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Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β

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The circadian clock acts at the genomic level to coordinate internal behavioural and physiological rhythms via the CLOCK–BMAL1 transcriptional heterodimer. Although the nuclear receptors REV-ERB-α and REV-ERB-β have been proposed to form an accessory feedback loop that contributes to clock function[1,2], their precise roles and importance remain unresolved. To establish their regulatory potential, we determined the genome-wide *cis*-acting targets (cistromes) of both REV-ERB isoforms in murine liver, which revealed shared recognition at over 50% of their total DNA binding sites and extensive overlap with the master circadian regulator BMAL1. Although REV-ERB-α has been shown to regulate *Bmal1* expression directly[1,2], our cistromic analysis reveals a more profound connection between BMAL1 and the REV-ERB-α and REV-ERB-β genomic regulatory circuits than was previously suspected. Genes within the intersection of the BMAL1, REV-ERB-α and REV-ERB-β cistromes are highly enriched for both clock and metabolic functions. As predicted by the cistromic analysis, dual depletion of *Rev-erb-α* and *Rev-erb-β* function by creating double-knockout mice profoundly disrupted circadian expression of core circadian clock and lipid homeostatic gene networks. As a result, double-knockout mice show markedly altered circadian wheel-running behaviour and deregulated lipid metabolism. These data now unite REV-ERB-α and REV-ERB-β with PER, CRY and other components of the principal feedback loop that drives circadian expression and indicate a more integral mechanism for the coordination of circadian rhythm and metabolism.

The circadian clock is a transcriptional mechanism that coordinates both behavioural and physiological processes such as the sleep–wake cycle and food intake. The existing model for the mammalian core molecular clock involves a transcriptional negative-feedback loop in which the transactivation of E-box-containing genes by CLOCK and BMAL1 is inhibited by the expression of *Per1* and *Cry1*, themselves under transcriptional control of E boxes[3]. As functional redundancy is common for core clock components, deletion of multiple paralogues in mice is often required to uncover relevant phenotypes such as perturbations in circadian gene expression, metabolism and wheel-running behaviour[4–7].

As REV-ERB-α, REV-ERB-β, ROR-α, ROR-β and ROR-γ bind to a common response element (the RORE), their intrinsic repressive and inductive activities, respectively, are believed to establish the rhythmic expression of target genes such as *Bmal1*. However, the partial penetrance and mild period phenotype of *Rev-erb-α*$^{-/-}$ mice[1] (essentially intact circadian wheel-running behaviour) and the modest rhythmic phenotype upon partial *Rev-erb-β* depletion of *Rev-erb-α*$^{-/-}$ cultured cells[2], suggested that they are not required for principal core clock function. Rather, REV-ERBs are proposed to form an accessory feedback loop that stabilizes the clock and has a role in receiving input signals or transmitting output pathways[2,8].

To clarify the regulatory potential of REV-ERB-α and REV-ERB-β in circadian regulation, we generated isoform-specific antibodies (Supplementary Methods and Supplementary Fig. 1) and determined their genome-wide *cis*-acting targets (cistromes) in the liver at Zeitgeber time (ZT) 8, the peak of their protein expression (data not shown and ref. 9). *De novo* motif analysis (Fig. 1a) revealed that *in vivo*, in addition to the classic REV-ERB direct repeat 2 (DR2) motifs, other nuclear receptor binding sites (particularly DR1) are predominant in the DNA elements bound by both REV-ERB-α and REV-ERB-β[10]. Although not surprising, extensive overlap was observed between the REV-ERB cistromes, with commonly bound peaks accounting for 54.8% and 60.7% of the total REV-ERB-α and REV-ERB-β peaks, respectively (Fig. 1b). The limited overlap of our REV-ERB-α cistrome with one that has been published previously[11] (<50%) can most likely be attributed to differences in the antibody specificities (Supplementary Figs 2 and 3). Pathway analyses of our REV-ERB-α and REV-ERB-β overlapping peaks[12] revealed an enrichment in lipid metabolism genes (Fig. 1c), consistent with the hyperlipidaemic phenotype previously observed in *Rev-erb-α* null mice[13]. Notably, loci encoding circadian clock genes (*Clk*, *Bmal1*, *Cry1*, *Cry2*, *Per1*, *Per2*; see Fig. 1d) were also enriched in the REV-ERB-α/β cistromic overlap, suggesting that the coordinated actions of both REV-ERB isoforms are directly linked with clock function. A comparison of the REV-ERB-α/β cistrome with published BMAL1 binding sites[14] revealed that 28% of BMAL1 peaks (at ZT6 and ZT10) were shared with the REV-ERB-α/β (ZT8) cistrome and 68% of these peaks (781) were occupied by all three transcription factors. Clear binding sites for each of these transcription factors were found on 'core clock' gene loci as well as on many clock-controlled target genes (*Rev-erb-α*, *Rev-erb-β*, *Ror*, *Dbp*, *Hlf*, *Tef*, *Nfil3*; see Fig. 1d, e). In addition to circadian annotated loci, the BMAL1, REV-ERB-α, REV-ERB-β triple intersection is highly enriched for genes in the receptor tyrosine kinase signalling pathway as well as those known for energy homeostasis (Supplementary Table 1). Their confluence at hundreds of clock and clock output genes indicates that beyond a simple 'binary relationship', REV-ERB-α/β and BMAL1 cooperate to regulate clock and clock output genes coordinately.

To test the above proposal, we used Cre/*lox* recombination to generate three genetically modified mouse lines in C57BL/6 backgrounds harbouring global, tissue-specific, or conditional knockouts of *Rev-erb-α* and *Rev-erb-β* loci. Global *Rev-erb-α*$^{-/-}$ and *Rev-erb-β*$^{-/-}$ single knockout mice were generated using a *CMV-cre* transgenic allele (detailed in Supplementary Methods). Homozygous deletion of *Rev-erb-β* did not cause overt gross abnormalities, lethality or infertility, whereas *Rev-erb-α*$^{-/-}$ mice, although viable, show frequent postnatal lethality (before 2 weeks of age) (Supplementary Table 2), with survivors exhibiting diminished fertility in both sexes. In contrast,

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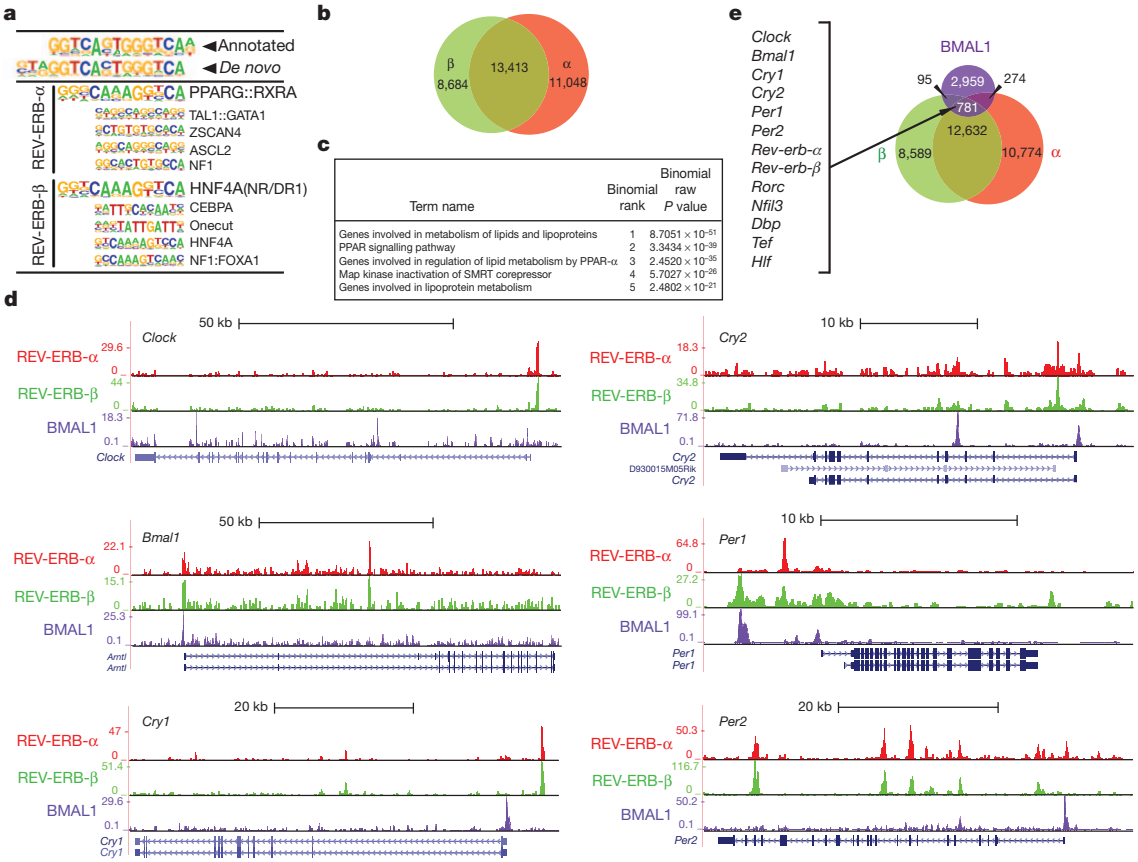

Figure 1 | Cistromic analyses of REV-ERB-α and REV-ERB-β in liver. **a**, *De novo* HOMER motif analysis of *in vivo* REV-ERB-α and REV-ERB-β binding. **b**, Venn diagram depicting the unique and common REV-ERB-α and REV-ERB-β bound peaks. **c**, Commonly bound REV-ERB-α and REV-ERB-β peaks are enriched for genes involved in lipid metabolism and associated with PPARs. **d**, REV-ERB-α, REV-ERB-β and BMAL1 binding at canonical circadian clock genes. The left axis indicates sequence-tag counts. **e**, BMAL1 cistrome significantly overlaps with REV-ERB-α and REV-ERB-β. Examples of clock-related genes in overlap are listed, and selected peaks are shown in Supplementary Fig. 4.

Rev-erb-α$^{-/-}$ mice on a mixed 129/Sv and C57BL/6 background were reported to have reduced fertility but no postnatal lethality[15].

Liver-specific *Rev-erb-α*$^{-/-}$ *Rev-erb-β*$^{-/-}$ mice were generated to assess combinatorial REV-ERB signalling in hepatic circadian gene expression via an albumin-Cre transgene[16]. These liver-specific double knockout mice (*Rev-erb-α*$^{lox/lox}$ *Rev-erb-β*$^{lox/lox}$ albumin-Cre (L-DKO)) were born at the expected Mendelian ratio, effectively bypassing the frequent lethality of the *Rev-erb-α*$^{-/-}$ global deletion. Depletion of both *Rev-erb-α* and *Rev-erb-β* (Fig. 2a, b) disrupted the circadian, and often total, expression of many hepatic core clock genes (Fig. 2c–f) and presumed output genes (Fig. 2g–i).

Microarray analysis of gene expression over a 24-h time course (presented as Zeitgeber time) revealed massive differences between the wild-type and L-DKO liver. Using CircWave v3.3 (ref. 17) software, we determined that of the ~900 genes showing circadian expression in wild-type liver, the rhythmicity of more than 90% was perturbed in the L-DKO liver (Fig. 3a). The severity of the circadian disruption was comparable to that described for mice deficient in core clock components[18,19], emphasizing the functional impact associated with the extensive intersection between REV-ERB-α/β and BMAL1 cistromes. Furthermore, the gene ontology of the REV-ERB-α/β-dependent circadian transcriptome (those genes that lose rhythm in L-DKO mice; Fig. 3b) also mirrors that of their cistromes (Fig. 1c and Supplementary Table 1). This is of interest as the disrupted oscillation of these genes in the liver occurred *in vivo* in the presence of otherwise wild-type entraining signals and fully intact extra-hepatic clockwork (Supplementary Fig. 5). Notably, residual rhythmic expression of some genes was maintained in the L-DKO mice, indicating that they may be controlled by systemic cues, as suggested previously[20].

To link the cistromic and transcriptomic data functionally, we examined the gene expression levels of REV-ERB/BMAL1 common cistromic sites. A comparison of the liver expression levels across ZT8 to ZT16 revealed that 45% of the co-occupied genes were significantly perturbed in the liver-specific DKO compared with wild-type liver (Supplementary Table 3). This strong correlation between REV-ERB occupancy and perturbed gene expression corroborates a direct role for REV-ERBs in maintaining rhythmic expression patterns for many of the above genes.

The benchmark assay for circadian dysfunction is wheel-running behaviour, where the persistence or fragmentation of running rhythm is assessed in constant darkness after prior entrainment to a 12-h light/dark cycle. Actograms recorded for *Rev-erb-α*$^{-/-}$ mice in a pure C57BL/6 background displayed a fully penetrant period shortening of 30 min (Fig. 4a, b), contrasting with the lack of fully penetrant phenotype reported in mixed background studies[1,15]. No changes in the activity levels, activity consolidation, or phase angle of entrainment (activity onset relative to dark onset) were observed for *Rev-erb-α*$^{-/-}$ mice. *Rev-erb-β*$^{-/-}$ mice showed no change in any of the parameters of circadian activity rhythms (Fig. 4a, b), raising the possibility that the circadian clock function is independent of REV-ERB-β. Given the functional redundancy demonstrated for the established components of the core clock, we sought to address whether REV-ERB-α and REV-ERB-β might cooperatively influence circadian rhythms in wheel running. Using the tamoxifen-activated *creER* transgene, inducible double knockout (iDKO) mice were generated[21], allowing ubiquitous deletion of both genes in adulthood (see Supplementary Methods). Whereas the wheel-running activity of *Rev-erb-α*$^{lox/lox}$ *Rev-erb-β*$^{lox/lox}$ mice treated with tamoxifen was normal, the phenotype of mice

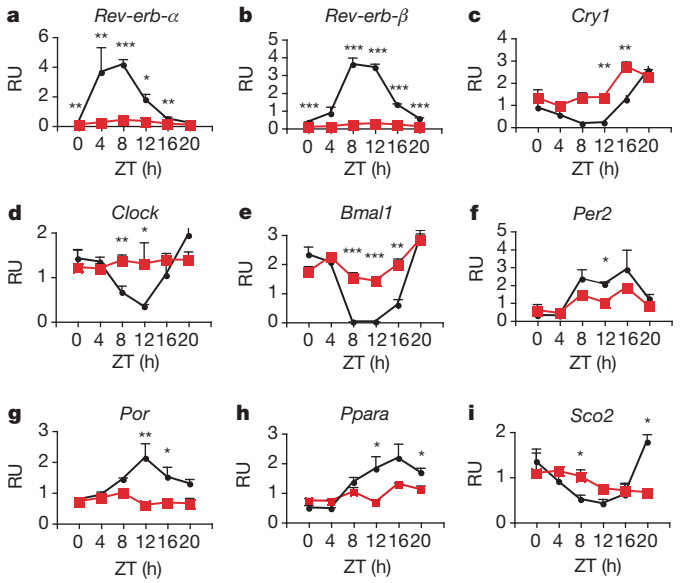

Figure 2 | Circadian gene expression of many canonical core clock genes and output genes are disrupted in livers of *Rev-erb-α*$^{lox/lox}$ *Rev-erb-β*$^{lox/lox}$ albumin-Cre (L-DKO) mice. **a–i**, The expression levels of *Rev-erb-α* (**a**), *Rev-erb-β* (**b**), canonical core clock genes (*Cry1*, *Clock*, *Bmal1* and *Per2*) (**c–f**) and presumed output genes (*Por*, *Ppara* and *Sco2*) (**g–i**) in livers from L-DKO (albumin-Cre positive, red labels) and wild-type (albumin-Cre negative, black labels) mice are shown. Livers ($n = 3$) were collected at each indicated ZT under a 12-h light/dark cycle. QPCR was performed in technical triplicates. Relative units (RU) normalized with *36B4*. Error bars indicate standard error of the mean; statistical significance was determined by Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

bearing the *creER* transgene was more severe than the additive effects of the individual knockouts (Fig. 4a–c). The iDKO mice showed reduced and severely fragmented activity, and advanced phase angle of entrainment, features also found in *Bmal1*$^{-/-}$ mice[22]. Furthermore, as shown in the periodogram in Fig. 4c, in mice with detectable activity rhythms, the free-running period length under constant darkness was shortened by as much as 2.5 h (see also Supplementary Tables 4 and 5). The severe activity defects in iDKO mice is unexpected when one considers the properties of the single knockouts, and supports a strongly selected, novel and cooperative role for REV-ERB-α and REV-ERB-β in rhythmic behaviour[4–7].

Mice and humans with disrupted circadian rhythms have been shown to develop metabolic disorders including hyperlipidaemia and hyperglycaemia, suggesting a link between proper clock function and metabolism[23]. To investigate if REV-ERB activity is similarly required for normal metabolic regulation, we compared metabolic parameters of tamoxifen-treated control and iDKO mice. Treated iDKO mice displayed increased circulating glucose and triglyceride levels, and a reduction in the level of free fatty acids compared to control littermates (Fig. 4d). The reduced fatty acids may reflect a more oxidative metabolism of the double knockout mice as exemplified by reduced RERs. We also note a distinct RER phase shift in constant dark conditions (Fig. 4e), indicating dysregulation of overall body metabolism. Although previous studies have linked REV-ERB-α with lipid homeostasis[13,24,25], the presence of both REV-ERB-α and REV-ERB-β on multiple key lipid and bile acid regulatory genes including *LXRα* (also called *Nr1h3*), *Fxr* (*Nr1h4*), *Apoc3*, *Cyp7a1*, *SHP* (*Nr0b2*), *Insig2* and SREBP (*Srebf1*) provides a molecular mechanism for rhythmic cholesterol and bile acid metabolism. The current cistromic studies elevate REV-ERB-β to equal prominence with REV-ERB-α in the transcriptional regulation of these pathways, and identify additional direct gene targets (Supplementary Fig. 6). The severity of the metabolic phenotype observed in the iDKO mice compared to the REV-ERB-α knockout mice is consistent with the cistromic analyses of the REV-ERBs.

Defining the genetic mechanisms that comprise the circadian clock is central to understanding how genomic rhythms are transformed into behavioural and metabolic physiology. In this study, using a combination of genome-wide cistromic profiling, mRNA expression analysis and three new targeted gene knockout mouse models, we unequivocally demonstrate a more critical and central role for REV-ERB-α/β circadian clock regulation than previously suspected. The genomic analysis confirms *Bmal1* as a direct REV-ERB-α/β target, while at the genome-wide level we find a predominance of both core clock and circadian output genes targeted by REV-ERBs, indicating a more integral role for this family in the core clock than previously considered. These findings indicate that a dynamic balance between BMAL1 and REV-ERB-α/β cistromes is used to regulate both core clock and clock output genes by co-localizing opposing epigenetic regulators to shared genomic targets. *In vivo*, using targeted and inducible double knockout mice, we demonstrate that REV-ERB-α and REV-ERB-β together function as integral drivers of the circadian clock, rather than simply as stabilizers of an output, thereby redefining the established paradigm for these receptors. The similarity of the DKO circadian phenotype to compound core clock mutants (*Per1*$^{-/-}$*Per2*$^{-/-}$, *Cry1*$^{-/-}$*Cry2*$^{-/-}$)[5–7] in severity, penetrance and strong period shortening is more reflective of a pacemaker rather than a stabilizer of rhythm. Together, this leads to a model of mutual direct regulation, with BMAL1 controlling one loop and REV-ERB-α/β a second loop of the core clock, with both loops using cistromic convergence to coordinate key clock and metabolic functions (Fig. 4f). The adaptive feature of the circadian clock enables its control of sleep–wake cycles, physiological rhythms, energy homeostasis and behaviour. In contrast, disruption of rhythm spawns a range of problems from jet lag, to more profound sleep disorders, obesity, metabolic disease, immune function and cancer[26–29]. As partnered regulators, the recent development of both potent REV-ERB agonists (that enhance repression)[25] and REV-ERB antagonists (that relieve repression)[30] provides a new therapeutic approach to both reset disrupted rhythms and re-establish metabolic balance.

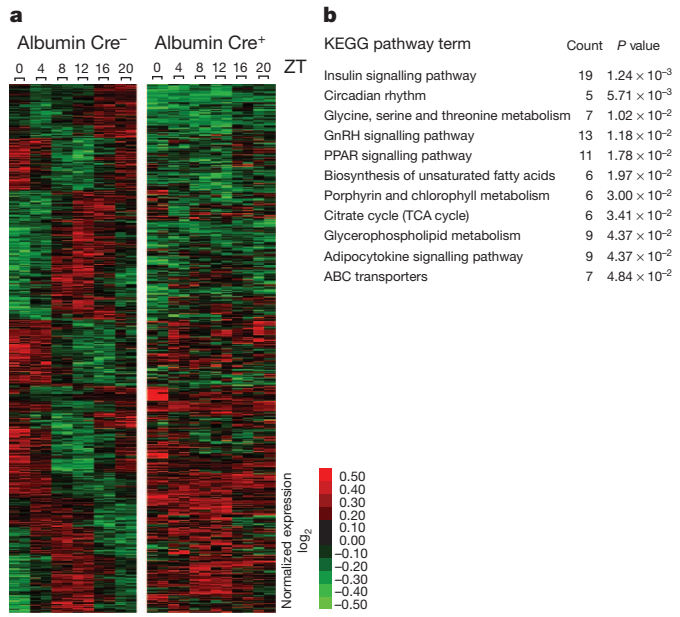

Figure 3 | Broad disruption of circadian transcriptome in the absence of *Rev-erb-α* and *Rev-erb-β*. **a**, Heat map of genes with circadian expression in wild-type (left panel) and L-DKO (right panel) livers. A total of 1,227 unique accession numbers were selected based on false discovery rate <0.05. **b**, Genes expressed in a circadian manner that lose rhythm are highly associated with circadian and energy homeostasis functions as assessed by KEGG pathway analysis.

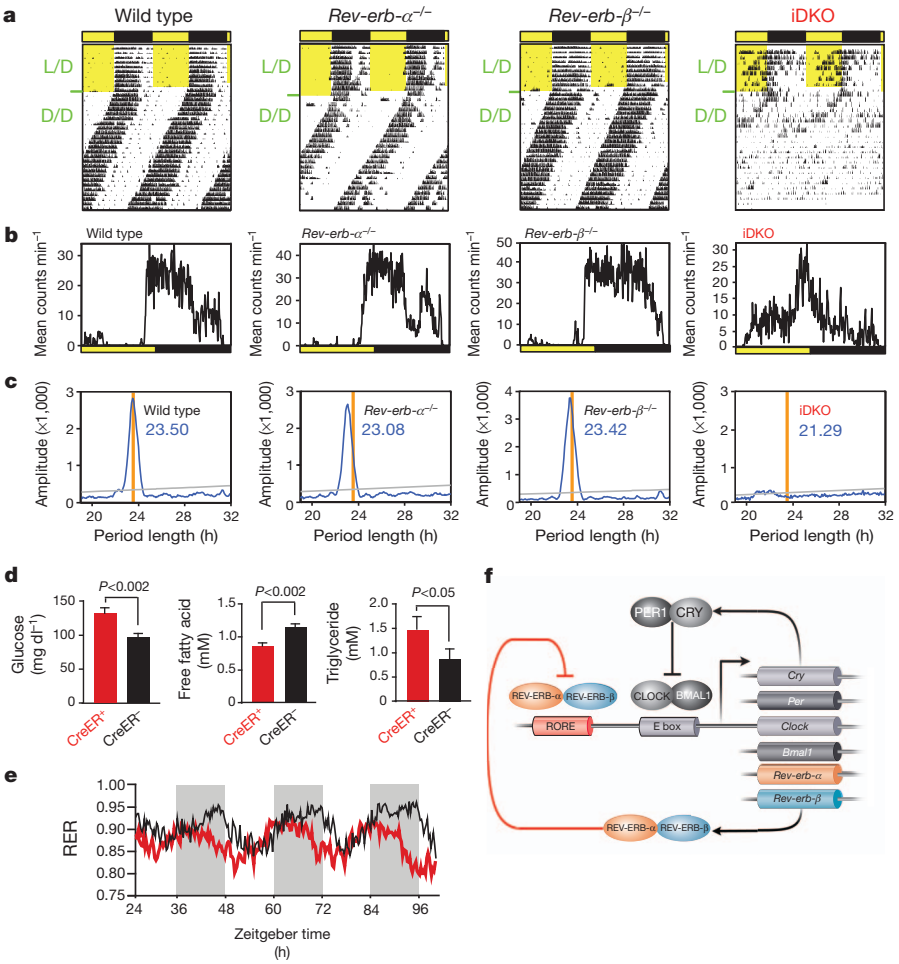

Figure 4 | Loss of both *Rev-erb-α* and *Rev-erb-β* results in disrupted circadian wheel-running behaviour and metabolic shift. **a–c**, Voluntary locomotor activity of wild-type, *Rev-erb-α*$^{-/-}$, *Rev-erb-β*$^{-/-}$ and *Rev-erb-α*$^{-/-}$ *Rev-erb-β*$^{-/-}$ (iDKO) mice. **a**, Actograms showing wheel-running activity in constant darkness after prior entrainment in light/dark. **b**, Activity profiles during light/dark cycles. **c**, Chi-squared periodogram of the initial 20 days in constant darkness ($n = 5$–9 for each mutant strain; $n = 5$–6 littermate controls). Representative actograms from individual mice are shown. The period length of wild-type mice is shown as a vertical orange line. **d**, Fasting glucose ($n = 6$), free fatty acid ($n = 6$) and triglyceride ($n = 6$) levels in iDKO and wild-type mice. **e**, Respiratory exchange ratio (RER, v_{CO_2}/v_{O_2}) for wild-type (black) and iDKO (red) mice ($n = 4$). **f**, Model depicting the activating (CLOCK–BMAL1) and repressive (REV-ERB-α–REV-ERB-β) transcriptional complexes, the coordinate actions of which generate rhythmic gene expression.

METHODS SUMMARY

For chromatin immunoprecipitation (ChIP), 5-month-old male C57BL/6J mice were killed by CO_2 asphyxiation at ZT8. Livers were removed and pooled for ChIP, processing and sequencing. Tamoxifen-induction of Cre recombinase activity was accomplished by daily intraperitoneal injection of 2 mg tamoxifen (Sigma) in 100 μl corn oil (Sigma) for 7 days. Three-month-old tamoxifen-treated animals were subjected to wheel-running assays for 7 days after the end of treatment. For gene expression analysis, 5–6-month-old males were killed by cervical dislocation at indicated ZT points for rapid dissection and snap freezing of the tissues. During the dark cycle, procedures were performed under red light. Detailed methods are provided in the Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions X.Z., G.D.B., R.T.Y., M.D. and C.L. performed and/or analysed the results from ChIP-seq. R.T.Y., M.D., M.T.L. and C.K.G. performed and/or analysed the results from the microarray experiment. M.H., L.D. and S.P. performed and/or analysed the wheel-running assay and the real-time luciferase assay. J.A. performed gene targeting. H.C. and L.-W.C. performed all experiments. H.C. and R.M.E. designed all experiments, analysed all results and H.C., R.T.Y., M.D., A.R.A., S.P. and R.M.E. wrote the manuscript.

Author Information Microarray and ChIP-seq data sets have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE34020. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.M.E. (evans@salk.edu).