Circadian REV-ERBs repress E4bp4 to activate NAMPT-dependent NAD\(^+\) biosynthesis and sustain cardiac function

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The heart is a highly metabolic organ that uses multiple energy sources to meet its demand for ATP production. Diurnal feeding–fasting cycles result in fluctuations in substrate availability, which, together with increased energetic demand during the active period, impose a need for rhythmic cardiac metabolism. The nuclear receptors REV-ER\(\alpha\) and REV-ER\(\beta\) are essential repressive components of the molecular circadian clock and major regulators of metabolism. To investigate their role in the heart, here we generated mice with cardiomyocyte (CM)-specific deletion of both Rev-erb genes (\(Nn1d1/2\)), which died prematurely due to dilated cardiomyopathy. Loss of REV-ERBs markedly downregulated expression of fatty acid–oxidation genes before overt pathology, which was mediated by induction of the transcriptional repressor E4BP4, a direct target of cardiac REV-ERBs. E4BP4 directly controls circadian expression of Nampt and its biosynthetic product NAD\(^+\) via distal cis regulatory elements. Thus, REV-ERB-mediated E4BP4 repression is required for Nampt expression and NAD\(^+\) production by the salvage pathway. Together, these results highlight the indispensable role of circadian REV-ERBs in cardiac gene expression, metabolic homeostasis and function.

Most organisms manifest diurnal phases of activity and inactivity, requiring switching between fuel sources for biologic functions. In mammals, substrate selection is tissue specific, and thus, while some organs are mainly fueled by glucose (for example, the brain), others such as the liver and heart predominantly rely on fatty acids. Food use depends not only on nutrient availability but also on the physiological context\(^1\). Disruption of circadian clocks can lead to a plethora of metabolic defects such as hyperglycemia, hypoinsulinemia, diabetes and dyslipidemia, sleep defects, impaired regenerative capacity and also predisposition to cancer\(^2\). Thus, understanding the precise contributions of the clock to organ-specific tissue homeostasis is of utmost importance.

Several cardiovascular parameters such as heart rate and blood pressure fluctuate over the course of the day, peaking in the early phase of the awake period\(^3\)\(^4\). Many circadian-regulated processes in the heart are governed by the CM circadian clock\(^6\). The underlying molecular pathway relies on a transcriptional–translational feedback loop that comprises a number of core clock factors, such as BMAL1, CLOCK and REV-ER\(\alpha\) and REV-ER\(\beta\). Their rhythmic function results in tissue-specific clock-controlled gene expression programs that maintain proper cellular physiology\(^1\). Nicotinamide phosphoribosyltransferase (Nampt), encoding the rate-limiting enzyme in the NAD\(^+\) salvage pathway, is one such clock-controlled gene shown to be under direct control of the BMAL1–CLOCK heterodimer\(^7\)\(^8\). NAD\(^+\) plays a central role in not only oxidative metabolism and bioenergetics but also as a cofactor for reactions that can generate signaling intermediates or alter post-translational modifications (for example, ADP ribosylation or deacetylation by sirtuins\(^9\)). Disturbing the positive output of the circadian clock via ablation of \(Clock\) or \(Bmal1\) (\(Arntl\)) in the whole body\(^10\), as well as in the heart specifically, results in disrupted metabolism\(^11\), ventricular arrhythmias\(^12\) and dilated cardiomyopathy\(^13\)\(^14\). REV-ERB nuclear receptors are key factors in the negative limb of the core clock and have emerged as a key mechanistic link to metabolism in extracardiac tissues\(^14\)\(^15\). As they lack the canonical activation domain of nuclear receptors, REV-ERBs act as potent and constitutive transcriptional repressors by recruiting nuclear receptor corepressor (NCoR) and histone deacetylase 3 (HDAC3)\(^16\). The latter facilitates histone deacetylation at enhancers, thereby contributing to transcriptional repression of target genes. How REV-ERBs...
regulate gene transcription in the healthy heart is mainly unknown, but several reports show that REV-ERB agonists can impact the cardiac transcriptome, hypertrophy and function (even after myocardial infarction)\(^\text{17-19}\). Although the compounds used in these studies were shown to display some non-REV-ERB-mediated effects\(^\text{16}\), this nonetheless highlights the importance of investigating the role and target genes of REV-ERBs to better understand how they modulate heart function in vivo under physiological conditions.

Here, we report that a mouse model in which both Rev-erb genes are deleted in CMs (CM-RevDKO mice) results in the development of age-onset dilated cardiomyopathy, leading to premature death. We provide evidence that, in the absence of REV-ERBs, inability to meet the high demands for energy production in the heart as a result of direct derepression (activation) of one set of genes and repression of an additional set via upregulation of transcriptional repressors leads to development of heart failure. In extracardiac tissues, circadian clocks have been reported to facilitate cyclic Nad\(^+\) levels via BMAL1–CLOCK-mediated circadian transcriptional activation of Nampt\(^\text{24}\). We discover that, in addition to regulation by the positive arm of the clock pathway, REV-ERBs are essential for proper Nampt expression. Despite increased Bmal1 expression, absence of REV-ERBs results in a drastic repression of Nampt, secondary to upregulation of E4BP4, which directly binds to cis regulatory elements (CREs) upstream of the gene. Consistent with a dominant effect of E4BP4, we show that repression of Nampt in Bmal1-knockout (KO) hearts is prevented by additionally knocking out E4bp4 (Njfl3), demonstrating a causal role for E4BP4-mediated Nampt downregulation in the circadian pathway. In conclusion, we show that circadian REV-ERBs are indispensable to establish the transcriptional program that controls cardiac metabolism, deregulation of which leads to dilated cardiomyopathy and premature death.

**Results**

**CM-specific depletion of REV-ERBs causes lethal dilated cardiomyopathy.** To interrogate the role of the nuclear receptors REV-ERBs (encoded by Nrf1\(\text{d1}\)) and REV-ERB\(\beta\) (encoded by Nrf1\(\text{d2}\)) in the heart, we crossed conditional Rev-erb\(\alpha\)\(^\text{21}\),Rev-erbf\(\beta\)\(^\text{21}\) with a CM-specific Cre deleter (αMHC-Cre\(^\text{11}\), C57BL/6 background) to generate CM-specific Rev-erb\(\alpha\),Rev-erbf\(\beta\)-double KO (DKO) (CM-RevDKO) mice (Fig. 1a). Reduction in Rev-erb\(\alpha\) and Rev-erbf\(\beta\) mRNA expression was observed in the heart across a 24-hour circadian cycle (Fig. 1b), and REV-ERB protein levels were constitutively reduced in hearts from CM-RevDKO mice (referred to as CM-RevDKO hearts) (Fig. 1c). Consistent with this, expression of the canonical REV-ERB target genes Bmal1 and Npas2 was markedly increased in CM-RevDKO hearts (Fig. 1d) and BMAL1 protein levels were constitutively high (Extended Data Fig. 1a). Rhythmic Per2 mRNA levels, on the other hand, were unaffected (Extended Data Fig. 1b). Of note, day–night differences in Rev-erb\(\alpha\) mRNA levels were dampened in 6-month-old versus 2-month-old floxed control mice (Extended Data Fig. 1c).

While CM-RevDKO mice were born at expected Mendelian ratios (Extended Data Fig. 2a), the CM-RevDKO model resulted in severely reduced lifespan with increased mortality around 6–9 months in both male and female mice (Fig. 1e). CM-RevDKO mice display normal circadian behavior, food intake and body weight (Extended Data Fig. 2b–d). In addition, glucose and insulin tolerance tests in 6-month-old mice yielded normal results (Extended Data Fig. 2e,f). Yet, compared to wild-type (WT) mice, heart weight was higher in CM-RevDKO males and females at 6 months of age (Fig. 1f) but not in 2-month-old mice (Extended Data Fig. 2g), indicating that CM-specific Rev-erb deletion causes cardiac enlargement over time. Consistent with increased biventricular weight, CM cross-sectional area was also larger in 6-month-old CM-RevDKO mice (Extended Data Fig. 2h). This is in line with a recent report describing a protective effect of REV-ERB activation against hyper trophy in neonatal ventricular rat CMs\(^\text{26}\). Echocardiography revealed that left ventricular (LV) systolic and end-diastolic diameters were significantly increased at 6 months of age, accompanied by reduced LV ejection fraction, LV fractional shortening (Fig. 1g,h) and other echocardiographic abnormalities reflective of the cardiomyopathic pathologic remodeling only in older mice (Extended Data Fig. 2i,j). Hematoxylin and eosin and trichrome staining revealed dilation in the CM-RevDKO heart, without signs of fibrosis (Fig. 1i and Extended Data Fig. 2k). In addition, we could not detect substantial cell death in CM-RevDKO or control hearts (Extended Data Fig. 2l). In conclusion, loss of REV-ERBs in CMs has major structural as well as functional consequences leading to premature death from dilated cardiomyopathy.

**CM-RevDKO disrupts metabolic transcriptional programs and causes mitochondrial defects.** To elucidate the molecular mechanism that underlies age-dependent heart failure upon KO of both Rev-erb genes in CMs, we performed transcriptomic analysis at 2 months of age (that is, before the onset of cardiomyopathy). Hearts were isolated at zeitgeber time (ZT)10, which corresponds to elevated expression of Rev-erb\(\alpha\) and Rev-erbf\(\beta\) as well as the trough of Bmal1 expression in WT hearts (Extended Data Fig. 3a). We detected 553 differentially expressed genes (DEGs) (log2 (fold change (FC))) > 0.58, adjusted P < 0.05), of which 253 were upregulated and 300 were downregulated in CM-RevDKO hearts (Fig. 2a and Supplementary Table 1). Comparison of these genes (n = 553) with transcripts differentially expressed at ZT10 upon KO of Rev-erb genes in the liver\(^\text{26}\) (n = 762, log2 (FC) > 0.58, adjusted P < 0.05) showed that REV-ERB target genes in the heart were generally distinct from those in the liver (Extended Data Fig. 3b), consistent with the established notion that REV-ERBs control gene expression in a tissue-specific manner with the exception of core clock factors\(^\text{26}\). Indeed, canonical circadian regulators such as Bmal1, Clock and
Npas2 were derepressed in both CM-RevDKO hearts and hepatocyte-specific RevDKO livers (Extended Data Fig. 3b). By contrast, several key cardiac genes involved in essential CM function (such as metabolism, signaling and ion channels, for example, p21 (Cdkn1a), Slc41a3 and Fhit) were dysregulated upon REV-ERB loss, as validated by reverse transcription quantitative PCR (RT–qPCR).
Fig. 2 | REV-ERBs control metabolic gene expression in the heart. 

a, Scatterplot showing 553 DEGs in CM-revDKO versus control hearts (n = 3 per genotype; cutoff, FC > 1.5 for upregulated (red) and downregulated (blue) genes; adjusted P (false discovery rate (FDR)) < 0.05). 

b, KEGG pathway and GO analysis on all DEGs from a. Analysis was performed using https://www.gsea-msigdb.org/gsea/index.jsp.

c, Schematic showing metabolic pathways with significantly upregulated (red) and downregulated (blue) genes in CM-revDKO mice, determined in a, b. Agpat9, Gpat3; IR, insulin receptor; TAG, triacylglycerol; TCA, tricarboxylic acid cycle. 

d, Electron micrographs of ventricular tissue from control and CM-revDKO hearts at 6 months. Open arrowheads denote mitochondria with normal appearance and dense cristae. Black arrows denote aberrant mitochondria with abnormal cristae. Scale bars, 1 µM.

e, Traces for oxidative phosphorylation (oxygen-consumption rate (OCR)) from Seahorse measurements in a mitochondrial stress test on Rev-erbαfl/fl;Rev-erbβfl/fl neonatal CMs transduced with Adeno-RFP (control) versus Adeno-Cre (RevDKO) vectors. Palmitate–BSA was added to CMs immediately before the assay (n = 11 wells with independently transduced cells for control and n = 12 for RevDKO samples). Oligo, oligomycin; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; AM, antimycin; rot, rotenone. n represents biologically independent replicates unless otherwise indicated. Data are shown as mean ± s.e.m. *P < 0.05 by two-sided Student’s t-test (exact P values are provided in the source data).
(Extended Data Fig. 3c). Overlap with previously published circadian cardiac gene expression data showed that ~31% of DEGs in CM-RevDKO hearts were rhythmically expressed in WT hearts. Moreover, the acrophases of the upregulated and downregulated genes mainly clustered anti-phase and in-phase, respectively, with expression of Rev-erb genes (Extended Data Fig. 3d). This suggests that REV-ERB normally represses the observed upregulated genes during the inactive period of the day and that the downregulated genes are most likely not direct REV-ERB targets. To further investigate the functionality of upregulated and downregulated genes in CM-RevDKO hearts, we performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Uregulated genes were enriched for terms such as circadian rhythms, insulin signaling and regulation of transport and cell–cell signaling, while downregulated genes were related to mitochondrial activity (e.g. Ppargc1a, Ppargc1b, Ppargc1c, Ppargc1d). These data suggest that the deletion of both Rev-erb genes in the heart resulted in transcriptional deregression of essential cardiac oxidative metabolic processes.

Indeed, mRNA for several components in the insulin signaling pathway, a process known to be under clock control in the heart, such as Iris (upregulated), Pde3a (upregulated) and Akt1 (downregulated), were differentially expressed in CM-RevDKO hearts as well as downstream insulin-targeted pathways, such as glycogen storage (for example, Pygl (upregulated)) and signaling pathways that modulate the activity of proteins encoded by these transcripts (for example, Ppp1r3a (downregulated), Ppp1r1b (upregulated) and Ppp1r3b and Ppp1r3c (upregulated)) (Fig. 2c). We validated the dynamic increase in levels of the phosphoprotein DARPP-32 (E4bp4 (upregulated)) (Fig. 2c). We validated the dynamic increase in levels of the phosphoprotein DARPP-32 (E4bp4 (upregulated)) (Fig. 2c). We validated the dynamic increase in levels of the phosphoprotein DARPP-32 (E4bp4 (upregulated)) (Fig. 2c).

REV-ERBs can mediate gene activation via direct repression of E4bp4 in the heart. REV-ERBs are known to function through binding to CRES such as enhancers and promoters, leading to transcriptional repression. To identify REV-ERB-responsive regulatory regions that might explain directly upregulated genes as well as putative regulatory sites involved in indirect gene downregulation in CM-RevDKO hearts, we performed cleavage under targets and release using nuclease (Cut&Run) for acetylated histone 3 lysine 27 (H3K27ac), a mark of active enhancers. Because downregulated genes are unlikely to be direct REV-ERB targets, we opted to perform Cut&Run on isolated CRES from CM and CM-RevDKO mice to increase specificity and search for binding sites of potential transcription factors involved in downregulated CRES (Fig. 3a). H3K27ac changes were integrated with RNA-seq data using integrated analysis of motif activity and gene expression (IMAGE), which revealed a number of transcription factors, such as REV-ERBs, BMAL1, NPAS2, CLOCK, NKX2-5 and E4BP4 (also called NFIL3) (Supplementary Table 2) as putative drivers of REV-ERB-dependent mRNA and enhancer activity changes. In addition, we performed binding similarity analysis using Cistrome Data Browser (Cistrome DB) matching regions with changes in H3K27ac with Cistrome from published heart, muscle and liver chromatin immunoprecipitation followed by sequencing (ChiP-seq) experiments. As expected, this analysis revealed that binding of REV-ERBα as well as known REV-ERBβ, HDAC3 and NCoR1 were enriched at H3K27ac sites that became active upon loss of REV-ERBs (Fig. 3b), supporting direct involvement of REV-ERBs in target derepression. By contrast, binding similarity...
analysis of differentially enriched H3K27ac regions that were
downregulated by at least twofold in CM-RevDKO CMs revealed
enrichment for glucocorticoid receptor (GR), TBX3, E4BP4 and
others (Fig. 3b).

To establish which of these factors is likely directly involved in
repression of the downregulated DEGs described here, we investi-
gated whether they were differentially expressed (FC > 1.5, adjusted
\( P < 0.05 \)) upon DKO of Rev-erbα and Rev-erbβ. Expression of E4bp4
Constitutive binding of E4BP4 contributes to downregulated gene expression in CM-RevDKO hearts. a, Heatmap showing E4BP4 ChIP–seq tag densities at 4,248 E4BP4 peaks in control and CM-RevDKO hearts at ZT10 and ZT22. b, Venn diagram showing overlap between DEGs in CM-RevDKO versus control hearts and annotated peaks from the control cardiac E4BP4 cistrome (at ZT22). c, ChiP–seq, Cut&run and RNA-seq read distribution for E4BP4 and H3K27ac near identified E4BP4-target genes in control and CM-RevDKO hearts. d, Relative E4BP4-target mRNA expression in control versus CM-RevDKO hearts from 2-month-old male mice (n = 3 hearts per genotype per time point, except for n = 2 at ZT2 and n = 4 at ZT18 for CM-RevDKO mice). e, CM-specific E4bp4-KO (CM-E4bp4-KO) hearts from 3-month-old male mice. n = 2 at ZT2 and n = 8 for control mice and 10 for CM-specific E4bp4-KO mice. f, Venn diagram showing overlap between DEGs in CM-RevDKO mice with published data of deregulated genes in cardiac-specific Gr (Nr3c1)−/− mice. g, Heatmap showing E4BP4 ChIP–seq tag densities at 4,248 E4BP4 peaks in control and CM-RevDKO hearts at ZT10 and ZT22.

was found to change significantly with an observed upregulation of approximately sixfold. While expression of Gr (Nr3c1) was moderately decreased (downregulated, FC = 1.4, Fig. 3c), overlap of genes downregulated in CM-RevDKO mice showed almost no overlap with published data of deregulated genes in cardiac-specific Gr−KO hearts (Extended Data Fig. 5a). We therefore turned our focus to E4bp4 and observed that the gene was constitutively derepressed across a full diurnal cycle at the mRNA level as well as at the protein level (Fig. 3d,e). To validate regulation of E4bp4 by REV-ERBs in the heart specifically, we performed ChIP–seq for REV-ERBs using an anti-hemagglutinin (HA) antibody at ZT10 in a 3×HA-Rev-erba mouse model as well as in WT (with no HA knockin) mice as control. In total, 1,486 high-confidence peaks were identified in three samples with high correlation between biological replicates (Pearson correlation >0.85; Fig. 3f and Extended Data Fig. 5b). Nine percent of the REV-ERB peaks were found at promoters, 51% were found at intronic sites, and 35% were found at intergenic regions (Extended Data Fig. 5c). A motif search confirmed binding enrichment at known motifs, such as RORE and RevDR2. In line with this, enhancers with twofold increased H3K27ac signal revealed similar motifs (Extended Data Fig. 5d). Overlap of annotated high-confidence peaks with DEGs in CM-RevDKO mice showed that upregulated genes were significantly enriched for REV-ERB binding in their vicinity, while downregulated genes were not (Extended Data Fig. 5e). Examples of DEGs with REV-ERBα binding and changed regulatory activity include both well-known canonical clock target genes, such as Bmal1 and Cry1 (Extended Data Fig. 5f,g), as well as important cardiac regulators, such as the ischemia–reperfusion injury modulator encoded by p21 and Fbn2 encoding the extracellular glycoprotein fibrillin 2 (Extended Data Fig. 5h).

Importantly, we found that REV-ERBα binds to the E4bp4 promoter in the heart (Fig. 3g), suggesting that REV-ERB directly...
represses E4bp4 expression. Indeed, overexpression of Rev-erbα with varying adenoviral titers caused a dose-dependent pattern of E4bp4 repression (Fig. 3h). To further verify REV-ERB-dependent functional transcriptional regulation of E4bp4, we constructed an E4bp4-luciferase (E4bp4-luc) construct containing the E4bp4 promoter region (−445 bp, +390 bp relative to the transcriptional start site) harboring an identified putative REV-ERB response element (REV-RE). Transient transfection experiments showed that Rev-erbα overexpression induced transcriptional repression of this WT E4bp4-luc construct (Fig. 3i and Extended Data Fig. 5i). Repression was abolished when the REV-RE was either mutated or deleted (Fig. 3i). These results demonstrate that REV-ERBs represses E4bp4 expression directly by binding to a REV-RE identified in its promoter-proximal region. In conclusion, loss of REV-ERBs results in derepression of a set of genes including the transcriptional repressor encoded by E4bp4. Upon Rev-erb DKO, E4bp4 is expressed at constitutively high levels and could contribute to the paradoxical downregulation of its targets by the loss of repressive REV-ERBs.

Derepression of E4bp4 causes downregulated lipid metabolism gene expression. To interrogate whether CM-RevDKO-mediated E4bp4 derepression has a role in downregulation of genes, we performed ChIP–seq for E4bp4 at ZT10 versus ZT22 in control as well as CM-RevDKO hearts. Both known and de novo motif searches for control heart peaks at ZT22 (n = 3,384) confirmed NFIL3 as the top enriched motif (Extended Data Fig. 6a), validating the quality of the ChIP-seq data. Peaks were annotated to promoters (~28%), intergenic (~25%) and intronic (~43%) regions (Extended Data Fig. 6b). In contrast to the circadian E4bp4 binding that we observed in control hearts, chromatin-binding rhythmicity was lost in CM-RevDKO mice, which displayed constant binding to CREs at ZT10 and ZT22 (Fig. 4a). Indeed, the majority (2,187 of 3,384) of E4bp4 binding sites at ZT22 in control hearts were bound at both ZT22 and ZT10 after CM-RevDKO (Extended Data Fig. 6c), indicating that E4bp4 constitutively binds to sites normally occupied only during the dark phase.

Intersection of these data with DEGs in CM-RevDKO hearts showed greater enrichment for E4bp4 binding near downregulated transcripts (Fig. 4b), consistent with transcriptional repression by E4bp4. A number of important metabolic genes that were repressed in CM-RevDKO hearts contained regulatory regions at which H3K27ac was reduced in the absence of REV-ERBs and colocalized with E4bp4 binding in the heart. For example, Cpt1a (encoding carnitine O-palmitoyltransferase, regulator of mitochondrial uptake of long-chain fatty acids and their subsequent β-oxidation), Ces1d (encoding the lipase carboxylesterase 1d) and Ces1d (encoding glycerol-3-phosphate acyltransferase 1, mitochondrial and CM-RevDKO hearts, respectively (Fig. 5a). Intersection of these data with previously published 3D chromatin architecture Hi-C data from the mouse heart revealed looping between these putative enhancers and the Nampt promoter (Fig. 5a), suggesting that these CREs directly regulate cardiac Nampt gene expression. In addition, in each of these three enhancers, we found a D-box motif (TTAYGTTA), a canonical sequence bound by E4bp440. ChIP–qPCR for E4bp4 at the most proximal CREs (50 kb upstream of the Nampt promoter) validated E4bp4 binding at both ZT10 and ZT22 in CM-RevDKO hearts compared to only ZT22 in the control (Fig. 5b). These epigenetic changes led to constant repression of Nampt mRNA as well as NAMPT protein levels across the full diurnal cycle (Fig. 5c,d). Diurnal Nampt mRNA expression has classically been attributed to BMAL1–CLOCK-mediated transcription, for which KO of Clock or Bmal1 causes drastic reductions in levels of Nampt as well as NAD+41,44. As Nampt levels were reduced and E4bp4 was induced in both CM-specific Bmal1-KO and Rev-erbα/Rev-erbβ-KO hearts, we hypothesized that E4bp4 would be the main driver of Nampt repression. Therefore, we compared Nampt as well as Bmal1, Rev-erbα, Rev-erbβ and E4bp4 mRNA levels in the repressive function of REV-ERBs in both models. In reverse, Rev-erb DKO results in the constitutive presence of Bmal1 (Fig. 1d and Extended Data Fig. 1a); therefore, a potential set of genes downregulated in CBK mice but upregulated in CM-RevDKO mice could also exist. We investigated this and indeed found that 105 of 253 upregulated genes in CM-RevDKO hearts were also upregulated in CBK hearts, while, of these 253 genes, only ten were downregulated in CBK hearts (Extended Data Fig. 6e). This suggests that the upregulated transcriptional changes could be a consequence of REV-ERB loss rather than Bmal1 loss (in CBK mice) or gain (in CM-RevDKO mice). Indeed, ~25% of these genes had a binding site for REV-ERBα in the heart (Extended Data Fig. 6f). In total, 121 of 300 downregulated genes in CM-RevDKO hearts were also downregulated in CBK hearts (Extended Data Fig. 6g).

Given that the potent repressor E4bp4 is upregulated in both Bmal1-KO and Rev-erbα/Rev-erbβ-KO models, we compared transcriptomic data from hearts from KO mice for these two transcription factors with E4bp4 ChIP–seq data. Approximately 45% of the commonly downregulated genes in both Bmal1-KO and Rev-erbα/Rev-erbβ-KO hearts had E4bp4 binding in close proximity (Extended Data Fig. 6h). This overlap between differentially enriched genes suggests that loss of REV-ERBs and constitutively high levels of E4bp4 as a result contribute to the observed phenotype in both models. Indeed, as loss of Bmal1 leads to marked reduction in REV-ERBs41, this suggests that Bmal1 KO has an indirect effect on E4bp4 via downregulation of REV-ERBs.
Fig. 5 | REV-ERBs control cardiac NAD⁺ biosynthesis via E4BP4-mediated repression of Nampt. a, Hi-C data from the heart (obtained from\(^{10}\)), H3K27ac Cut&run data from isolated hearts of adult control and CM-RevDKO mice (at ZT10) and E4BP4 ChIP-seq data (at ZT10 and ZT22) from control and CM-RevDKO hearts. Green dotted lines delineate three-dimensional contacts. Identified CREs are highlighted in faint yellow; three of them were found to be bound by E4BP4 and are marked by black rectangles, and their respective distance to the transcriptional start site of Nampt is shown. Data were visualized with Juicebox\(^{66}\). b, E4BP4 ChIP-qPCR at the CRE identified in a, 50 kb upstream of the Nampt transcriptional start site (at ZT10 and ZT22), from control and CM-RevDKO hearts. A random locus in the genome was chosen as the negative (neg.) control (\(–50\) kb) Neg. control. c, Nampt mRNA levels in different genetic models. d, Immunoblot and protein quantification for NAMPT levels from control versus CM-RevDKO hearts from 2-month-old male mice. e, Relative mRNA expression in hearts from control versus CM-RevDKO 2-month-old male mice (\(n=3\) per condition per genotype). f, Relative Nampt mRNA expression in hearts from multiple genetic CM-specific KO models: CBK (\(n=7\) for control and \(n=8\) for KO), CM-RevDKO (\(n=5\) for control and \(n=6\) for KO), E4bp4 (\(n=8\) for control and \(n=10\) for KO) and CBK-E4bp4 DKO (\(n=6\) for control and \(n=9\) for DKO) and in control hearts from 2-month-old male mice for CM-RevDKO and control mice at ZT10 and from 3-month-old male mice for the rest at ZT22. Cartoon of summarized experimental data and proposed mechanism of transcriptional regulation of Nampt in different genetic KO models. g, NAD⁺ levels from hearts from control versus CM-RevDKO 2-month-old mice (\(n=6\) hearts per time point except for \(n=5\) for ZT6 in control mice; \(n=5\) except for \(n=4\) for ZT6; \(n=6\) for ZT18; and \(n=7\) for ZT22 in CM-RevDKO mice). **(\(P<0.01\)); ***\((P<0.001); ****\((P<0.0001)\) by two-way ANOVA in c,d,g, by one-way ANOVA followed by Tukey’s multiple-comparisons test in b and by two-sided Student’s \(t\)-test in e (exact \(P\) values are provided in the source data).
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expression (Fig. 5e,f). Protein levels were in line with this, with reduction of NAMPT levels in Bmal1–KO hearts, which was par-
reduced -KO and Bmal1 mice as well. In addition, genes such as Ppp1r1b, encoding DARPP-32, could be linked to both the core clock and metabolism (Fig. 2). Future investigations will be needed to disen-
tangle this putative contribution of different genes to the phenotype.

In addition, we find that the transcriptional repressor E4BP4 is a direct REV-ERBβ target that is constitutively derepressed in CM-RevDKO hearts. Expression of E4bp4 across a full diurnal cycle leads to occupancy at its cognate binding sites at ZT22 as well as at ZT10, rather than at ZT22 only. At ZT22, we observed a modest decrease in E4BP4 binding in DKO hearts compared to control hearts, which could potentially be due to deregulated competing transcription factors. The contribution of downregulated genes to the cardiac phenotype is demonstrated by decreased expression of a set of important metabolic genes (for example, Cpt1a, Ces1d and Gpam; Fig. 4c and Extended Data Fig. 6d). In addition, this study reveals that the marked downregulation of NAMPT in CM-RevDKO hearts is facilitated through constitutive E4BP4 binding and repression at three CREs 160–50 kb upstream of the transcriptional start site of Nampt. Concomitantly, cardiac NAD+ levels were reduced across a full diurnal cycle. However, although both Nampt mRNA and NAMPT protein levels were constitutively lower in CM-RevDKO hearts, we still noted a residual circadian pattern in the lower NAD+ levels. Because CM-RevDKO mice still show rhythmic activity and feeding pat-
terns, it is likely that systemic rhythmic cues (for example, food intake), other NAD+ biosynthesis pathways (de novo or kynure-
mine or Preiss–Handler) or remaining NAMPT protein are contrib-
tuting to residual rhythms. These data suggest that reduced cardiac NAD+ levels together with impaired metabolic gene expression can result in age-onset development of dilated cardiomyopathy and premature lethality (Fig. 6).

We find that a substantial number of the genes downregulated in CM-RevDKO hearts were also found to be downregulated in CBK hearts. As these genes are enriched for E4BP4 binding, this suggests that E4BP4 could contribute to gene repression in CBK hearts as well. We found that downregulated Nampt levels in CBK hearts could be rescued by additional KO of E4bp4. From these data, we conclude that E4BP4 is an essential mediator of circadian Nampt regulation and suggest that the REV-ERB–E4BP4 repres-
sive axis is more dominant than the previously described activating BMAL1–CLOCK axis in circadian transcriptional Nampt regu-
lation. In hepatocyte-specific Rev-erbβ-DKO livers we also noted downregulated Nampt and derepressed E4bp4. As the Nampt gene is downregulated in Clock-KO and Bmal1–KO livers and also contacts the upstream CREs, this suggests that E4BP4-mediated repression of Nampt is not tied to the heart only and is likely to

to dilated cardiomyopathy; FA, fatty acid; FAO, fatty acid oxidation.

Discussion

The prevalence of heart disease in association with metabolic disor-
ders stresses the importance of understanding how cardiac energy metabolism is maintained with constantly changing external cues. Here, we have uncovered an essential role for circadian REV-ERB nuclear receptors as major transcriptional contributors to meta-

Fig. 6 | Scheme of metabolic deregulation in hearts of CM-RevDKO mice. DCM, dilated cardiomyopathy; FA, fatty acid; FAO, fatty acid oxidation.

CM-specific Bmal1-KO, Rev-erbrα;Rev-erbrβ-KO and E4bp4-KO mice as well as Bmal1;E4bp4-DKO mice (Extended Data Fig. 7b). While both Bmal1-KO and Rev-erbrα;Rev-erbrβ-KO mice showed reduced Nampt levels, hearts from E4bp4-KO mice displayed increased Nampt expression (Fig. 5e). Strikingly, Bmal1;E4bp4-DKO mice did not show reduced Nampt levels, suggesting that, in Bmal1-single KO mice, E4BP4 was necessary to repress Nampt expression (Fig. 5e,i). Protein levels were in line with this, with reduction of NAMPT levels in Bmal1-KO hearts, which was partially restored by additionally knocking out E4bp4 (Extended Data Fig. 7c). Altogether, these results demonstrate that the repressive REV-ERB–E4BP4 axis is dominant and essential in regulating diur-
nal Nampt expression.

To investigate the consequences of deregulated NAMPT levels for NAD+, we compared CM-RevDKO to control hearts and observed severely reduced NAD+ levels at each time point during the diurnal cycle (Fig. 5g). While consistent reduction over 24 h was significant, we still noted an oscillatory pattern of NAD+ levels in the heart. Similar observations were made in Bmal1-KO livers, which also show lower levels across a circadian cycle but maintain rhythmicity. As CM-RevDKO mice have no systemic clock defect, activity of remaining NAMPT protein and substrate availability could contribute to remaining NAD+ rhythmicity. However, we show that constitutive E4BP4-mediated Nampt repression in the absence of REV-ERBs results in lower NAD+ levels throughout the entire 24-h cycle.

We find that a substantial number of the genes downregulated in CM-RevDKO hearts were also found to be downregulated in CBK hearts. As these genes are enriched for E4BP4 binding, this suggests that E4BP4 could contribute to gene repression in CBK hearts as well. We found that downregulated Nampt levels in CBK hearts could be rescued by additional KO of E4bp4. From these data, we conclude that E4BP4 is an essential mediator of circadian Nampt regulation and suggest that the REV-ERB–E4BP4 repres-
sive axis is more dominant than the previously described activating BMAL1–CLOCK axis in circadian transcriptional Nampt regu-
lation. In hepatocyte-specific Rev-erbβ-DKO livers we also noted downregulated Nampt and derepressed E4bp4. As the Nampt gene is downregulated in Clock-KO and Bmal1–KO livers and also contacts the upstream CREs, this suggests that E4BP4-mediated repression of Nampt is not tied to the heart only and is likely to

Fig. 6 | Scheme of metabolic deregulation in hearts of CM-RevDKO mice. DCM, dilated cardiomyopathy; FA, fatty acid; FAO, fatty acid oxidation.
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Immunoblotting. Samples were lysed in RIPA buffer supplemented with complete protease inhibitor cocktail (Roche) and PhosSTOP (Roche). Lysates were resolved by gel electrophoresis (Bio-Rad), transferred to PVDF membranes (Immobilon-P, Millipore) and probed with the following antibodies: anti-REV-ERBα (1:1,000, Abcam, ab174309), anti-BMAL1 (1:1,000, Bethyl Laboratories, A302-616A), anti-REV-ERBβ (1:500, Cell Signaling Technology (CST), 14312S (D5K80)), anti-NAMP (1:1,000, Bethyl Laboratories, A300-372A-M), anti-DARPP-32 (1:1,000, SCBT, sc-271111H (H)), anti-FLAG (1:1,000, Invitrogen, PAI-984A), anti-vinulin–HRP (1:1,000, CST, E18799), anti-HSP90 (1:1,000, CST, 48745), anti-rabbit IgG, HRP linked (1:1,000, CST, 70675).

Quantitative PCR with reverse transcription. Total RNA was extracted from cells (RLT buffer) and tissues (TRizol) using the RNeasy kit (Qiagen) according to the manufacturer’s instructions and treated with DNase (Qiagen) before reverse transcription. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with specific primers on a QuantStudio 6 Flex instrument (Applied Biosystems). mRNA expression was normalized to the housekeeping gene Pibb for all samples. Importantly, detecting REV-erb deletion through qPCR was performed with primers located in the deleted exons, as exons outside the deleted region are upregulated due to the normal auto-repression of REV-erb gene expression by REV-ERB proteins. Primer sequences for RT–qPCR were as follows: Pibb-fw, 5′-GCAAGCTTCAAGGCTCAGTC-3′; Pibb-rev, 5′-GTCATTGGAGTTGAGTTTTTC-3′; E4BP4-fw, 5′-CGACTTTGGTCAGTCTCCTG-3′; E4BP4-rev, 5′-GGTCAATTGCAACGCTACCTGTT-3′; Bmal1-fw, 5′-TAGGAGTGTGACCGGAAAGGAA-3′; Bmal1-rev, 5′-TCAAACAGCTGTCGCAATG-3′; Npsa-fw, 5′-TGGCCCTGACGGCAGCTC-3′; Npsa-rev, 5′-GTCATTCAGTCAAAAGTCTGT-3′; Nampt-fw, 5′-GGATTCTATTGGCAACAGGTAG-3′; Nampt-rev, 5′-CCAGCTTGATGCGCATCG-3′. Data were obtained for 3.5 d in 12-h–12-h LD cycles (84 h). Data acquisition and analysis were performed with the OxyMax and Prism software.

Glucose tolerance test. Six-month-old mice were fasted for 6 h before the glucose tolerance test. Blood was obtained from a tail cut and was assessed for fasting glucose levels using a OneTouch Ultra 2 (LifeScan, Johnson & Johnson) glucometer. For the test, briefly, mice were intraperitoneally injected with a glucose solution (0.2 g/ml)2 g/kg body weight), and then subsequent measurements of blood glucose levels were made at 15, 30, 60 and 120 min after injection.

Insulin tolerance test. Six-month-old mice were fasted for 5 h before the insulin tolerance test. After measurement of baseline blood glucose levels, insulin (0.5 U per kg) (Novolin, Novo Nordisk) was injected intraperitoneally, and blood glucose levels were measured again at 15, 30, 60 and 120 min after injection.

NAD+ measurements. Hearts from two-month-old male and female mice were collected and snap frozen in liquid nitrogen. Hearts were powdered, and subsequently, cardiac cells were lysed in 0.6 M perchloric acid. Cardiac NAD+ levels were measured by a cycling enzymatic assay as previously described15.

RNA sequencing. RNA-seq reads were aligned to the mouse genome (mm10) using HISAT2 with default parameters. Only unique mapped reads were counted for the study.
considered for further analysis. Normalized expression values, fragments per kb of exon per million reads mapped (FPKM), were calculated for each gene using StringTie\(^{32}\). Genes with FPKM greater than 1 in at least one sample were considered for further analysis. For differential expression analysis, the read counts were measured within Ensembl genes (NCBI: M37.67) using featureCounts\(^{53}\), and then DESeq2\(^{54}\) was used for data with adjusted \(P < 0.05\) and log(FC) > 0.8.

Cleavage under targets and release using nucleases. H3K27ac Cut&Run in isolated adult CMs was performed as previously described\(^{32}\). Cardiac cells were dissociated from hearts of 2-month-old adult mice by Langendorff perfusion between Z77 and ZT10. The CM fraction was enriched by centrifuging at low speed (300 r.p.m.) for 3 min. In total, 200,000 CMs were used for each Cut&Run reaction. Anti-H3K27ac (CST, 8173) or anti-rabbit IgG (CST, 3900) antibodies at 1:100 were incubated with CMs, which were attached to concanavalin A-coated magnetic beads (BANGS Laboratories, BP531) overnight at 4 °C. The next day, 2.5 μl pA-MNase from a 1:10 dilution of the original stock provided by the Henikoff laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) was applied to the cell–bead mixture and incubated at room temperature for 10 min. Pulled down DNA was extracted in place by phenol–chloroform extraction. The DNA library was prepared using the HyperPrep kit (Kapa Biosystems, KK8502) and subjected to sequencing on the Illumina NextSeq 500 platform at the Next-Generation Sequencing Core at the University of Pennsylvania. Paired-end read sequencing at 40 bp × 40 bp was adopted.

MAQ version 2.1.0\(^{32}\) was used for peak calling with the following parameters: \(P = 10^{-10}\), \(\text{extend} = 300\) and local lambda = 100,000 using IgCg as input controls. Peaks were extended to a minimum size of 2,000 bp, and overlapping peaks were merged into one nonredundant list using WT and CM-RevDKO peaks. To calculate FC in peak enrichment between WT and CM-RevDKO CMs, read coverage within peaks was determined using bedtools coverage. Reads were normalized for the total number of reads within peaks, and peaks with a control/CM-RevDKO ratio of >2 or <0.5 were defined to be down or up, respectively, in WT versus CM-RevDKO. Bigwig files were RPM normalized.

ChIP–seq and ChIPmentation and data analysis. In general, one to two mouse hearts were homogenized with 15 ml cold swelling buffer (10 mM HEPES, 2 mM MgCl\(_2\), 3 mM CaCl\(_2\), 1x protease inhibitor cocktail) 20–30 times with piston A; the homogenate was centrifuged at 400 and 4°C for 10 min, washed once with DPBS and resuspended in 10 ml 1% formaldehyde in DPBS for 20 min at room temperature. Next, cells were quenched with 0.125 M glycine for 10 min at room temperature. Following this, cells were resuspended in 15 ml cold swelling buffer and dounced again by piston B 10–40 times after 80–90% of single cells or nuclei were released from clumps. The homogenate was filtered with a 100-μm cell strainer and centrifuged at 400g and 4°C for 10 min. The pellet was resuspended in 10 ml cold swelling buffer containing 10% glycerol, followed by slow addition of 10 ml cold lysis buffer (swelling buffer with 10% glycerol and 1% IGEPAL). After incubation on ice for 5 min, 30 ml cold lysis buffer was added, and the sample was centrifuged at 600g and 4°C for 5 min. The pellet was washed once with 25 ml cold lysis buffer and 10 ml DPBS and then resuspended in 10 ml 1.5 M EG5 (Thermo Fisher Scientific, 21365), incubated at 37°C for 45 min with 0.125 mM MgCl\(_2\) for 10 min at room temperature. Cross-linked CMs were kept at -80°C until the ChIP experiment.

HA and E4BP4 ChIP–seq were performed according to a previous protocol\(^{2}\). In short, two cross-linked heart samples from 3 WT HA-Rev-erbα/Rev-erbβ\(^{2+/-}\) or WT on MUMC-3Reb-αI/Rev-erbβ\(^{2+/-}\) mice were used for ChIP pull down with anti-HA Magnetic Beads (Thermo Fisher Scientific, 88837) or anti-E4BP4 antibody (CST, 14312). Immunoprecipitated DNA was subjected to Tn5 (Illumina, 20034197) tagmentation and PCR amplification using (illuDNA, FC-121-1031). PCR products were subjected to the SPRIselect procedure (Beckman Coulter, B23318) for size selection and paired-end sequencing on the Illumina HiSeq platform.

ChIP–seq data were processed by a standard ChIP–seq pipeline: in brief, FastQ files were trimmed by cutadapt\(^{2}\) and aligned by BWA-MEM\(^{2}\). Reads with low MAPQ scores (≤10) and read duplicates were removed by SAMtools\(^{2}\) and picard (‘Picard Toolkit’; 2019, Broad Institute, GitHub Repository, [https://broadinstitute.github.io/picard/]). Filtered BAM files were used for peak calling with MACS2\(^{2}\) for HA–REV-erbB (FDR < 0.05 and FC > 2 over DKO samples) and E4BP4 (FDR < 0.05). ChIPseeker\(^{2}\) and Homer\(^{2}\) were used for peak annotation and motif search, and plots were visualized with customized R–Python scripts.

Extracellular flux assay. Neonatal mouse CMs were cultured on Seahorse 96-well plates (Agilent Technologies) in primary CM isolation medium. The night before the assay, the medium was switched to substrate-limited starvation medium (DMEM, 1% D-glucose, 0.5 mM HEPES, 0.2 mM L-glutamine, 0.5 mM taurine and 1% FBS), and the cells were incubated for an additional 24 h to deplete endogenous substrates within the cells. The medium was switched to fatty acid oxidation assay buffer (KHb, supplemented with 2.5 mM glucose, 0.5 mM taurine and 5 mM HEPES, pH 7.4), and immediately before the assay, XF Palmitate–BSA (167 μM) was added. For the mitochondrial stress test, oligomycin (1 μM), carbonyl cyanide 4-trifluoromethoxy)-phenylhydrazone (0.8 μM) and antimycin–rotenone (1 μM) were serially injected using a Seahorse XF96 extracellular flux analyzer (Agilent Technologies). Oxygen consumption rate values as 11–12 replicates per condition were assayed. Negative values were not included in the analysis.

Echocardiography. Ultrasound examination of the left ventricle was performed by the Mouse Cardiovascular Phenotyping Core at the University of Pennsylvania (Cavendish Laboratory, University of Pennsylvania). Paired-end read sequencing was obtained from featureCounts for previously identified DEGs. For enhancer activity, we supplied IMAGE with normalized reads-in-peak counts for H3K27ac in control and CM-RevDKO samples. The program was then executed with default parameters and the multi-threading option.

GIGGLE score calculation. For transcription factor–binding similarity screening analysis, twofold up and down H3K27ac–enriched coordinates were used separately. Cistrome DB\(^{2}\) using GIGGLE\(^{2}\) was then applied to determine the similarity between these genomic coordinates and published cistromes. The following settings were used: genome assembly, mm10; data type in Cistrome, transcription factor; chromatin regulator and peak number of Cistrome sample to use, top 1,000 peaks according to enrichment. Cistromes from WT and untreated mouse liver, heart and muscle tissues were selected for downstream analysis.

Statistics and reproducibility. All data are reported as mean ± s.e.m., and individual data points are shown. Data distribution was assumed to be normal, but this was not formally tested. Statistical analyses were performed using Prism 9 (GraphPad Software). No statistical methods were used to determine sample size. When appropriate, statistical analyses were performed using an unpaired, two-tailed t test (for comparison of two groups), one-way ANOVA (for comparison of three or more groups) or two-way ANOVA (grouped analysis). Survival data were analyzed using Kaplan–Meier survival analysis with a log-rank (Mantel–Cox) test. Multiple–comparisons analysis was performed using Tukey’s methods (method was selected based on the recommendation of Prism 9 for a given comparison). \(P\) values less than 0.05 were considered significant. For RNA-seq analysis, \(P\) and adjusted \(P\) (FDR) values were calculated with DESeq2\(^{2}\). All experiments are represented by multiple biological replicates or independent experiments. The number of replicates per experiment is indicated in the legends.

Contact for reagent and resource sharing. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact M.A.L. (lazar@pennmedicine.upenn.edu).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions

P.D. and M.A.L. conceived and designed the overall study. P.D., K.Z., Y.X., C.J., M.W.V., under the supervision of G.A.B., J.Z., under the supervision of L.P., and H.C.B.N. contributed to next-generation sequencing experiments and bioinformatic analyses. T.S.L. performed NAD+ measurements under the supervision of J.A.B. P.D., B.J.C., A.K.H. and B.K. helped with circadian sampling. P.D. and B.J.C. performed gene expression measurements, western blotting, cell culture experiments, animal caretaking, reporter assays and metabolic experiments. T.Y., under the supervision of D.P.K., and I.M.-P. assisted with echocardiography experiments. S.M. and M.L. (under the supervision of M.E.Y.) performed RT–qPCR and western blots on CM-specific Bmal1;KO, E4bp4;KO and Bmal1;E4bp4;DKO hearts. A.D. assisted with the generation of CM-specific E4bp4;KO mice. P.D. and M.A.L. wrote the manuscript with input from all authors.

Competing interests

M.A.L. receives research support from Pfizer for unrelated work; serves as an advisory board member for Pfizer and Flare Therapeutics; has consulted for Novartis, Madrigal, Calico and Third Rock; and holds equity in KDAC Therapeutics and Flare Therapeutics. D.P.K. serves as an advisory board member for Pfizer and Amgen. J.A.B. is an inventor on a patent for using NAD+ precursors in liver injury, is a consultant for Pfizer and Cytokinetiks and has received research funding and materials from Elysium Health and Metro International Biotech, both of which have an interest in NAD+ precursors. No funds or materials from Elysium Health and Metro International Biotech were used to generate any data for this study. The remaining authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Additional validation of CM-RevDKO. a, Immunoblot for BMAL1 with quantification from control (αMHC-Cre-) vs CM-RevDKO (αMHC-Cre+) hearts from 2-month-old male mice (n = 2/timepoint/genotype). b, Relative mRNA expression in control vs CM-RevDKO hearts from 2-month-old male mice (n = 3 hearts/genotype/timepoint except for n = 2 for ZT2 and n = 4 for ZT22 in CM-RevDKO) and c, young and old WT mice (n = 3/genotype). n represents biologically independent replicates. Data are presented as mean ± SEM, except for a, ns, non significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 by 2-way ANOVA (exact P values are provided in the Source Data).
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Age-dependent impairment of cardiac structure and function in CM-RevDKO mice. a, Birth rates for CM-RevDKO and littermate control animals. b, Locomotor activity and c, food consumption of 2-month-old male control (n = 3) and CM-RevDKO (n = 5) mice housed under 12:12 light:dark conditions. d, Body weight (in grams) of male control and CM-RevDKO mice at 2 (n = 17 for control and n = 34 for CM-RevDKO)–4 (n = 8 for control and n = 14 for CM-RevDKO)–6 (n = 8 for control and n = 11 for CM-RevDKO) months of age. e, Blood glucose concentrations of 6-months old male control (n = 6) and CM-RevDKO (n = 11) mice that underwent glucose tolerance test (GTT) and f, insulin tolerance test (ITT) (n = 6 for control and n = 10 for CM-RevDKO). g, Biventricular to body weight (BVW/BW) ratios of male control (n = 5) and CM-RevDKO (n = 6) hearts. h, Cardiomyocyte size assessment of 6-month old control and CM-RevDKO hearts. Representative images (cardiac sarcolemma stained by WGA in red, nuclei by DAPI = blue) of 11 control and 9 CM-RevDKO hearts are shown. Scale bars, 50μM. i, Cardiac structure (LVIDd/s: Left ventricular internal diameter during diastole/systole) and function (EF: Ejection fraction and FS: Fractional shortening) data from 2-month-old control and CM-RevDKO mice obtained through echocardiography (n = 4/genotype). j, Echocardiographic parameters from control vs CM-RevDKO mice age 2 months (n = 4/genotype) versus 6 months (n = 11 for control and n = 8 for CM-RevDKO). (HR) Heart rate; (LVPWs/d) Left ventricular posterior wall during systole/diastole; (IVSs/d) Interventricular septum thickness during systole/diastole; (rWT) Relative wall thickness. k, Masson’s trichrome and l, TUNEL staining (green) on hearts of 6-month-old control and CM-RevDKO mice. Nuclei are stained with DAPI (blue). Representative images of n = 11 hearts for control and n = 9 hearts for CM-RevDKO are shown. Scale bars, (k) 50μM and (l), 100μM. n represents biologically independent replicates unless otherwise indicated. Data are presented as mean ± SEM. ns, non significant; *P < 0.05; ***P < 0.001 by 2-sided Student’s t test (exact P values are provided in the Source Data).
Extended Data Fig. 3  |  CM-RevDKO causes tissue-specific deregulated expression of circadian genes. a, Circadian Rev-erbα/β and Bmal1 mRNA expression in 2-month-old male control hearts (n=5 timepoint, except for ZT7, n=4 and ZT10, n=6). b, Left: Venn diagram showing overlap between DEGs in CM-RevDKO hearts and hepatocyte-specific Rev-erb DKO (HepDKO) livers22 (at ZT10). Right: relative mRNA expression of commonly (in both CM-RevDKO and Hep-revDKO) deregulated clock genes in CM-RevDKO vs control hearts (n=3 hearts/genotype, harvested at ZT10). c, qRT-PCR validation of genes derepressed upon CM-RevDKO in the heart of 2-month-old male mice (n=5 for control and n=6 for CM-RevDKO). d, Left: Venn diagram showing overlap between cardiac oscillators published in24 and all DEGs in CM-RevDKO that were assessed in24. Right: phase plots of rhythmic, differentially expressed genes identified on the left. n represents biologically independent replicates. Data are presented as mean ± SEM. Adj. P values in b were calculated by DESeq2. ***P < 0.001; ****P < 0.0001 by 2-sided Student’s t-test (exact P values are provided in the Source Data).
Extended Data Fig. 4 | Mitochondrial size is affected in CM-RevDKO cardiomyocytes. a, Relative Ppp1r1b mRNA expression in CM-RevDKO (n = 6) vs control (n = 5) hearts from 2-month-old male mice. b, Immunoblot for DARPP-32 in CM-RevDKO vs control hearts. c, Scatter plot and histogram of mitochondria area for ventricular CMs in control and CM-RevDKO hearts from 6-month-old male mice based on electron microscopy images (n = 86 mitochondria for control, measured from 5 images, n = 90 mitochondria for CM-RevDKO, measured from 7 images). d, Left: scatter plot of mitochondria area for ventricular CMs in control and CM-RevDKO hearts at 2 months (n = 108 mitochondria for control, measured from 7 images, n = 100 mitochondria for CM-RevDKO, measured from 9 images). Scale bars, 10 μM. e, Relative levels of mitochondrial DNA quantified by RT-qPCR. mtCo1/2 and mtNd1 levels were normalized to nuclear genomic βActin (n = 3/genotype). n represents biologically independent replicates unless otherwise indicated. Data are presented as mean ± s.e.m. ns, non-significant; *P < 0.05; ***P < 0.001; ****P < 0.0001 by 2-sided Student’s t-test (exact P values are provided in the Source Data).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Characterization of the 3xHA-REV-ERBα cistrome in the heart. 

a, Venn diagram showing the overlap between the downregulated differentially expressed genes (DEGs) in CM-RevDKO hearts and up/downregulated genes in cardiomyocyte-specific glucocorticoid receptor (GR) KO hearts32. 

b, Pearson correlation plots comparing 3xHA ChIP-seq replicate samples. 

c, Pie chart of annotated cardiac 3xHA-REV-ERBα ChIP-seq peaks. 

d, Results of motif search at 3xHA-REV-ERBα ChIP-seq peaks and enhancers that displayed increased H3K27ac Cut&Run signal (FC > 2) in CM-RevDKO vs control cardiomyocytes as reported by HOMER. 

e, Venn diagram showing overlap between DEGs in CM-RevDKO vs control hearts and annotated peaks from the cardiac 3xHA-REV-ERBα cistrome (at ZT10). 

f, ChIP-seq, Cut&Run and RNA-seq read distribution for REV-ERBα and H3K27ac near derepressed REV-ERBα canonical target genes Bmal1 and Cry1 and output genes p27 and Fbn2. 

i, Immunoblot and quantification for FLAG and REV-ERBα from DsRed (control) vs FLAG-Rev-erbα overexpressing plasmid transfected C2C12 cells (n=2 independently transfected wells/condition). 

Significance of overlap in e is calculated via a hypergeometric test without multiple testing correction.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | E4BP4-based repression is a unifying mechanism to explain transcriptional changes and cardiomyopathy common to the cardiac-specific loss of BMAL1 or REV-ERBs. 

a, Results of motif search at cardiac E4BP4 ChIP-seq peaks (Control at ZT22) as reported by HOMER.

b, Pie chart of annotated cardiac E4BP4 ChIP-seq peaks (control ZT22).

c, Venn diagram showing overlap between annotated peaks from the cardiac control (at ZT22) and CM-RevDKO (at ZT10 and ZT22) E4BP4 cistromes.

d, ChIP-seq, Cut&Run and RNA-seq read distribution for E4BP4 and H3K27ac near identified E4BP4 target genes in control and CM-RevDKO hearts.

e, Overlap between upregulated and g, downregulated genes in CM-RevDKO hearts and cardiomyocyte-specific Bmal1 KO (CBK) hearts. Proposed models for normal (Norm) and experimental (Exp.) conditions are depicted on the right.

f, Overlap between commonly upregulated and h, downregulated genes in CBK/CM-RevDKO hearts (identified in e and g respectively) and cardiac REV-ERBa/E4BP4 cistromes. CBK data in (e,f,g,h) was obtained from35.
Extended Data Fig. 7 | Deletion of Rev-erb genes in cardiomyocytes derepresses key metabolic regulators leading to mitochondrial abnormalities and loss of normal heart function. a, Relative Pgc-1α/β, Naprt1 and Nampt mRNA levels in CM-RevDKO and control hearts (n = 3/genotype) from 2-month-old male mice. b, Relative Bmal1, Rev-erbα/β, and E4bp4 mRNA levels in CBK (n = 7 for control and n = 8 for KO), CM-RevDKO (n = 5 for control and n = 6 for KO), E4bp4 (n = 8 for control and n = 10 for KO) and CBK/E4bp4 (double) KO (n = 6 for control and n = 9 for DKO) and control hearts from 2-month-old male mice for CM-RevDKO and control at ZT10 and from 3-month-old male mice for the rest at ZT12. c, Representative immunoblots and relative protein quantification for NAMPT in hearts from 3-month-old male mice with the following genetic background: CBK (n = 6/genotype), E4bp4 (n = 7/genotype), and CBK/E4bp4 (double) KO (n = 6/genotype), harvested at ZT12. n represents biologically independent replicates. All data are presented as mean ± SEM. Adj. P values in a were calculated by DESeq2, while ns, non significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 by 2-sided Student’s t-test in b and one-way ANOVA followed by a Tukey’s multiple comparisons test in c (exact P values are provided in the Source Data).
Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- Wave 2.6, QuantStudio 6, Oxymax, Biotek Gen5, Vevo

Data analysis
- Prism9, Vevo LAB, Wave 2.6, QuantStudio 6, IGV2.7.2, R-Studios, Excell, www.gsea-msigdb.org, www.string-db.org,

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The RNA, Cut&Run and ChiP-Sequencing datasets reported in this paper have been deposited in GEO (GSE153014) and are publicly available.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No power calculations were performed. Sample sizes were determined based on previous experimental observations and literature. |
|-------------|-------------------------------------------------------------------------------------------------------------------|
| Data exclusions | The only data points not included were because of technical error: negative values for Seahorse-based experiment as indicated in the method section. |
| Replication | The experimental findings were reliably reproduced as described in the figure legends. Data is displayed as a representation of one experiment or as a combination of multiple independent replicated experiments, as indicated in the text. |
| Randomization | Age-and sex-matched animals were randomly assigned to groups before the start of each experiment. |
| Blinding | All experiments, data acquisition and analyses were performed in a blinded manner. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☐ | Palaeontology and archaeology |
| ☐ | Animals and other organisms |
| ☒ | Human research participants |
| ☒ | Clinical data |
| ☒ | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | ChIP-seq |
| ☐ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Antibodies

| Antibodies used |
|-----------------|
| - H3K27ac (Cell Signaling Technology #8173) |
| - Rabbit IgG (Cell Signaling Technology #3900) |
| - HA Magnetic Beads (Thermo Fisher Scientific, #88837) |
| - E4BP4 (Cell Signaling Technology #14312) |
| - REV-ERBa (Abcam #ab174309) |
| - BMAL1 (Bethyl Laboratories # A302-616A) |
| - NAMPT (Bethyl Laboratories #A300-372A-M) |
| - DARPP-32 (Santa Cruz Biotechnology #sc-271111(H3)) |
| - FLAG (Invitrogen PA1-984B) |
| - Vinculin-HRP (Cell Signaling Technology #E18799) |
| - HSP90 (Cell Signaling Technology #48745S) |
| - Rabbit IgG, HRP-linked (Cell Signaling Technology #7074S) |
| - Mouse IgG, HRP-linked (Cell Signaling Technology #7076S) |

Validation

All antibodies used in this study were validated for Western blotting or ChIP by the manufacturer. We selected antibodies that have been commonly used in the field. For anti-REV-ERBa, we validated specificity through the use of knockout material.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) C2C12 cells (ATCC:CRL-1772)
Authentication | C2C12 cells were visually authenticated
Mycoplasma contamination | C2C12 cells were not tested for mycoplasma
Commonly misidentified lines | No commonly misidentified cell lines were used

### Animals and other organisms

Policy information about [studies involving animals](https:// ARRIVE guidelines](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153014) recommended for reporting animal research

#### Laboratory animals
Cardiomyocyte-specific Rev-erba/b double KO (CM-RevOKO) mice were generated by breeding Rev-erbafl/fl (with floxed alleles spanning exon 3-4-5); Rev-erbbfl/fl (with floxed alleles spanning exon 3) animals (Institut Clinique de la Souris, Illkirch, France) to an αMHC-Cre mouse line (Jacks: 011038), on C57BL/6 background. Male and female mice at the age of 2-12 months were used for all experiments, unless stated otherwise in figures and figure legends. They were housed under 12h light/12h dark conditions and fed a standard chow diet (Rodent Diet 5010, LabDiet) ad libitum with free access to water. All animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania (IACUC protocol: 804747) and University of Alabama at Birmingham in accordance with the NIH guidelines.

#### Field-collected samples
The study did not involve samples collected from the field

#### Ethics oversight
All animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania (IACUC protocol: 804747) in accordance with the NIH guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### ChIP-seq

#### Data deposition
- [Confirm that both raw and final processed data have been deposited in a public database such as GEO.](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153014)
- [Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153014)

#### Data access links
May remain private before publication.

- https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153014

#### Files in database submission

- Reverb.WT.Rep1.R1.fastq.gz
- Reverb.WT.Rep1.R2.fastq.gz
- Reverb.WT.Rep2.R1.fastq.gz
- Reverb.WT.Rep2.R2.fastq.gz
- Reverb.WT.Rep3.R1.fastq.gz
- Reverb.WT.Rep3.R2.fastq.gz
- Reverb.KO.Rep1.R1.fastq.gz
- Reverb.KO.Rep1.R2.fastq.gz
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- Reverb.KO.Rep2.R2.fastq.gz
- Reverb.KO.Rep3.R1.fastq.gz
- Reverb.KO.Rep3.R2.fastq.gz
- H3K27ac.RevWT.R1.fastq.gz
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- H3K27ac.igg.R2.fastq.gz
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- HA-REV-ERBa_WT_Rep1.R2.fastq.gz
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- HA-REV-ERBa_WT_Rep2.R2.fastq.gz
- HA-REV-ERBa_WT_Rep3.R1.fastq.gz
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- HA-REV-ERBa_Input.R2.fastq.gz
- E4BP4_RevFloxed_Control_ZT10_Rep1.R1.fastq.gz
- E4BP4_RevFloxed_Control_ZT10_Rep1.R2.fastq.gz
- E4BP4_RevFloxed_Control_ZT10_Rep2.R1.fastq.gz
- E4BP4_RevFloxed_Control_ZT10_Rep2.R2.fastq.gz
Methodology

Replicates
- For bulk RNA-Sequencing 3 replicates per genotype were taken
- For Cut&Run 1 replicate per genotype was taken. IgG was used as a control
- For HA-REV-ERBa ChIP-Seq 3 replicates per condition were taken. WT samples served as control. Input was also sequenced.
- For E4BP4 ChIP-Seq 3 replicates per condition were taken. IgG was used as a control.

Sequencing depth
For H3K27ac Cut&Run in Rev-erba/b floxed:
- H3K27ac, 39.9 million paired-end reads were sequenced and 31.8 million paired-end reads were uniquely mapped. The read length is 40 bp.
- IgG, 36 million paired-end reads were sequenced, 21.6 million paired-end reads were uniquely mapped. The read length is 40 bp.

For H3K27ac Cut&Run in CM-RevDKO:
- H3K27ac, 40.2 million paired-end reads were sequenced and 31.9 million paired-end reads were uniquely mapped. The read length is 40 bp.
- IgG, 33.6 million paired-end reads were sequenced, 20 million paired-end reads were uniquely mapped. The read length is 40 bp.

For HA-REV-ERBa ChIP-Seq:
- HA-REV-ERBa, ~50-90 million paired-end reads were sequenced and ~50 million paired-end reads were uniquely mapped. The read length is 150 bp.
- Input, ~35 million paired-end reads were sequenced and ~30 million paired-end reads were uniquely mapped. The read length is 150 bp.

For E4BP4 ChIP-Seq:
- E4BP4, ~25-60 million paired-end reads were sequenced and ~20-40 million paired-end reads were uniquely mapped. The read length is 150 bp.
- IgG, ~7-10 million paired-end reads were sequenced and ~5.5-7.5 million paired-end reads were uniquely mapped. The read length is 150 bp.

Antibodies
- H3K27ac (Cell Signaling Technology #8173)
- HA Magnetic Beads (Thermo Fisher Scientific, #88837)
- E4BP4 (Cell Signaling Technology#14312)
- Rabbit IgG (Cell Signaling Technology #3900)

Peak calling parameters
Mapping:
- Cut&Run:
  MACS2 (version 2.1.0) was used for peak calling with the following parameters: P = 10^{-5}, extsize = 300 and local lambda = 100,000 using IgG as input controls. Peaks were extended to a minimum size of 2000 base pairs and overlapping peaks were merged into one non-redundant list using WT and CM-RevDKO peaks.
- ChIP-Seq:
  Filtered bam files were used for peak calling by MACS2 for HA-REV-ERBa (FDR < 0.05 and FC > 2 over DKO) and E4BP4 (FDR < 0.05).

Data quality
- For Cut&Run data:
  At the beginning, fastp was used to remove reads with low quality, too short, too many N. Then bowtie2 was used to map reads to the mouse genome (mm10). Only uniquely mapped reads were considered. Duplicate reads were removed using samtools. MACS2 version 2.1.057, was used for peak calling with the following parameters: P = 10^{-5}, extsize = 300 and local lambda = 100,000 using
IgG as input controls. Peaks were extended to a minimum size of 2000 base pairs and overlapping peaks were merged into one non-redundant list using WT and CM-RevDKO peaks. To calculate fold change in peak enrichment between WT and CM-RevDKO cardiomyocytes, read coverage within peaks was determined using bedtools coverage. Reads were normalized for the total number of reads within peaks and peaks with a control/CM-RevDKO ratio of >2 or <0.5 were defined to be down, and up respectively in WT versus CM-RevDKO. Bigwigs were RPM normalized.

For ChIP-Seq data:
the fastq files were first trimmed by cutadapt and aligned by BWA mem, low MAPQ (<=10) and read duplicates were removed by samtools and picard (“Picard Toolkit.” 2019. Broad Institute, GitHub Repository. http://broadinstitute.github.io/picard/; Broad Institute.). The filtered bam files were used for peak calling by MACS2 for HA-REV-ERBa (FDR < 0.05 and FC > 2 over DKO) and E4BP4 (FDR < 0.05). ChiPseeker and Homer were used for peak annotation and motif search and plots were visualized by customized R/python scripts.

| Software |
|----------|
| fastp was used to remove reads with low quality, too short, too many N |
| bowtie2 was used to mapping reads to mouse genome |
| samtools was used to extract uniquely mapped reads, and remove duplicated reads |
| bedtools was used for determining read coverage within peaks |
| MACS2, ChiPseeker and Homer were used for peak calling, peak annotation and motif search. |