LETTERS

Transcriptional coactivator PGC-1α integrates the mammalian clock and energy metabolism

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The mammalian clock regulates major aspects of energy metabolism, including glucose and lipid homeostasis and mitochondrial oxidative metabolism[1,2]. The biochemical basis for coordinated control of the circadian clock and diverse metabolic pathways is not well understood. Here we show that PGC-1α (Ppargc1a), a transcriptional coactivator that regulates energy metabolism[3–9], is rhythmically expressed in the liver and skeletal muscle of mice. PGC-1α stimulates the expression of clock genes, notably Bmal1 (Arntl) and Rev-erbα (Nr1d1), through coactivation of the ROR family of orphan nuclear receptors. Mice lacking PGC-1α show abnormal diurnal rhythms of activity, body temperature and metabolic rate. The disruption of physiological rhythms in these animals is correlated with aberrant expression of clock genes and those involved in energy metabolism. Analyses of PGC-1α-deficient fibroblasts and mice with liver-specific knockdown of PGC-1α indicate that it is required for cell-autonomous clock function. We have thus identified PGC-1α as a key component of the circadian oscillator that integrates the mammalian clock and energy metabolism.

Most living organisms exhibit behavioural and physiological rhythms, including activity, sleep, metabolism and body temperature. These rhythms are controlled by a circadian clock and are subject to regulation by light–dark cycles. In mammals, the central and peripheral clocks are controlled by a common transcriptional circuitry that generates rhythmic patterns of gene expression, most notably those encoding metabolic enzymes[10–14]. Perturbations of circadian rhythms have been associated with an increased risk for metabolic disorders in humans[15]. Remarkably, mice with defective clock function develop obesity and have impaired glucose homeostasis[16,17], suggesting that the regulation of circadian clocks is linked to the pathways of energy metabolism and potentially to the pathogenesis of metabolic diseases. PGC-1α is an inducible transcriptional coactivator that regulates adaptive energy metabolism in multiple tissues[4,6]. The critical role of this factor in linking the environment to metabolism prompted us to examine whether PGC-1α regulates the circadian clock and serves as a link between the clock and energy metabolism.

The clock oscillator consists of transcriptional activators and repressors that are assembled into feedback loops[18,19]. To determine the effects of PGC-1α on clock genes, we transduced primary hepatocytes and C2C12 myotubes with recombinant adenoviruses expressing green fluorescent protein (GFP) or PGC-1α. Quantitative PCR (qPCR) analyses indicate that PGC-1α induces the expression of Bmal1 and Clock in both cell types (Fig. 1a). Consistent with messenger RNA expression, Bmal1 and Clock protein levels are elevated in hepatocytes in response to PGC-1α (Fig. 1b). Clock and Bmal1 are two basic helix–loop–helix transcription factors that activate the transcription of period (Per) and cryptochrome (Cry) genes[18,19]. Per and Cry proteins in turn inhibit their own expression by repressing Clock/Bmal1 activity, forming the critical feedback loop within the clock circuitry. Interestingly, the effects of PGC-1α on Per and Cry gene expression seem to be cell-type-specific. Previous studies have implicated orphan nuclear receptors of the ROR and Rev-erb families in the control of circadian clock function[13,20–22]. Although PGC-1α exerts only modest effects on the expression of RORα (Rora) and RORγ (Rorc), it significantly induces Rev-erbα and Rev-erbβ expression in both cell types (Fig. 1a).

Recent expression profiling studies have identified a large number of genes that show rhythmic expression in the liver[11–13]. To assess the role of PGC-1α in the regulation of circadian gene expression at the transcriptome level, we performed microarray analysis on total RNA isolated from primary hepatocytes transduced with GFP or PGC-1α adenoviruses. We integrated the PGC-1α microarray data set with a previously described set of 335 oscillating hepatic genes[11] to determine global effects of PGC-1α on circadian genes. As shown in Fig. 1c, 47 of these circadian-regulated genes are induced by PGC-1α by more than 1.8-fold, with the expression of many clustering at the onset and during early dark phase (Supplementary Table 1). Analysis of PGC-1α mRNA levels in mouse liver revealed a diurnal rhythm that peaks at CT13 (CT0 is the onset at hour 0 of subjective light period), gradually declines thereafter, and reaches a nadir at CT1 (Fig. 1d). PGC-1α expression is similarly rhythmic in skeletal muscle, though to a lesser degree. The expression of other PGC-1 coactivators, that is, PGC-1β and PRC, also show circadian rhythms in these tissues. Immunoblotting analyses indicate that PGC-1α protein reaches peak levels in early dark phase (Fig. 1e), coinciding with the activation of Bmal1 transcription and its mRNA accumulation. Interestingly, the short isoform of PGC-1β shows a diurnal rhythm that peaks several hours before PGC-1α. In contrast, the protein levels of Brm (also known as Smarca2), a component of the SWI/SNF chromatin-remodelling complexes, remain unchanged. Taken together, these data indicate that rhythmic activation of PGC-1α, and possibly other PGC-1 coactivators, may have an important role in the regulation of clock function.

To identify transcription factors that mediate the regulation of clock genes by PGC-1α, we examined the ability of PGC-1α to synergize with these factors in the regulation of Bmal1 transcription. PGC-1α dramatically augments the transcriptional activity of RORα and RORγ, but not C/EBPα, on a Bmal1 promoter reporter (Fig. 2a and Supplementary Fig. 1). Coactivation of RORα by PGC-1α was previously observed for genes involved in muscle lipid metabolism[23]. In contrast, PGC-1α exerts modest effects on the transcriptional activity of Clock/Bmal1 (data not shown). The synergistic effects of ROR and PGC-1α are abolished when the ROR-binding sites (RORE) on the proximal Bmal1 promoter are mutated. Physical interaction assays indicate that PGC-1α binds to RORα and RORγ through the LXXLL motifs (Supplementary Fig. 1). This functional synergy between
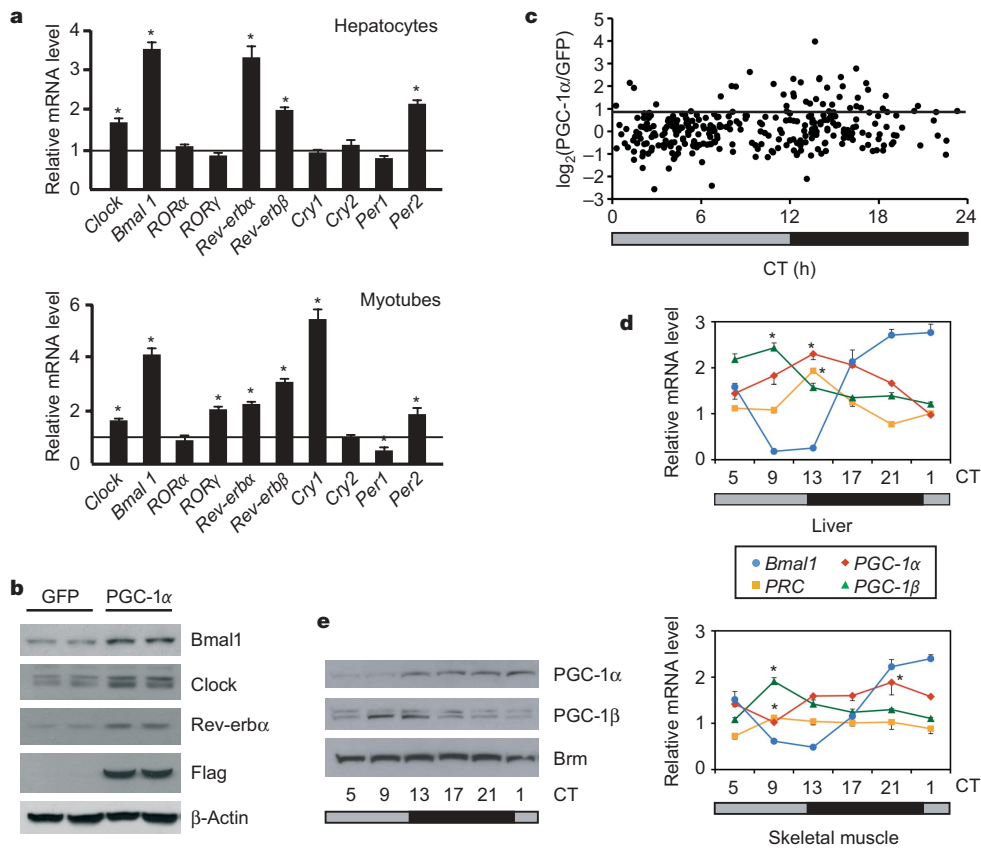
Figure 1 | Regulation of clock genes by PGC-1α. a, qPCR analysis of clock genes in primary hepatocytes and C2C12 myotubes. Shown is fold-induction by PGC-1α. Data represent mean ± s.d. *$P < 0.02$, PGC-1α versus GFP. **b,** Immunoblots of hepatocyte lysates using the indicated antibodies. **c,** Effects of PGC-1α on circadian-regulated genes in the liver. Fold induction by

PGC-1α of the circadian gene set was calculated on the basis of microarray data, log-transformed and plotted against the respective peak timepoints of expression in the liver (solid line, 1.8-fold). **d,** qPCR analysis of liver and skeletal muscle gene expression. Data represent mean ± s.d. *$P < 0.01$, peak versus nadir. **e,** Immunoblots of liver nuclear extracts using the indicated antibodies.

RORα and PGC-1α is also observed for the endogenous *Bmal1* gene (Fig. 2b). Previous studies indicate that Rev-erbα negatively regulates *Bmal1* transcription by recruiting corepressor proteins[22], indicating that the induction of Rev-erb factors may provide a negative feedback mechanism for PGC-1α function. Consistent with this, both Rev-erbα and Rev-erbβ drastically repress the stimulatory effects of PGC-1α on the *Bmal1-luc* reporter (Fig. 2c). These results illustrate that the ability of PGC-1α to activate *Bmal1* transcription is modulated by the relative abundance of the ROR and Rev-erb families of orphan receptors.

Chromatin immunoprecipitation (ChIP) assays in HepG2 cells indicate that PGC-1α is present near RORE on the proximal *Bmal1* promoter (Fig. 2d). In addition, PGC-1α recruits histone acetyltransferases, including p300 and GCN5, but not Clock, a recently described DNA-binding histone acetyltransferase[24], to this site. Histone hyperacetylation is associated with transcriptional activation. On the other hand, trimethylation of lysine 4 of histone 3 (H3K4me3) is a hallmark for actively transcribed genes, whereas H3K9 dimethylation (H3K9me2) is typically found in heterochromatin and silenced genes[25]. Remarkably, PGC-1α expression leads to a robust increase in histone H3 acetylation and H3K4me3 levels with a corresponding reduction in H3K9me2 levels on the proximal *Bmal1* promoter. These results indicate that PGC-1α activates *Bmal1* transcription by altering the local chromatin environment from a repressive to an active state.

The ability of PGC-1α to regulate the expression of clock genes indicates that this factor may impact on the circadian clock, thereby linking clock oscillators to energy metabolism. To test whether PGC-1α is required for clock function *in vivo*, we monitored locomotor activity and body temperature in wild-type and *PGC-1α* null mice
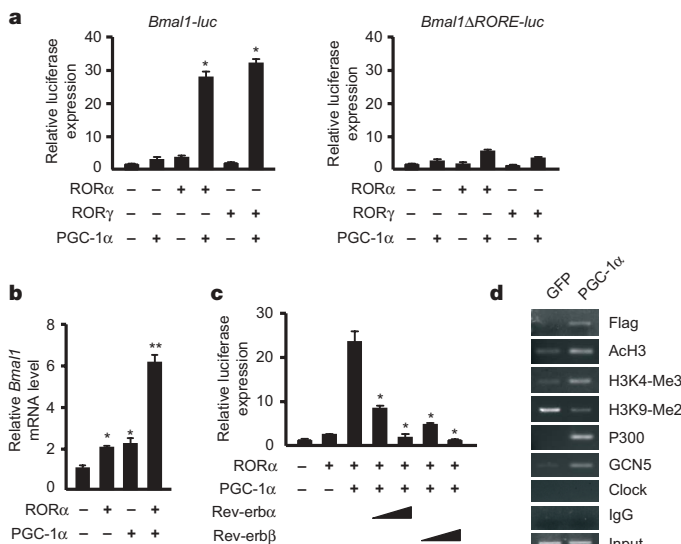

Figure 2 | Regulation of *Bmal1* gene transcription by PGC-1α and ROR. a, Reporter gene assays using wild-type (*Bmal1-luc*) or RORE-mutant (*Bmal1ΔRORE-luc*) *Bmal1*-luciferase reporters. *$P < 0.01$, PGC-1α versus control. **b,** qPCR analysis of *Bmal1* expression in transduced hepatocytes. **a, b,** Error bars, s.d. *$P < 0.05$, RORα or PGC-1α versus Control; **$P < 0.01$, RORα plus PGC-1α versus RORα. **c,** Reporter gene assays using *Bmal1-luc* in the presence of Rev-erbα or Rev-erbβ. Shown with mean ± s.d. *$P < 0.001$, Rev-erb versus control. **d,** ChIP assay with the indicated antibodies using HepG2 cells transduced with *GFP* or *Flag–PGC-1α* adenoviruses. PCR primers amplify a fragment flanking the proximal RORE.

using telemetry. Wild-type mice exhibit a free-running period of approximately 23.7 h in constant darkness, whereas the free-running period is approximately 24.0 h in PGC-1α null mice (Fig. 3a). In addition, locomotor activity is evenly distributed throughout the subjective night in the null mice. These results indicate that PGC-1α regulates locomotor behaviour in mice and is required for maintaining normal circadian periods. Diurnal oscillation of body temperature is also distinct between these two genotypes (Fig. 3b). Because PGC-1α is an important regulator of energy metabolism, we next examined whether the daily oscillation of metabolic rate is altered in the null mice. Using indirect calorimetry, we measured oxygen consumption rate (VO_2) in wild-type and PGC-1α null mice over a period of three days[6]. Consistent with locomotor activity and body temperature rhythms, VO_2 is significantly higher in the dark phase (Fig. 3c). Compared with wild-type mice, PGC-1α null mice have elevated VO_2 in both light and dark phases, though the diurnal oscillation of VO_2 is severely blunted. These results indicate that PGC-1α is a critical component of the clock circuitry that may link the clock oscillator to metabolism.

Analysis of clock gene expression indicates that the rise of Bmal1 mRNA levels is significantly impaired (37% lower at CT19), although the rhythmic expression of Bmal1 is nearly intact in PGC-1α null livers (Fig. 3d). In contrast, peak Bmal1 mRNA level is higher in PGC-1α-deficient skeletal muscle (Fig. 3e), indicating that PGC-1α deficiency differentially perturbs clock genes in different tissues. Diurnal rhythms of Clock and Per1 mRNA are also disrupted in the liver and skeletal muscle of PGC-1α null mice, respectively. We next examine whether PGC-1α is required for the cyclic expression of various enzymes involved in energy metabolism. PEPCK (PCK1), a rate-limiting enzyme of hepatic gluconeogenesis, shows a daily cycle that peaks at CT13 in the wild-type liver. PGC-1α seems to be dispensable for the rhythmic expression of this enzyme. Nevertheless, the mRNA levels of PEPCK are constitutively elevated at CT1 and CT13 (Fig. 3d), in a similar way to the previous studies[6]. Pyruvate dehydrogenase kinase 4 (PDK4) is a key regulator of the pyruvate

dehydrogenase complex that controls the flux of pyruvate into the tricarboxylic acid cycle. The diurnal PDK4 expression is severely impaired in PGC-1α-deficient liver and skeletal muscle, although the phase of PDK4 oscillation seems to be preserved. Similarly, the cyclic expression of fatty acid binding protein 3 (Fabp3/H-FABP) as well as other mitochondrial genes involved in oxidative phosphorylation (OXPHOS), such as aconitase (Aco2) and Cox4a (Cox4i1), is also disrupted in PGC-1α null skeletal muscle. Together, our results illustrate that PGC-1α is essential for oscillatory expression of clock and metabolic genes in vivo.

The defects in circadian rhythms of clock and metabolic gene expression in PGC-1α null mice could potentially be affected by altered central clock function. Previous work indicates that peripheral oscillators can be reset by restricted feeding, essentially uncoupling them from the master pacemaker in the suprachiasmatic nucleus[26]. To determine whether PGC-1α is required for phase resetting of peripheral clocks in response to feeding, we subjected wild-type and null mice to night feeding and then switched them to day feeding. Blood glucose concentrations show robust oscillation when the mice were fed exclusively at night (Fig. 4a). The phase of glucose oscillation is shifted by approximately 12 h after the feeding switch in both groups. However, the amplitude of blood glucose rhythms is significantly dampened in mice lacking PGC-1α. qPCR analyses revealed that the expression of PGC-1α and clock genes are reversed in response to restricted feeding in wild-type livers (Fig. 4b). In contrast, feeding-induced resetting of clock gene expression is significantly disrupted in PGC-1α null liver and skeletal muscle (Fig. 4b and Supplementary Fig. 2). The expression of Bmal1 in the suprachiasmatic nucleus is also reduced in PGC-1α null mice (Supplementary Fig. 2); however, the feeding switch does not seem to alter Bmal1 expression patterns. Interestingly, PGC-1β mRNA levels are elevated at some timepoints in these tissues.

To rule out the possibility that the perturbation of peripheral clocks in PGC-1α null mice may be secondary to a compromised central clock, we examined cell-autonomous clock function in vivo
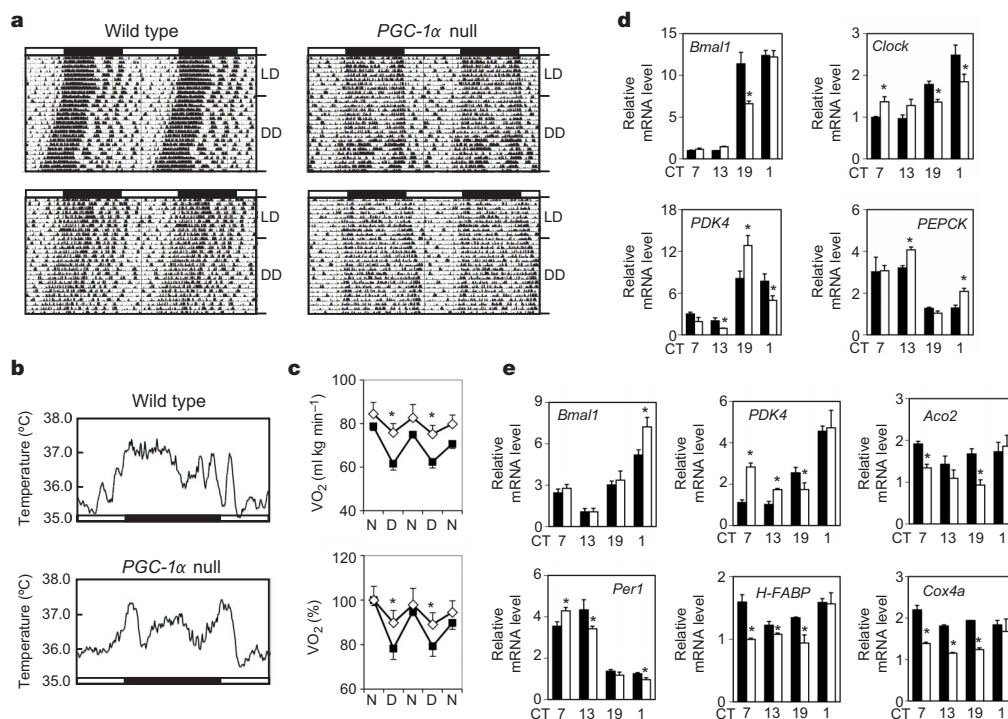

Figure 3 | Disruption of clock function in PGC-1α null mice. a, Locomotor activity in wild-type and PGC-1α null mice under normal light:dark cycles 12:12 h (LD) or constant darkness (DD). Data were plotted in duplicate columns in each panel. **b,** Representative traces of core body temperature in wild-type and PGC-1α null mice. **c,** Metabolic rate in wild-type (filled square, n = 5) and PGC-1α null (open diamond, n = 6) mice. Shown are average and normalized VO_2 during subjective night (N) and day (D). Error bars, s.e.m. **d, e,** qPCR analysis of gene expression in pooled liver (**d**) and skeletal muscle (**e**). Error bars, s.d. of the mean. *P < 0.05, wild-type (filled box) versus null mice (open box).
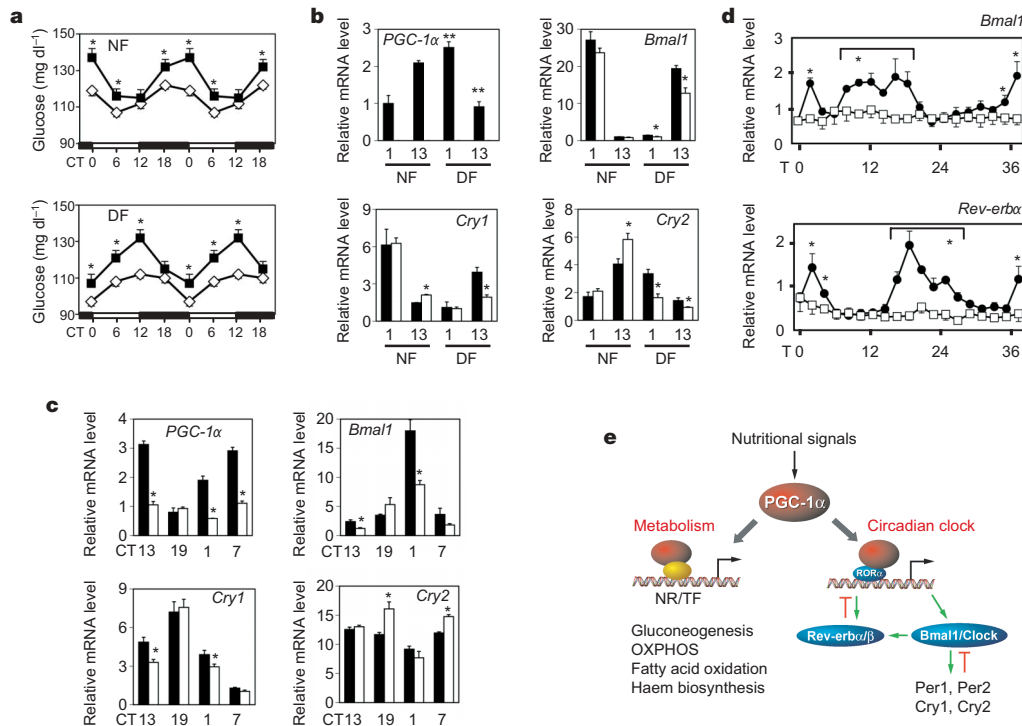
Figure 4 | Cell-autonomous role of PGC-1α in clock regulation. a, Plasma glucose concentrations in wild-type (filled square, $n = 16$) and *PGC-1α* null mice (open diamond, $n = 14$) subjected to night (NF) or day (DF) feeding. Data were plotted in duplicate columns. **b,** qPCR analysis of total liver RNA isolated from wild-type (filled box) and *PGC-1α* null (open box) mice. **c,** qPCR analyses of hepatic gene expression in mice transduced with control (filled box) or *PGC-1α* RNAi (open box) adenoviruses. Error bars in **a–c** indicate s.e.m. **a, b, c,** $*P < 0.05$ wild-type versus null mice; $**P < 0.01$ NF versus DF. **d,** Time course expression of clock genes in wild-type (filled circle) and *PGC-1α* null (open square) fibroblasts following serum shock. Error bars, s.d. $*P < 0.05$ wild-type versus null cells. **e,** Model for coordinated regulation of circadian clock and energy metabolism through PGC-1α.

as well as in cultured cells. C57/Bl6J mice were transduced through the tail vein with adenoviruses expressing random or short hairpin RNA (shRNA) directed towards *PGC-1α*[27]. RNA interference (RNAi) knockdown of *PGC-1α* in the liver significantly disrupts rhythmic expression of *Bmal1*, *Cry1* and *Cry2* in this tissue, but not in the suprachiasmatic nucleus (Fig. 4c and Supplementary Fig. 2). Serum shock has been demonstrated to induce rhythmic clock gene expression in cultured fibroblasts[28]. We exposed immortalized brown pre-adipocytes to brief serum shock and analysed expression of clock genes over a period of 36 h. Serum shock leads to robust oscillation of *Bmal1* and *Rev-erbα* expression in wild-type cells (Fig. 4d). In contrast, rhythmic expression of these genes is essentially abolished in cells lacking PGC-1α. Consistent with these results, the ability of RORα to induce the expression of *Bmal1* and *Rev-erbα* is impaired in hepatocytes lacking PGC-1α (Supplementary Fig. 3), indicating that PGC-1α is required for the transcriptional activity of RORα in this context. We conclude from these studies that PGC-1α exerts its effects on the circadian clock largely in a tissue- and cell-autonomous manner.

We have identified PGC-1α, a major metabolic regulator, as a critical component of the mammalian clock. PGC-1α stimulates the expression of *Bmal1* through coactivating the ROR family of orphan nuclear receptors and is essential for normal circadian rhythms. Because PGC-1α expression is highly responsive to nutritional signals and potentially light, our findings support a mechanism through which energy metabolism and circadian clock can be directly coupled at the transcriptional level (Fig. 4e). *PGC-1α* null mice are resistant to diet-induced obesity and are more insulin-sensitive[6,17], whereas *Clock* mutant mice develop obesity. These differences are probably due to the fact that *PGC-1α* null mice are hyperactive and have a higher metabolic rate in the absence of increased food intake. Our findings raise an intriguing possibility that the expression and/or activity of PGC-1α itself may be regulated

by components of the clock oscillator. Because feeding and loco-motor activity are regulated by circadian clocks, it is possible that rhythmic PGC-1α expression is controlled by these physiological and behavioural rhythms. Alternatively, PGC-1α is associated with the SirT1 histone deacetylase complex[29] and may directly sense the metabolic state of the cell, in a similar way to the regulation of the clock homologue NPAS2 by redox status[30]. Disruption of circadian rhythms has been implicated in the pathogenesis of metabolic disorders. Our studies have uncovered a potential molecular target that could simultaneously modulate circadian clocks and energy metabolism.

METHODS SUMMARY

Transcriptional analyses. Adenoviral transduction, reporter gene assays, and ChIP experiments were performed as previously described[6,9]. For gene expression analyses, total RNA was isolated from tissues or cultured cells using Trizol reagents (Invitrogen), reversed transcribed and analysed by quantitative PCR using SYBR-Green.

Animal experiments. For activity and body temperature monitoring, wild-type and *PGC-1α* null mice were implanted with a G2 Minimiter II probe in the abdominal cavity (MiniMitter) and allowed to recover for two weeks prior to the initiation of monitoring using telemetric receivers. Voluntary activity and body temperature were recorded under light:dark cycles 12:12 h for two weeks before switching to constant darkness for three more weeks. For restricted feeding, male wild-type and *PGC-1α* null mice were fed exclusively at night for ten days. Blood glucose levels were measured at the end of night feeding as well as four days following the switch to day feeding. Tissues were harvested at CT1 and 13 for both feeding groups for qPCR analysis of gene expression. For liver-specific PGC-1α knockdown, wild-type C57/Bl6J male mice were administered control or *PGC-1α* RNAi adenoviruses via the tail vein. Tissues were harvested from transduced animals five days after adenoviral transduction.

Serum shock. Immortalized fibroblasts from wild-type and *PGC-1α* null mice were established and maintained in DMEM supplemented with 10% FBS. For serum shock, media of confluent cultures was replaced with DMEM plus 50% horse serum ($t = 0$) and, after 1 hour, the cells were washed once with PBS and

incubated with serum-free DMEM. Total RNA was extracted at the indicated time points and processed for qPCR analysis. More details are provided in Methods.

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METHODS

Adenoviral transduction. The maintenance and adenoviral transduction of primary hepatocytes and C2C12 myotubes was performed, as previously described[6]. Total RNA was isolated using Trizol reagents (Invitrogen) 48 h following transduction, reverse transcribed, and analysed by qPCR using SYBR Green. A complete list of PCR primers is shown in Supplementary Table 2. Primers for ribosomal protein 36B4 were included for normalization. Microarray analysis was performed on total RNA isolated from hepatocytes transduced with *GFP* or *PGC-1α* adenoviruses using U74av2 chips (Affymetrix).

Animals. All animal procedures were approved by the University Committee on Use and Care of Animals. For analysis of PGC-1α expression in tissues, male C57/Bl6J mice of 12 weeks of age were kept under light:dark (LD) 12:12 h and subsequently subjected to constant darkness for 36 h. Tissues from five mice were dissected every 4 h for a total of 24 h thereafter. Total RNA was isolated and analysed by qPCR using gene-specific primers. For analysis of gene expression in wild-type and *PGC-1α* null mice, three to five mice of each genotype were euthanized every six hours. Tissues were immediately frozen and subsequently processed for qPCR analysis and immunoblotting analyses.

For activity and body temperature monitoring, 3-month old wild-type and *PGC-1α* null mice were implanted with a G2 Minimiter II probe in abdominal cavity (MiniMitter). Animals were allowed to recover for two weeks prior to the initiation of monitoring using telemetric receivers. Voluntary activity and body temperature were recorded under LD 12:12 h for two weeks before switching to constant darkness for three more weeks. Data were analysed using software supplied by the manufacturer. For restricted feeding, male wild-type and *PGC-1α* null mice were fed exclusively at night for ten days. Blood glucose levels were measured at the end of night feeding as well as four days following the switch to day feeding. Tissues were harvested at CT1 and 13 for both feeding groups for qPCR analysis of gene expression. For liver-specific PGC-1α knockdown, a total of 32 C57/Bl6J male mice were administered control or *PGC-1α* RNAi adenoviruses (0.1 absorbance units per mouse), as previously described. Five days following tail vein injection, tissues were harvested from transduced animals at CT1, 7, 13 and 19 (four mice per treatment).

Reporter gene assays. Reporter gene assays were performed in BOSC 293 cells, as previously described. In a typical experiment, 25 ng of reporter plasmids were mixed with 10 ng of expression constructs for transcription factors in the presence or absence of PGC-1α expression construct. Equal amounts of DNA were used for all transfection combinations by adding appropriate vector DNA. Relative luciferase activities were determined 48 h following transfection. All transfection experiments were performed in triplicates.

Serum shock. Immortalized fibroblasts from wild-type and *PGC-1α* null mice were established and maintained in DMEM supplemented with 10% FBS. For serum shock, media of confluent cultures was replaced with DMEM plus 50% horse serum ($t = 0$), and after 1 h, the cells were washed once with PBS and incubated with serum-free DMEM. Total RNA was extracted at the indicated time points and processed for qPCR analysis using 36B4 as a normalization control.

ChIP assay. Chromatin immunoprecipitation was performed essentially as described by the Upstate Biotechnology (www.upstate.com). Briefly, HepG2 hepatoma cells were transduced with *GFP* or *Flag–PGC-1α* adenoviruses for 48 h. Chromatin lysates were prepared, pre-cleared with Protein-A agarose beads, and immunoprecipitated with antibodies against Flag (Sigma), K9-dimethylated and K4-trimethylated histone H3 (Abcam), acetylated histone H3 (Upstate Biotechnology), p300 and Clock (Santa Cruz Biotech), GCN5 (BioLegend), or normal mouse IgG (Sigma) in the presence of BSA and salmon sperm DNA. Beads were extensively washed before reverse cross-linking. DNA was purified using a PCR purification kit (Qiagen) and subsequently analysed by PCR using primers flanking the proximal RORE on the human *Bmal1* promoter, as detailed in Supplementary Table 2.