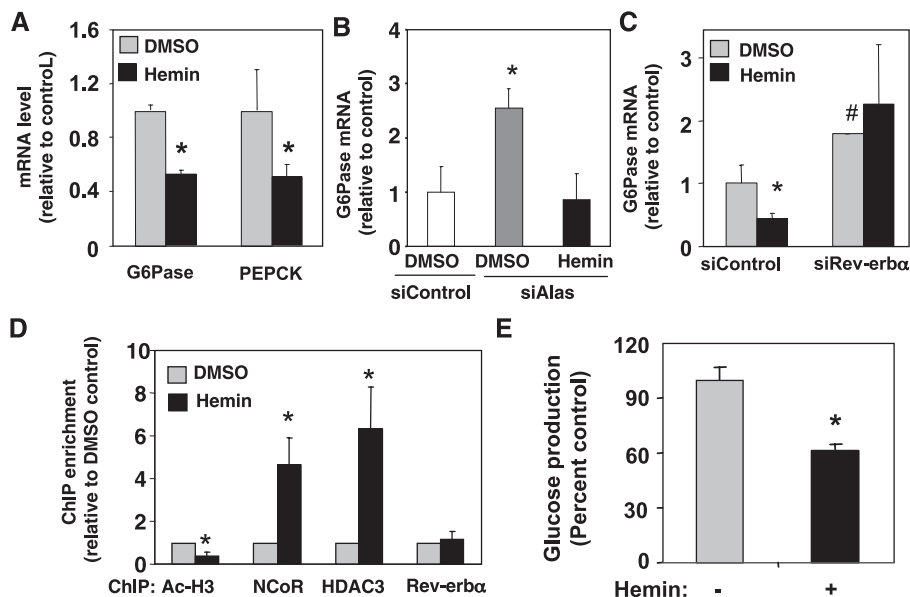
**Fig. 3.** Impaired transcriptional repression by the Rev-*erb* $\alpha$  602HF mutant and failure to recruit the NCoR-HDAC3 corepressor complex. (A) Stability of Flag-tagged full-length Rev-*erb* $\alpha$  WT and 602HF mutant transfected into 293T cells. Proteins were detected by immunoblotting. (B) Repression of *Bmal1*-luciferase reporter in cells transfected with empty vector (pcDNA), WT Rev-*erb* $\alpha$ , or Rev-*erb* $\alpha$ -602HF. Results are expressed as mean  $\pm$  SD of at least three independent experiments. (C) ChIP assay for recruitment of Rev-*erb* $\alpha$ , NCoR, and HDAC3 performed 36 hours after transfection of 293T cells with Flag-tagged Rev-*erb* $\alpha$  WT or 602HF vector and the *Bmal1* luciferase plasmid. (D) Protein-protein interaction between Rev-*erb* $\alpha$  WT or 602HF and NCoR-HDAC3 corepressor complex in HeLa Tet-on cells stably transfected with an inducible expression vector expressing either WT or 602HF Flag-tagged Rev-*erb* $\alpha$ . Cells were lysed and subjected to immunoprecipitation (IP) with FlagM2-conjugated agarose beads. The presence of NCoR and HDAC3 was examined by immunoblotting.



**Fig. 4.** Effect of alteration of heme concentration on the interaction between Rev-*erb* $\alpha$  and NCoR-HDAC3 corepressor complex. (A) HeLa cells stably expressing WT Flag-tagged Rev-*erb* $\alpha$  were treated with SA (5 mM) for 16 hours to deplete intracellular heme. Amounts of NCoR and HDAC3 copurified with Flag-Rev-*erb* $\alpha$  were determined by immunoblotting. (B) HeLa cells expressing Rev-*erb* $\alpha$  were first treated with SA for 16 hours and then treated with either dimethyl sulfoxide (DMSO), hemin (6  $\mu$ M), or VitB12 (5 mg/ml) for 6 hours. Cells were then lysed and assayed for coimmunoprecipitation of NCoR-HDAC3 with Rev-*erb* $\alpha$ . (C) Time course of restoration of Rev-*erb* $\alpha$  binding to NCoR-HDAC3 after treatment with hemin for various times before immunoprecipitation. (D) Concentration dependence of hemin effect of Rev-*erb* $\alpha$  binding to NCoR-HDAC3.

sis kinase 3 $\beta$ -mediated phosphorylation and stabilization (28). We have demonstrated that alteration of heme modulates the interaction between Rev-*erb* $\alpha$  and the NCoR-HDAC3 corepressor complex. Heme concentrations oscillate

in a circadian manner (10, 20), and heme is also required by proteins that control various metabolic pathways and biological processes, making it a candidate for integrating circadian clock and metabolic systems. Heme negatively affects



**Fig. 5.** Suppression of the expression of gluconeogenic genes and glucose production in liver cells treated with hemin. **(A)** Expression of G6Pase and PEPCK genes in HepG2 cells treated with either DMSO or hemin (6  $\mu$ M) for 6 hours. Amounts of mRNA were quantitated by RT-PCR and normalized to cyclophilin. Mean  $\pm$  SD of three independent experiments is shown. \* $P$  < 0.05 compared with control by Student's  $t$  test. **(B)** Expression of G6Pase gene in HepG2 cells treated with control or ALAS1-targeting siRNA and then stimulated with DMSO or hemin for 16 hours. Mean  $\pm$  SD ( $n$  = 3), \* $P$  < 0.05 compared with DMSO. **(C)** Effect of hemin on the expression of G6Pase gene in cells depleted of Rev-erb $\alpha$ . Mean  $\pm$  SD ( $n$  = 3), \* $P$  < 0.05 compared with DMSO-treated cells transfected with control siRNA. Pound symbol indicates  $P$  < 0.05 compared with DMSO-treated cells transfected with control siRNA. **(D)** Effect of hemin on the occupancy of Rev-erb $\alpha$ , HDAC3, NCoR, or acetylated H3 (Ac-H3) at the G6Pase promoter in cells. Results of hemin-treatment are normalized to DMSO results. Mean  $\pm$  SD ( $n$  = 4). \* $P$  < 0.05 compared with control. **(E)** Primary mouse hepatocytes were treated with dexamethasone and 8-(4-chlorophenyl-thio)-adenosine 3',5'-cyclic monophosphate along with DMSO or hemin for 16 hours, then glucose in the medium was measured. Mean  $\pm$  SD of triplicate samples is shown, and four independent experiments gave similar results. \* $P$  < 0.05 compared with control.

BMAL1-NPAS2-dependent transcription activation (19–21) while enhancing Rev-erb $\alpha$ -mediated transcription repression, providing a potential means of maintaining the amplitude of circadian rhythms.

Expression of the gene encoding ALAS1, the rate-limiting enzyme in heme biosynthesis, increased in response to peroxisome proliferator activated receptor coactivator-1 $\alpha$  (29), a regulator of mitochondriogenesis that increases flux through the Krebs cycle (30). This first and rate-limiting enzyme in heme biosynthesis requires succinyl CoA, a Krebs cycle intermediate (17, 29). Gluconeogenesis competes with the Krebs cycle for metabolic intermediates whose depletion compromises heme biosynthesis as well as mitochondrial oxidative metabolism (fig. S13). The ability of Rev-erb $\alpha$  to function as a receptor for heme could provide a general mechanism for coordinating these processes.

#### References and Notes

1. F. Gachon, E. Nagoshi, S. A. Brown, J. Ripperger, U. Schibler, *Chromosoma* **113**, 103 (2004).
2. S. M. Reppert, D. R. Weaver, *Annu. Rev. Physiol.* **63**, 647 (2001).
3. P. L. Lowrey, J. S. Takahashi, *Annu. Rev. Genomics Hum. Genet.* **5**, 407 (2004).
4. A. Kohsaka, J. Bass, *Trends Endocrinol. Metab.* **18**, 4 (2007).

5. M. Stratmann, U. Schibler, *J. Biol. Rhythms* **21**, 494 (2006).
6. M. Akashi, T. Takumi, *Nat. Struct. Mol. Biol.* **12**, 441 (2005).

7. N. Preitner *et al.*, *Cell* **110**, 251 (2002).
8. T. K. Sato *et al.*, *Neuron* **43**, 527 (2004).
9. L. Yin, M. A. Lazar, *Mol. Endocrinol.* **19**, 1452 (2005).
10. M. F. Ceriani *et al.*, *J. Neurosci.* **22**, 9305 (2002).
11. K. Oishi *et al.*, *J. Biol. Chem.* **278**, 41519 (2003).
12. S. Panda *et al.*, *Cell* **109**, 307 (2002).
13. K. Oishi *et al.*, *FEBS Lett.* **580**, 127 (2006).
14. R. D. Rudic *et al.*, *PLoS Biol.* **2**, e377 (2004).
15. F. W. Turek *et al.*, *Science* **308**, 1043 (2005); published online 21 April 2005 (10.1126/science.1108750).
16. H. Atamna, *Ageing Res. Rev.* **3**, 303 (2004).
17. P. Ponka, *Am. J. Med. Sci.* **318**, 241 (1999).
18. A. S. Tsiftoglou, A. I. Tsamadou, L. C. Papadopoulou, *Pharmacol. Ther.* **111**, 327 (2006).
19. E. M. Dioum *et al.*, *Science* **298**, 2385 (2002); published online 20 November 2002 (10.1126/science.1078456).
20. K. Kaasik, C. C. Lee, *Nature* **430**, 467 (2004).
21. D. Boehning, S. H. Snyder, *Science* **298**, 2339 (2002).
22. E. de Rosny *et al.*, *Biochemistry* **45**, 9727 (2006).
23. J. Reinking *et al.*, *Cell* **122**, 195 (2005).
24. S. Sassa, A. Kappas, *J. Clin. Invest.* **71**, 625 (1983).
25. Y. Zhu, T. Hon, W. Ye, L. Zhang, *Cell Growth Differ.* **13**, 431 (2002).
26. J. A. Ripperger, *Chronobiol. Int.* **23**, 135 (2006).
27. G. Adelman, A. Begue, D. Stehelin, V. Laudet, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3553 (1996).
28. L. Yin, J. Wang, P. S. Klein, M. A. Lazar, *Science* **311**, 1002 (2006).
29. C. Handschin *et al.*, *Cell* **122**, 505 (2005).
30. S. C. Burgess *et al.*, *J. Biol. Chem.* **281**, 19000 (2006).
31. We thank R. Gampe, D. Steger, T. Stanley, M. Walker, J. Williams, and T. Willson for helpful discussions. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant R01 DK45586 (M.A.L.).

#### Supporting Online Material

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## The *Arabidopsis* Circadian Clock Incorporates a cADPR-Based Feedback Loop

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Transcriptional feedback loops are a feature of circadian clocks in both animals and plants. We show that the plant circadian clock also incorporates the cytosolic signaling molecule cyclic adenosine diphosphate ribose (cADPR). cADPR modulates the circadian oscillator's transcriptional feedback loops and drives circadian oscillations of Ca<sup>2+</sup> release. The effects of antagonists of cADPR signaling, manipulation of cADPR synthesis, and mathematical simulation of the interaction of cADPR with the circadian clock indicate that cADPR forms a feedback loop within the plant circadian clock.

Circadian clocks are adaptations to the daily rotation of the planet. In plants and cyanobacteria, benefits occur when the clock is resonant with the environment (1–3). This requires the oscillator to be robust yet flex-

ible, which may explain the evolution of molecular clocks with multiple feedback loops (4–6). We tested the hypothesis that plant circadian oscillators also incorporate cytosolic signaling molecules because there are circadian rhythms in the