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Sobuj Mia
University of Alabama, Birmingham
David R Rawnsley
Washington University School of Medicine in St. Louis
Sumanth D Prabhu
Washington University School of Medicine in St. Louis
Abhinav Diwan
Washington University School of Medicine in St. Louis
et al.

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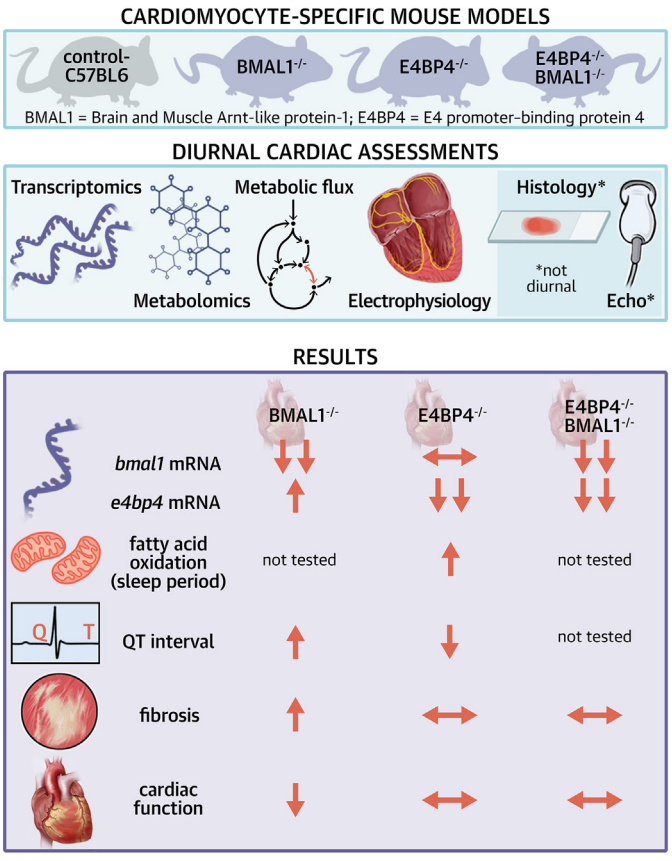
ORIGINAL RESEARCH - PRECLINICAL

Novel Roles for the Transcriptional Repressor E4BP4 in Both Cardiac Physiology and Pathophysiology

Sobuj Mia, PhD,[a,*] Ravi Sonkar, PhD,[a,*] Lamario Williams, BSc,[a,*] Mary N. Latimer, PhD,[a] David R. Rawnsley, PhD,[b] Samir Rana, PhD,[a] Jin He, PhD,[a] Pieterjan Dierickx, PhD,[c,d] Teayoun Kim, PhD,[e] Min Xie, MD, PhD,[a] Kirk M. Habegger, PhD,[e] Masato Kubo, PhD,[f,g] Lufang Zhou, PhD,[a] Morten B. Thomsen, PhD,[h] Sumanth D. Prabhu, MD,[b] Stuart J. Frank, MD,[e,i] Paul S. Brookes, PhD,[j] Mitchell A. Lazar, PhD,[c,k] Abhinav Diwan, MD,[b,l] Martin E. Young, DPhil[a]

VISUAL ABSTRACT



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HIGHLIGHTS

- Circadian disruption secondary to behaviors (eg, shift work, night eating, etc) and single nucleotide polymorphisms (within circadian clock genes) increases risk for cardiometabolic and cardiovascular diseases, through as yet undefined mechanisms.
- The current study reveals that genetic disruption of the cardiomyocyte circadian clock results in age-onset cardiac dysfunction secondary to chronic induction of E4BP4 and that this transcriptional repressor is a novel regulator of both cardiac metabolism (eg, fatty acid oxidation) and electrophysiology (eg, QT interval).
- Future studies are required to elucidate the extent to which E4BP4 plays a role in the pathogenesis of heart disease in nongenetic models.

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ABBREVIATIONS AND ACRONYMS

ANOVA = analysis of variance

BHq = Benjamin-Hochberg method

BMAL1 = brain and muscle ARNT-like 1

bp = base pair

CBK = cardiomyocyte-specific BMAL1 knockout

CEK = cardiomyocyte-specific E4BP4 knockout

CLOCK = circadian locomotor output cycles kaput

E4BP4 = E4 promoter binding protein 4

REV-ERBα/β = nuclear receptor reverse-ERB α/β

RT-PCR = reverse transcription polymerase chain reaction

ZT = zeitgeber time

SUMMARY

Circadian clocks temporally orchestrate biological processes critical for cellular/organ function. For example, the cardiomyocyte circadian clock modulates cardiac metabolism, signaling, and electrophysiology over the course of the day, such that, disruption of the clock leads to age-onset cardiomyopathy (through unknown mechanisms). Here, we report that genetic disruption of the cardiomyocyte clock results in chronic induction of the transcriptional repressor E4BP4. Importantly, E4BP4 deletion prevents age-onset cardiomyopathy following clock disruption. These studies also indicate that E4BP4 regulates both cardiac metabolism (eg, fatty acid oxidation) and electrophysiology (eg, QT interval). Collectively, these studies reveal that E4BP4 is a novel regulator of both cardiac physiology and pathophysiology. (J Am Coll Cardiol Basic Trans Science 2023;8:1141–1156)

Cardiovascular physiology and pathophysiology both fluctuate as a function of time of day.[1] In healthy individuals, blood pressure and heart rate peak in the early hours of the morning, associated with increased risk of myocardial infarction and arrhythmias.[2,3] Classically, 24-hour rhythms in both cardiovascular physiology and pathophysiology have been attributed to factors that are extrinsic to the cardiovascular system, such as behaviors (eg, sleep-wake and fasting-feeding cycles) and associated neurohumoral influences (eg, autonomic and sympathetic tone).[3] Interestingly, striking changes are observed within the kidney, vasculature, and myocardium over the course of the day at both cellular and molecular levels.[4-6] Moreover, it has become increasingly clear that intrinsic factors play critical roles in temporal governance of cardiovascular parameters. For example, when healthy volunteers are subject to 20- or 28-hour routines (in terms of environmental factor and behavioral cycle duration), 24-hour oscillations in multiple cardiovascular parameters persist (eg, heart rate).[7] Animal model-based studies support the concept that cell autonomous circadian clocks serve as the intrinsic timekeeping mechanism driving 24-hour rhythms in critical cardiovascular parameters.[4-6] Circadian clocks consist of numerous transcription/translation feedback loops that generate self-sustained oscillations with a periodicity close to 24 hours.[8] In addition to self-regulating core clock components, this mechanism modulates a diverse array of transcripts/proteins involved in processes ranging from transcription/translation to signaling, ion transport, and metabolism.[9] Importance of this timekeeping mechanism for cardiovascular physiology is underscored by observations that 24-hour rhythms in both heart rate and blood pressure are abolished when key circadian components (eg, circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 [BMAL1]) are genetically disrupted in mice.[10]

Despite appreciation for clock control of cardiovascular physiology, critical gaps in knowledge persist. Given their ubiquitous nature, coupled with

From the [a]Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA; [b]Departments of Medicine, Cell Biology and Physiology, Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Missouri, USA; [c]Institute for Diabetes, Obesity, and Metabolism, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA; [d]Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany; [e]Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA; [f]Research Institute for Biomedical Science, Tokyo University of Science, Chiba, Japan; [g]Laboratory for Cytokine Regulation, RIKEN Center for Integrative Medical Sciences (IMS), RIKEN Yokohama Institute, Kanagawa, Japan; [h]Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Demark; [i]Endocrinology Section, Birmingham VAMC Medical Service, Birmingham, Alabama, USA; [j]Department of Anesthesiology and Perioperative Medicine, University of Rochester, Rochester, New York, USA; [k]Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA; and the [l]John Cochran VA Medical Center, St. Louis, Missouri, USA. *Drs Mia, Sonkar, and Williams contributed equally to this work.

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the fundamental importance of the processes they influence, unanswered questions include: 1) which cell-specific clocks impact key cardiovascular processes; and 2) what are the mechanistic links between circadian clocks and clock controlled cardiovascular processes?[11] Progress has been made to address both these questions. Through the use of cell-specific genetic approaches, roles for circadian clocks within vascular smooth muscle cells, endothelial cells, and cardiomyocytes have begun to emerge. In the case of the cardiomyocyte circadian clock, genetic disruption of the central CLOCK/BMAL1 heterodimer (through mutation of CLOCK or deletion of BMAL1) has revealed that this mechanism temporally partitions key aspects of cardiac metabolism, signaling, electrophysiology, and contractility.[12-17] For example, expression of a dominant negative CLOCK mutant protein (lacking a transactivation domain) specifically in cardiomyocytes abolishes 24-hour rhythms in myocardial glucose oxidation, triglyceride synthesis, and contractile force.[12,14,15] The importance of the cardiomyocyte circadian clock in maintenance of normal cardiac function is highlighted further by studies reporting that genetic disruption of this mechanism leads to age-onset dilated cardiomyopathy (and decreased lifespan).[13,18,19] However, what is less clear are the precise mechanisms by which the cardiomyocyte circadian clock regulates cardiac physiology, as well as the mechanisms leading to pathology following clock disruption. Given the transcriptional nature of the circadian clock mechanism, unbiased transcriptomic approaches have been taken to identify putative candidate mediators; these studies have revealed that the cardiomyocyte circadian clock regulates 5% to 10% of the cardiac transcriptome.[12,13,19] This includes E4 promoter binding protein 4 (E4BP4; encoded by the Nfil3 gene).

E4BP4 is a transcriptional repressor that is regulated directly by the circadian clock.[20-23] More specifically, the central CLOCK/BMAL1 heterodimer within the mechanism induces nuclear receptor reverse-ERB α and β (REV-ERBα and REV-ERBβ; established negative feedback components that inhibit Bmal1 expression).[20-22] REV-ERBα/β directly repress a number of additional target genes, the majority of which do not feedback on the clock mechanism (so-called clock output genes).[20-22] E4BP4 is one such target. The function of E4BP4 has been studied largely in extracardiac tissues, including liver, adipose tissue, and immune cells, where it regulates metabolic and inflammatory processes.[22,24-26] With regard to the heart, Dierickx et al[19] recently reported that cardiomyocyte REV-ERBα/β regulates the metabolic enzymes nicotinamide phosphoribosyltransferase (NAMPT) and carboxylesterase 1d (CES1d), likely through an E4BP4-dependent mechanism. Consistent with the just-described CLOCK/BMAL1-REV-ERBα/β-E4BP4 relationship, E4BP4 levels are increased in the heart following genetic disruption of CLOCK, BMAL1, or REV-ERBα/β, leading to speculation that a chronic elevation of E4BP4 may be responsible for the age-onset cardiomyopathy observed in these murine models.[12,13,19] The overarching goal of the current study was to investigate the roles of E4BP4 on cardiac physiology and pathophysiology. Here, we report that adverse remodeling and cardiomyopathy following BMAL1 deletion is dependent on E4BP4, and provide direct evidence that E4BP4 regulates cardiac metabolism and electrophysiology.

METHODS

ANIMAL MODELS. The present study utilized 4 mouse lines: 1) cardiomyocyte-specific Bmal1 knockout (CBK; $Bmal1^{flox/flox}$/MHCαCre$^{+/-}$) and littermate control ($Bmal1^{flox/flox}$/MHCαCre$^{-/-}$) mice; 2) cardiomyocyte-specific E4bp4 knockout (CEK; $E4bp4^{flox/flox}$/MHCαCre$^{+/-}$) and littermate control ($E4bp4^{flox/flox}$/MHCαCre$^{-/-}$) mice; 3) cardiomyocyte-specific Bmal1/E4bp4 double knockout (CBK/CEK; $Bmal1^{flox/flox}$/$E4bp4^{flox/flox}$/MHCαCre$^{+/-}$) and littermate control ($Bmal1^{flox/flox}$/$E4bp4^{flox/flox}$/MHCαCre$^{-/-}$) mice; and 4) Cre recombinase control (MHCαCre$^{+/-}$) and littermate wild-type mice. Individual $Bmal1^{flox/flox}$, $E4bp4^{flox/flox}$, and MHCαCre$^{+/-}$ mouse lines were generated as described previously.[27-29] In addition, E4BP4 levels were enriched in hearts through either retro-orbital (telemetry studies) or tail vein (reverse transcription polymerase chain reaction [RT-PCR] studies) injection of mice with AAV9 vectors containing the E4bp4 gene driven by the CMV promoter (AAV-E4BP4; 3.5×10^{11} viral genomes); AAV9 vectors containing the Gfp gene driven by the CMV promoter served as controls (AAV-CON; 3.5×10^{11} viral genomes). All experimental mice were male, and were housed at the Center for Comparative Medicine at the University of Alabama at Birmingham, under temperature-, humidity-, and light-controlled conditions. A strict 12-hour light/12-hour dark cycle regime was enforced (lights on at 6 AM; zeitgeber time [ZT] 0); the light/dark cycle was maintained throughout these studies, facilitating investigation of diurnal variations (as opposed to circadian rhythms). Mice were housed in standard micro-isolator cages, except when whole-body metabolism, physical activity. and food intake were monitored; in the latter case, mice were housed in Comprehensive Laboratory Animal Monitoring

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System (CLAMS) cages, as described previously.[30] All mice received food and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

QUANTITATIVE RT-PCR. RNA was extracted from biventricular samples using standard procedures. Candidate gene expression analysis was performed by quantitative RT-PCR, using previously described methods.[31,32] For quantitative RT-PCR, either Thermo Fisher (Mm00474816, Mm01622471, Mm01205281, Mm01143659, and Mm00518915 for *ces1d, acot1*, *oplah*, *rhobtb1*, and *kcnip2*, respectively) or custom-designed (*acsl6*, *bmal1*, *col1a1*, *e4bp4*, *nampt*, *rev-erbα*) TaqMan assay were utilized; sequences for custom-designed assays have been reported previously.[12,13,33,34] All quantitative RT-PCR data are presented as fold change from an indicated control group.

RNA SEQUENCING. Transcriptomic analysis was performed through the use of RNA sequencing (RNA-seq) in the University of Alabama at Birmingham Genomics Core facility. Following initial testing of RNA sample using an Agilent BioAnalyzer, RNA with RIN values >7.0 were subsequently utilized for library preparation (after DNAse treatment). RNA-sequencing libraries were next generated using the NEBNext Ultra II RNA kit (NEB); resulting libraries were sequenced on the Illumina NextSeq 500 (Illumina) using paired end 75-base pair (bp) sequencing reads, per standard methods.

HISTOLOGIC ASSESSMENT. Cross sections from the middle region of the left ventricle were taken immediately upon removal of the heart, and were fixed in formalin for 24 hours (followed by storage in 70% ethanol at 4°C before embedding and sectioning). Wheat germ agglutinin staining was utilized for measurement of myocyte cross-sectional area; at least 45 myocytes were assessed per heart using ImageJ software (NIH), as described previously.[18] Assessment of left ventricular interstitial fibrosis employed Picrosirius Red staining of collagen fibers, followed by semiquantitative analysis using ImageJ software, as described previously.[35]

WORKING MOUSE HEART PERFUSIONS. Myocardial fatty acid oxidation was measured ex vivo through isolated working mouse heart perfusions, as described previously.[12,14,15,36] All hearts were perfused in the working mode (nonrecirculating manner) for 40 minutes with a preload of 12.5 mm Hg and an afterload of 50 mm Hg. Standard Krebs-Henseleit buffer was supplemented with 8 mmol/L glucose, 0.4 mmol/L oleate conjugated to 3% bovine serum albumin (fraction V, fatty acid-free; dialyzed), 10 μU/mL insulin (basal/fasting concentration), 0.05 mmol/L L-carnitine, and 0.13 mmol/L glycerol. Fatty acid oxidation was assessed through inclusion of [9,10-^{3}H]-oleate (0.067 mCi/L). Measures of cardiac metabolism (ie, oleate oxidation) and function (ie, cardiac power and heart rate) were monitored as described previously.[12,14,15,36] Data are presented as steady state values (ie, values during the last 10 minutes of the perfusion protocol).

ECHOCARDIOGRAPHY. Cardiac function was assessed by echocardiography using a VisualSonics VeVo 3100 Imaging System (VisualSonics), and was analyzed by VisualSonics software. Briefly, mice were anesthetized with 1.5% to 2% isoflurane in an oxygen mix; heart rate, respiratory rate, and body temperature (35°C to 37°C) were monitored continuously throughout the procedure to ensure adequate depth of anesthesia.

TELEMETRY. Telemetry devices (ETA-F10, DataSciences International [DSI]) were implanted in anesthetized mice (2% isoflurane in O_2) into a subcutaneous pouch on the back region just below the scapulae. The negative and positive leads were anchored subcutaneously in a lead II configuration as described previously.[37] Mice were allowed to recover from the surgery for 7 days, after which electric (at 1 kHz) and movement counts were recorded in individually housed mice in the horizontal plane continuously for 3 days in an environmental chamber (Columbus Instruments). Following acquisition of data using DSI software Dataquest A.R.T., activity and electrocardiograms were analyzed using LabChart as described previously.[38] QT intervals (an important marker of ventricular repolarization and arrhythmia risk) were mathematically corrected to a heart rate of 600 beats/min [$QTc = QT/(RR/100)^{0.5}$], as proposed previously; QTc essentially corrects for any variation that may be present in heart rate between mice.[39]

METABOLOMICS. Heart samples (n = 96; 6 hearts each for 16 experimental conditions) were sent to Metabolon Inc., for assessment of metabolites in an untargeted manner. Briefly, following sample preparation using established methodologies at Metabolon Inc., mass spectrometry was performed; spectra were compared with those of purified standards for compound identification. Of the 805 annotated metabolites, 755 remained after removing those with poor coverage across the data set. Outliers were next identified as those values falling outside the 99.99% CIs within any group of 6 samples within a specific experimental group, resulting in further removal of 27 metabolites (due to <3 values in an experimental group). After outlier removal, the

remaining 69,888 data points (728 metabolites × 96 samples) contained 1,500 missing values (2.15%), which were imputed as medians of remaining values per experimental group, as described previously.[40]

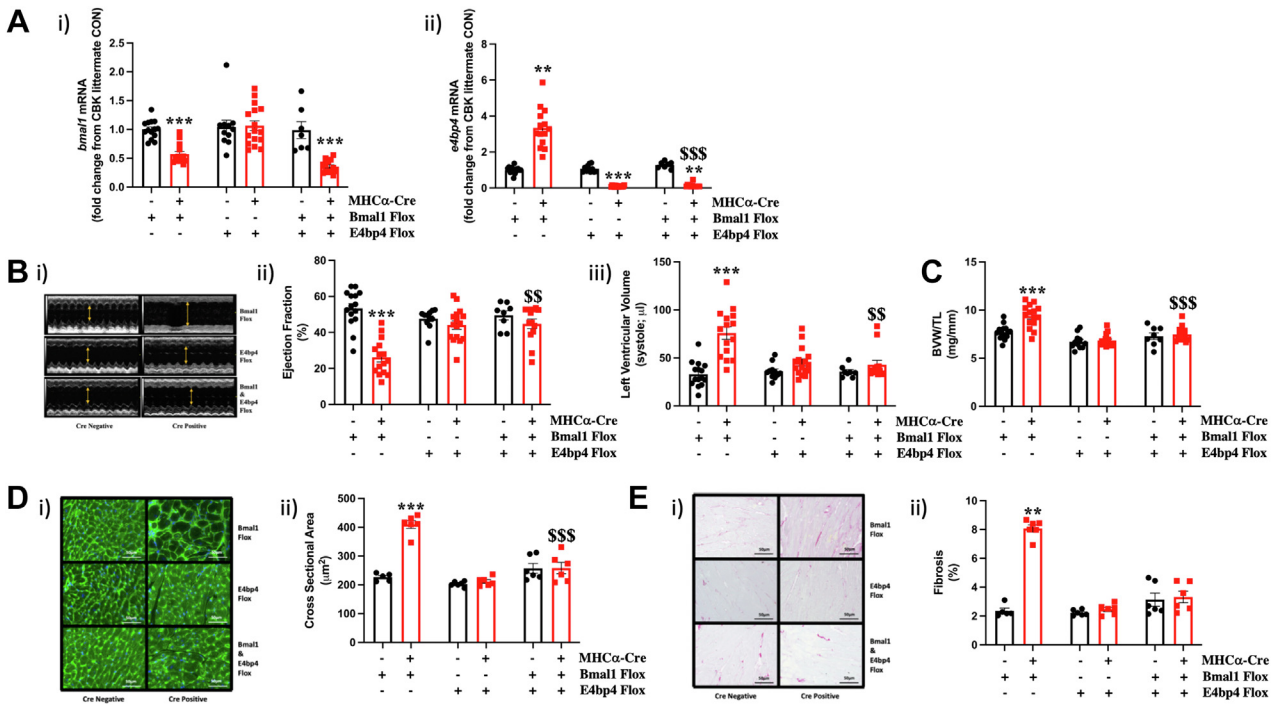
STATISTICAL ANALYSIS. All data are presented as mean ± SEM. For non-omics data (ie, not RNA-seq or metabolomics), comparisons among groups were analyzed by 2-way or 3-way analysis of variance (ANOVA) through use of Prism statistical software to investigate main effects of genotype (eg, floxed alleles, MHCα-Cre, AAV) and time. Normality of data was assessed through use of the Shapiro-Wilks test, followed by either parametric (*t*-tests for only 2 experimental groups or Sidak's post-hoc test for multiple pairwise comparisons) or nonparametric (Mann-Whitney for only 2 experimental groups or Kruskal-Wallis with Dunn's correction for multiple pairwise comparisons). Cosinor analyses were performed to determine whether 24-hour time series data significantly fit a cosine curve; if they did, then mesor (daily average value), amplitude (peak-to-mesor difference), and acrophase (timing of the peak) were calculated and compared between experimental groups, as described previously.[41] RNA-seq data analysis was performed on transcripts that had an average (all heart samples, independent of experimental group) expression value >10 counts. Two-way and 3-way ANOVAs were performed on RNA-seq data using DESeq2.[42] Time series RNA-seq data were analyzed for rhythmicity using the MetaCycle package in R software (R Foundation for Statistical Computing).[43] Transcripts were considered to have a 24-hour oscillation if they significantly fit a cosine curve ($P < 0.05$). Mesor, amplitude, and acrophase were calculated within MetaCycle, and Student *t*-tests were employed to determine significant differences between genotype for these cosine parameters. Enrichr was utilized for performing pathway analysis within the Reactome pathway database.[44,45] Metabolomics data were initially analyzed by 2-way ANOVAs using R package "omu"; presented *P* values are corrected for false discovery rate using the Benjamin-Hochberg method (BHq) with the null hypothesis rejected at $P < 0.05$. Time series metabolomics data were analyzed for rhythmicity in R with the MetaCycle Package utilizing false discovery rate-corrected JTK values.[43] Metabolites were considered to have a 24-hour oscillation if they significantly fit a cosine curve ($P < 0.05$). If they did, mesor, amplitude, and acrophase were calculated with SPSS, and Student *t*-tests were employed to determine whether significant differences between genotype existed for these cosine parameters. In all analyses, the null hypothesis of no model effects was rejected at $P < 0.05$.

RESULTS

CARDIOMYOCYTE-SPECIFIC E4BP4 DELETION PREVENTS AGE-ONSET CARDIOMYOPATHY IN CBK MICE. Previous studies report that genetic disruption of the cardiomyocyte circadian clock, either through deletion of BMAL1 or REV-ERBα/β, leads to a severe age-onset cardiomyopathy.[13,19] In both models, cardiac *e4bp4* mRNA levels are chronically elevated, leading to the hypothesis that E4BP4 contributes towards cardiomyopathy development. Such a hypothesis has not been tested previously. We therefore investigated whether cardiomyocyte-specific deletion of E4BP4 prevented age-onset cardiomyopathy in CBK mice. More specifically, hearts of CBK, CEK, and CBK/CEK mice were studied at functional, gravimetric, histologic, and molecular levels, at both 16 weeks and 36 weeks of age. Consistent with previously published studies, CBK hearts exhibit decreased *bmal1* mRNA levels regardless of age, concomitant with elevated *e4bp4* mRNA levels (**Figure 1A**, Supplemental Figure 1A). As predicted, *e4bp4* mRNA levels are markedly decreased in both CEK and CBK/CEK hearts (**Figure 1A**, Supplemental Figure 1A). At 16 weeks of age, echocardiographic parameters, as well as biventricular weight to tibia length ratio, did not differ between the experimental groups investigated (Supplemental Figures 1B and 1C). Conversely, histologic analysis revealed increased cardiomyocyte size and fibrosis in CBK hearts (Supplemental Figures 1D and 1E). Although not altered in CEK hearts, cardiomyocyte size was significantly attenuated in CBK/CEK hearts (relative to CBK hearts) (Supplemental Figure 1D). At 36 weeks of age, echocardiographic analysis revealed dramatic cardiac functional and structural changes in CBK mice, including a 51% decrease in ejection fraction and a 129% increase in left ventricular volume (relative to littermate controls) (**Figure 1B**, **Table 1**). By contrast, CEK and CBK/CEK mice did not exhibit significant differences in echocardiographic parameters when compared with littermate controls (**Figure 1B**, **Table 1**). Gravimetric analysis revealed increased biventricular weight to tibia length ratio in CBK (22%), but not CEK or CBK/CEK, mice (**Figure 1C**). Similarly, cardiomyocyte size and fibrosis were markedly increased in CBK hearts (81% and 241%, respectively); these histologic parameters were not altered in CEK or CBK/CEK hearts (**Figures 1D and 1E**). Collectively, these data reveal that the age-onset cardiomyopathy observed in CBK mice is dependent on E4BP4.

AUGMENTED E4BP4 LEVELS FOLLOWING CARDIOMYOCYTE CIRCADIAN CLOCK DISRUPTION ARE RESPONSIBLE FOR REPRESSION OF A SUBSET OF CARDIAC GENES. We have previously reported that disruption of the CLOCK/BMAL1 heterodimer in cardiomyocytes dramatically alters the cardiac transcriptome.[12,13] Given that

FIGURE 1 Age-Onset Cardiomyopathy in CBK Hearts is Dependent on E4BP4



(A) Reverse transcription polymerase chain reaction validation of *bmal1* **(i)** and *e4bp4* **(ii)** mRNA levels in cardiomyocyte-specific BMAL1 knockout (CBK), cardiomyocyte-specific E4BP4 knockout (CEK), and CBK/CEK double knockout hearts collected from 36-week-old mice at zeitgeber time (ZT)12 (relative to littermate floxed control [CON] hearts; n = 7-16). **(B)** Representative echocardiograms **(i)**, as well as quantification of ejection fraction **(ii)** and left ventricular volume during systole **(iii)** at ZT6 (n = 8-16). **(C)** Biventricular weight to tibia length ratio (BVW/TL) at ZT12 (n = 8-16). **(D)** Representative images **(i)** and quantification **(ii)** of cardiomyocyte size for hearts collected at ZT12 (n = 5-6). **(E)** Representative images **(i)** and quantification **(ii)** of fibrosis for hearts collected at ZT12 (n = 5-6). Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ for MHCα-Cre-positive vs MHCα-Cre-negative littermate flox control hearts; $\dagger\dagger P < 0.01$ and $\dagger\dagger\dagger P < 0.001$ for CBK/CEK vs CBK hearts. E4BP4 = E4 promoter binding protein 4.

E4BP4 is a transcriptional repressor, we reasoned that E4BP4 likely drives cardiomyopathy through repression of transcripts in CBK hearts. In order to test this hypothesis, RNA-seq was performed on hearts collected from 8 groups of 16-week-old mice (ie, before CBK heart failure development): 1) wild-type; 2) MHCα-Cre control; 3) Bmal1 flox control; 4) CBK; 5) E4bp4 flox control; 6) CEK; 7) Bmal1/E4bp4 flox control; and 8) CBK/CEK. A 3-way ANOVA (and subsequent post hoc pairwise comparisons) was performed to identify direct E4BP4 targets that potentially contribute toward the cardiomyopathy observed in CBK hearts. In line with the known relationship between BMAL1, E4BP4, and E4BP4 target genes (**Figure 2A**), a transcript was considered a candidate mediator if: 1) it was significantly repressed in CBK (vs littermate control) hearts; 2) it was significantly increased in CBK/CEK (vs CBK) hearts; and 3) interrogation of a recently published E4BP4 ChIP-seq study in the heart[19] confirmed that E4BP4 binds to DNA regions within −1,000 bp to +100 bp from the transcriptional start site. This analysis identified 55 E4BP4 target genes that potentially contribute toward cardiomyopathy in CBK mice (**Figure 2A**, Supplemental Table 1). RT-PCR validation for select transcripts involved in metabolism (*oplah*; 5-oxoprolinase), cellular signaling (*rhobtb1*; rho related BTB domain containing 1), and ion transport (*kcnip2*; potassium voltage-gated channel interacting protein 2) confirmed decreased levels in CBK hearts (39%, 74%, and 43% lower compared with littermate controls, respectively), which were either partially or fully normalized in CBK/CEK hearts (**Figure 2B**). Moreover, E4BP4 binds to the promoters of these targets at ZT22 (but not ZT10, when E4BP4 levels are low in control hearts) (**Figure 2C**). To confirm E4BP4-mediated repression of these target genes in the heart, AAV-mediated E4BP4 delivery was employed (tail vein injection into wild-type mice), resulting in an ∼23-fold increase in cardiac *e4bp4* mRNA levels,

TABLE 1 Echocardiographic Parameters in 36-Week-Old Mice

Transgene	Genotype					
Bmal1 Flox	+	+	−	−	+	+
E4bp4 Flox	−	−	+	+	+	+
MHCα-Cre	−	+	−	+	−	+
IVSd, mm	0.86 ± 0.03	0.82 ± 0.03	0.70 ± 0.02	0.72 ± 0.02	0.82 ± 0.04	0.88 ± 0.04
IVSs, mm	1.22 ± 0.04	1.00 ± 0.04***	0.96 ± 0.03	0.96 ± 0.02	1.12 ± 0.05	1.20 ± 0.05††
LVIDd, mm	4.04 ± 0.08	4.62 ± 0.13**	3.95 ± 0.08	4.22 ± 0.09	4.03 ± 0.11	4.13 ± 0.09†
LVIDs, mm	2.95 ± 0.11	4.06 ± 0.16***	3.02 ± 0.08	3.30 ± 0.11	3.05 ± 0.07	3.22 ± 0.12††
LVPWd, mm	0.78 ± 0.02	0.85 ± 0.03	0.74 ± 0.02	0.74 ± 0.02	0.90 ± 0.06	0.90 ± 0.03
LVPWs, mm	1.04 ± 0.03	0.95 ± 0.04	0.98 ± 0.03	0.96 ± 0.02	1.13 ± 0.08	1.09 ± 0.04
EF, %	52.8 ± 2.78	26.8 ± 2.62***	47.6 ± 1.57	44.1 ± 2.32	48.4 ± 2.65	44.7 ± 2.71††
FS, %	27.1 ± 1.70	12.5 ± 1.33***	23.6 ± 0.89	21.8 ± 1.35	24.2 ± 1.61	22.1 ± 1.51††
LV Vold, μL	72.3 ± 3.47	99.8 ± 6.34**	68.4 ± 3.52	80.4 ± 4.15	71.6 ± 4.88	76.2 ± 4.28†
LV Vols, μL	34.6 ± 3.19	74.7 ± 6.86***	36.0 ± 2.48	45.4 ± 3.70	36.6 ± 2.21	42.9 ± 4.50††

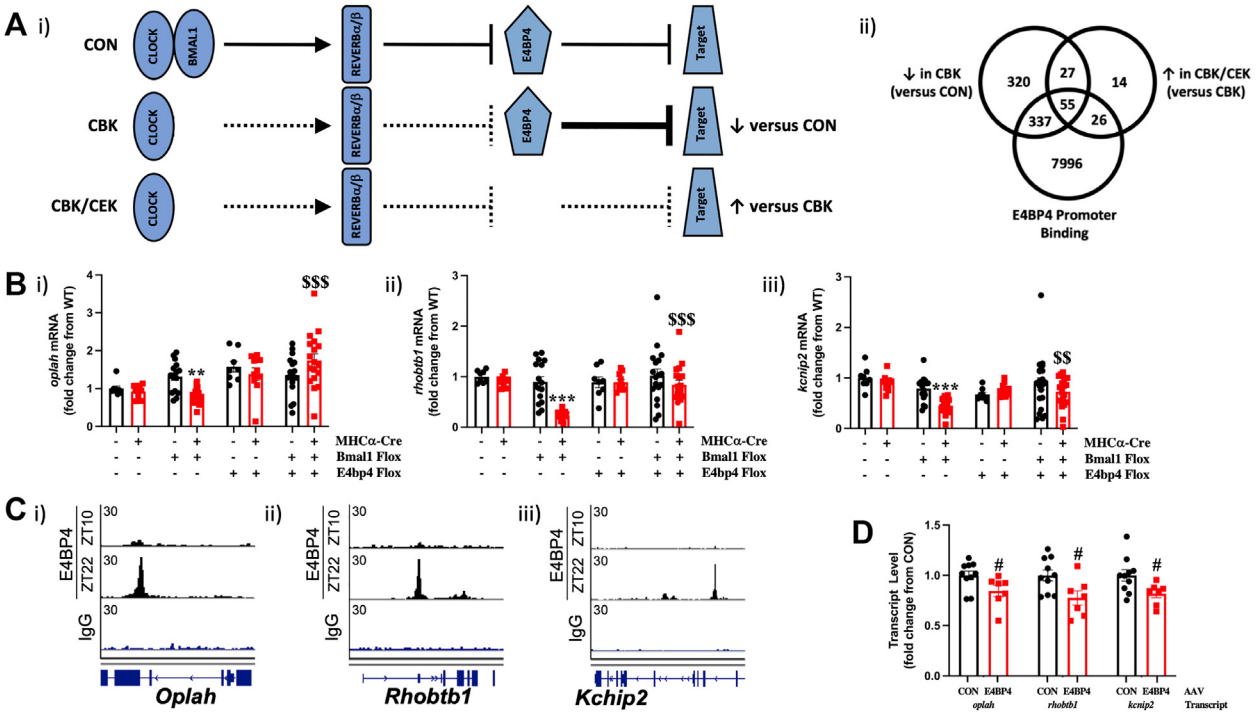
Echocardiographic parameters in 36-week-old cardiomyocyte-specific BMAL1 knockout (CBK), cardiomyocyte-specific E4BP4 knockout (CEK), CBK/CEK double knockout, and littermate flox control mice are shown. All data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ for MHCα-Cre–positive vs MHCα-Cre–negative littermate floxed control. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ for CBK vs CBK/CEK double knockout.

EF = ejection fraction; FS = fractional shortening; IVSd = inner ventricular septal wall thickness during diastole; IVSs = inner ventricular septal wall thickness during systole; LVIDd = left ventricular inner diameter during diastole; LVIDs = left ventricular inner diameter during systole; LVPWd = left ventricular posterior wall thickness during diastole; LVPWs = left ventricular posterior wall thickness during systole; LV Vold = left ventricular volume during diastole; LV Vols = left ventricular volume during systole.

concomitant with repression of previously established E4BP4-target genes (ie, previously published studies reveal direct binding to E4BP4 to the promoters of Nampt and Ces1d genes[19]) (Supplemental Figure 2). Importantly, E4BP4 overexpression resulted in decreased *oplah*, *rhobtb1*, and *kcnip2* mRNA levels in the heart (15%, 22%, and 18% lower compared to AAV controls, respectively) (**Figure 2D**). Collectively, these data suggest that chronic activation of E4BP4 in the heart following disruption of the cardiomyocyte circadian clock is directly responsible for 55 repressed genes in CBK, that are involved in processes such as metabolism, cellular signaling, and electrophysiology.

CARDIOMYOCYTE-SPECIFIC E4BP4 DELETION IMPACTS THE DIURNAL CARDIAC TRANSCRIPTOME. Given that E4BP4 is directly regulated by the circadian clock, we next sought to investigate whether this transcriptional repressor contributes toward daily fluctuations in the cardiac transcriptome. Accordingly, hearts were collected from CEK and littermate control mice at 3-hour intervals across the 24-hour light/dark cycle. Consistent with prior studies,[19,46] *e4bp4* mRNA levels exhibit 24-hour oscillations in control hearts (**Figure 3A**, Supplemental Table 2). Importantly, independent of the time of day, *e4bp4* mRNA levels were significantly lower in CEK hearts relative to littermate controls (ie, genotype main effect) (**Figure 3A**). By contrast, *e4bp4* mRNA diurnal variations did not differ with respect to genotype in liver and skeletal muscle (Supplemental Figures 3A and 3B, Supplemental Table 2). We next investigated whether genetic deletion of E4bp4 impacted the circadian clock in the heart. Consistent with the concept that E4BP4 is downstream of the clock mechanism, neither *bmal1* nor *rev-erbα* mRNA 24-hour oscillations differed between CEK and littermate control hearts (**Figure 3B**, Supplemental Table 2). By contrast, expression of *nampt* and *ces1d*, recently established direct cardiac E4BP4 targets,[19] were induced in CEK hearts independent of the time of day (ie, genotype main effect) (**Figure 3C**). RNA-seq was next utilized to interrogate the entire cardiac transcriptome. A 2-way ANOVA (DESeq2 analysis) was performed initially, revealing significant time-of-day- and genotype-dependent main effects for 1,149 and 1,104 transcripts, respectively (Supplemental Tables 3 and 4). With regard to genotype main effects, 611 transcripts were significantly increased in CEK hearts, whereas 493 transcripts were significantly decreased (Supplemental Table 4); significantly increased transcripts included *oplah*, *rhobtb1*, and *kcnip2* (Supplemental Figure 3C). Pathway analysis for differentially expressed genes in CEK hearts revealed an enrichment for metabolic pathways (**Figure 3D**), suggesting that E4BP4 deletion may perturb cardiac metabolism. RT-PCR validation was performed subsequently for a repressed transcript encoding for the metabolic enzyme acyl-CoA synthetase long chain family member 6 (*acsl6*) (Supplemental Figure 3D). Next, MetaCycle analysis was performed to identify transcripts with a time-of-day–dependent pattern that significantly fit a cosine oscillation; this analysis revealed that 1,512 and 1,230 transcripts oscillated in

FIGURE 2 Identification of Direct E4BP4 Target Genes Potentially Contributing Toward CBK Cardiomyopathy

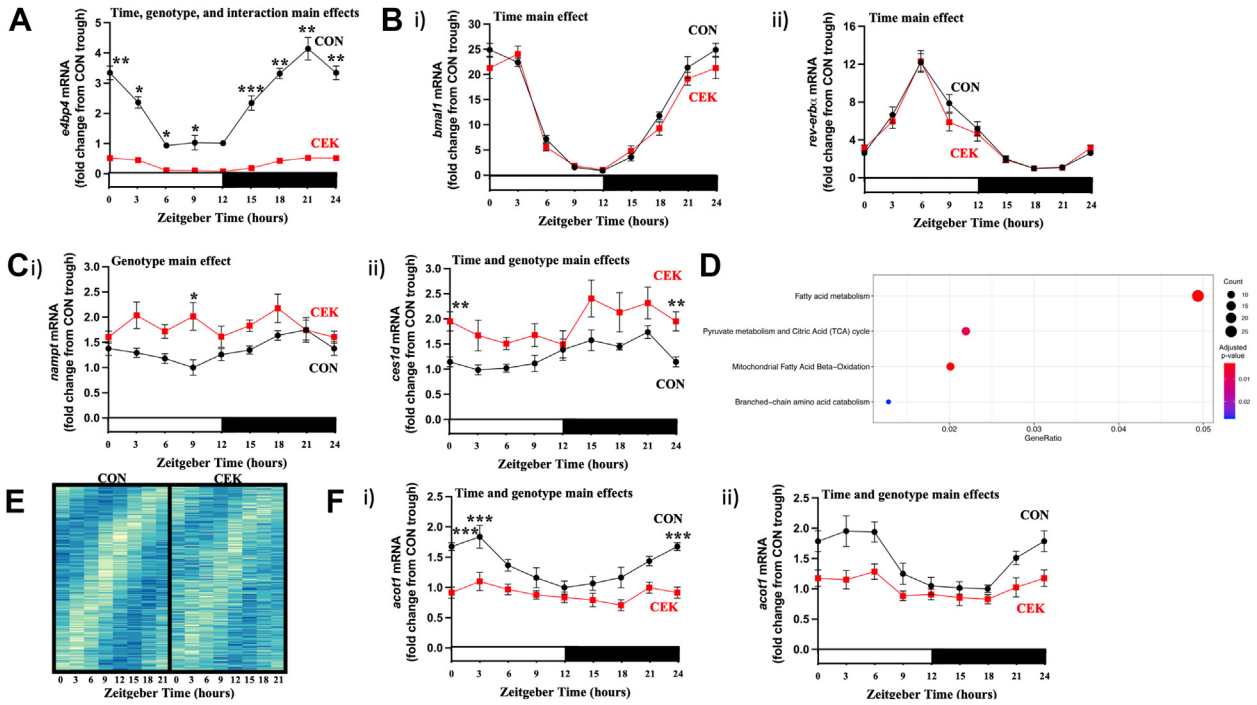


(A) Mechanistic relationship between CLOCK/BMAL1, REVERBα/β, E4BP4, and E4BP4 target genes in hearts of CON, CBK, and CBK/CEK mice **(i)**. Bioinformatic analysis of both RNA-seq and previously published ChIP-seq data identified 55 direct E4BP4 target genes that are repressed in CBK hearts (relative to littermate CON hearts) and partially/fully normalized in CBK/CEK double knockout hearts **(ii)**. **(B)** Reverse transcription polymerase chain reaction validation of *oplah* **(i)**, *rhobtb1* **(ii)**, and *kcnip2* **(iii)** mRNA levels in MHCα-Cre, CBK, CEK, and CBK/CEK double knockout hearts collected at ZT12 (relative to littermate floxed control hearts; n = 8-22). **(C)** Analysis of previously published cardiac ChIP-seq data reveal E4BP4 binding at ZT22 (but not ZT10) within the promoter/enhancer regions of Oplah **(i)**, Rhobtb1 **(ii)**, and Kcnip2 **(iii)**. **(D)** Levels of *oplah*, *rhobtb1*, and *kcnip2* mRNA in hearts of mice 4 weeks after tail vein injection of E4BP4-AAV or CON-AAV; hearts were collected at ZT12 (n = 7-10). Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ for MHCα-Cre–positive vs MHCα-Cre–negative littermate flox control hearts; $\dagger\dagger P < 0.01$ and $\dagger\dagger\dagger P < 0.001$ for CEK/CBK vs CBK hearts;§$P < 0.05$ for E4BP4-AAV vs CON-AAV hearts. WT = wild-type; other abbreviations as in Figure 1.

control and CEK hearts, respectively (Supplemental Tables 5 and 6). Interestingly, 705 transcripts (ie, 46.6% of the transcripts that oscillate in control hearts) either exhibited a significant amplitude attenuation or complete abolition of oscillation in CEK hearts; of these transcripts, 197 oscillate in control hearts with a peak-to-mesor ratio >1.4 (**Figure 3E**, Supplemental Table 7). Consistent with this analysis, RT-PCR confirmed that *acot1* (acyl-CoA thioesterase 1, involved in fatty acid metabolism) exhibited a time-of-day-dependent oscillation in control hearts, which was markedly attenuated in CEK hearts (**Figure 3F**, Supplemental Table 2). Conversely, 423 transcripts oscillated only in CEK hearts or exhibited augmented oscillations in CEK relative to control hearts (Supplemental Table 8). Collectively, these data suggest that E4BP4 plays an important role in maintenance of the diurnal transcriptome in the heart, particularly for transcripts involved in metabolic processes.

ALTERATIONS IN CARDIAC METABOLISM FOLLOWING CARDIOMYOCYTE-SPECIFIC E4BP4 DELETION. Circadian clocks have established roles in regulation of cellular metabolism. Given that our transcriptomic analysis suggested that several metabolic pathways are likely perturbed in CEK hearts (**Figure 3D**), we next performed metabolomics in hearts collected from 16-week-old mice at 3-hour intervals over the course of the day. Partial Least Squares Discriminant Analysis was performed for the 728 detected metabolites at each individual time of the day, indicating that metabolite patterns were distinct in CEK and control hearts at almost all times of the day (not significant for ZT15); common metabolites driving genotype differences across time points included bile acids, glycolytic intermediates, and

FIGURE 3 Alterations in the Cardiac Transcriptome of CEK Mice Identify Putative Roles for E4BP4 in the Heart

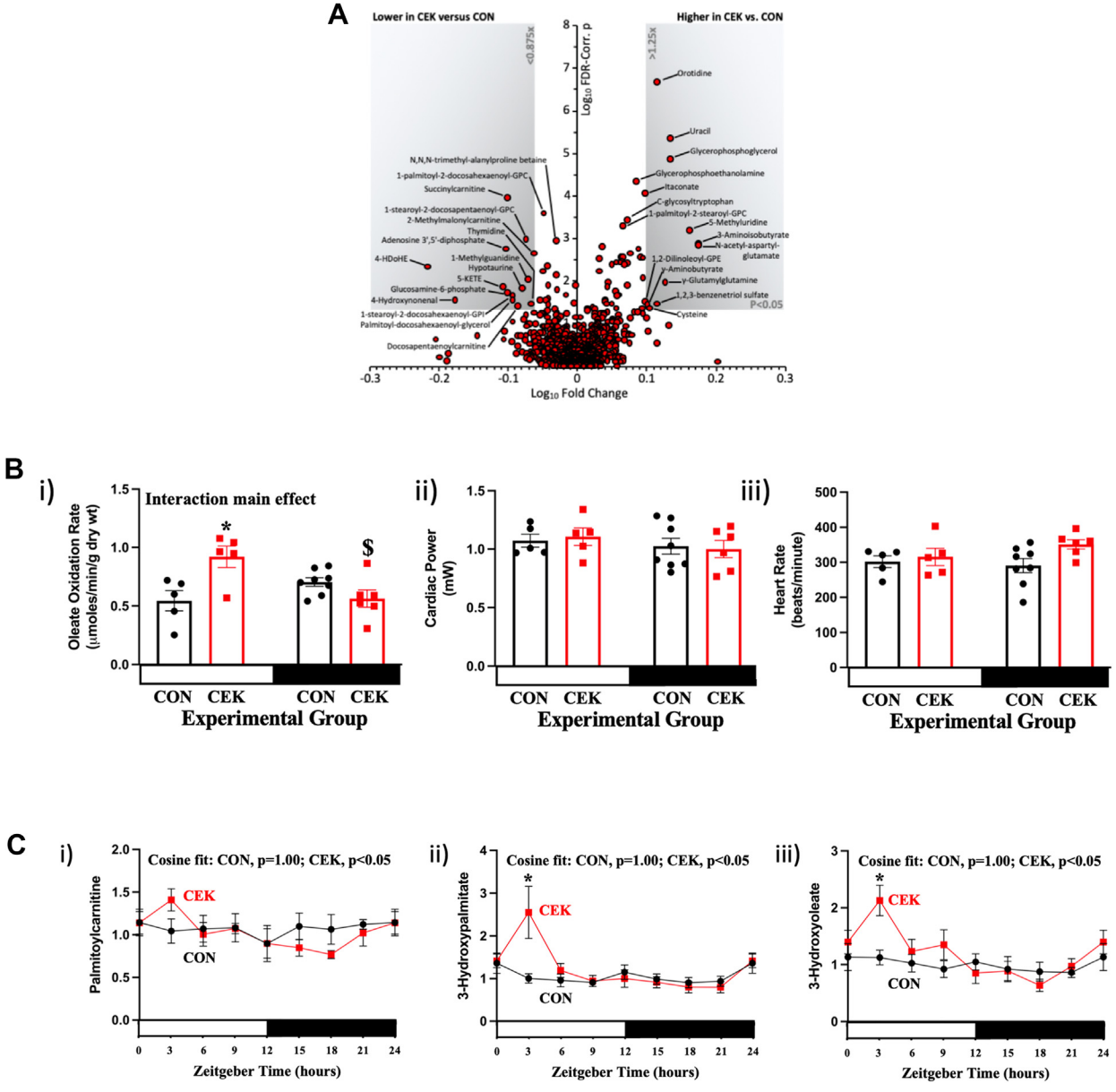


(A) Diurnal variations in *e4bp4* mRNA levels for CEK and CON hearts collected at 8 distinct times of the day (n = 4-13). **(B)** Diurnal variations in *bmal1* **(i)** and *rev-erbα* **(ii)** for CEK and CON hearts collected at 8 distinct times of the day (n = 8-13). **(C)** Diurnal variations in *nampt* **(i)** and *ces1d* **(ii)** mRNA levels for CEK and CON hearts collected at 8 distinct time of the day (n = 8-13). **(D)** Pathway analysis of RNA-seq data indicating processes for which differentially expressed genes in CEK hearts are enriched; x-axis represents the number of differentially expressed transcripts in CEK hearts divided by the total number of transcripts in the respective Reactome pathway. **(E)** Heat map for transcripts exhibiting either attenuated or abolished time-of-day–dependent oscillations in CEK hearts (relative to CON heart oscillations; n = 4). **(F)** Diurnal variations in *acot1* mRNA in CEK and littermate CON hearts, assessed by RNA-seq (**i**; n = 4) and reverse transcription polymerase chain reaction (**ii**; n = 8-13). For data presented in **A**, **B**, **C**, and **F**, ZT0 and ZT24 are identical (the data are double plotted purely for the sake of presentation). Data are presented as mean ± SEM. Main effects of genotype, time of day, and/or interaction are reported at the **top** of the figure panels. *$P < 0.05$ and **$P < 0.01$, and ***$P < 0.001$ for CON vs CEK hearts (at the same time of day). Abbreviations as in **Figure 1**.

fatty acid species (Supplemental Figure 4). A 2-way ANOVA was next performed, revealing significant time-of-day- and genotype-dependent main effects for 156 and 78 metabolites, respectively (Supplemental Tables 9 and 10). With regard to genotype main effects, 49 metabolites were significantly increased in CEK hearts, whereas 29 metabolites were significantly decreased (Supplemental Table 10). Many of these metabolites are common in lipid, nucleotide, and glutathione metabolism (**Figure 4A**). For example, uridine levels are significantly decreased (with a trend for decreased deoxyuridine; BHq corrected $P = 0.44$, unadjusted ANOVA $P = 0.18$, Mann-Whitney $P = 0.017$) in CEK hearts, concomitant with a 36.5% increase in uracil levels; inspection of the RNA-seq data revealed that transcript levels encoding for the enzyme catalyzing uridine/deoxyuridine conversion to uracil (uridine phosphorylase 1; *upp1*) is significantly induced in CEK hearts (Supplemental Figure 5A). Collectively, these observations suggest that cardiomyocyte E4BP4 deletion may impact cardiac metabolism.

Next, cosine (JTK cycle) analysis was performed on the metabolomics data, to identify metabolites that significantly oscillate in control and/or CEK hearts over the course of the day. This analysis revealed that 78 metabolites oscillate in both control and CEK hearts, 45 metabolites oscillate only in control hearts, whereas 94 metabolites oscillate only in CEK hearts (**Figure 4A**, Supplemental Tables 11 to 13). Particularly intriguing was the large number of metabolites that oscillate only in CEK hearts, many of which are involved in lipid metabolism. Given that the RNA-seq analysis similarly suggested that lipid metabolism is altered in CEK hearts (**Figure 3D**), we next assessed diurnal variations (ie, day-night differences) in oxidative metabolism through the use of radiolabeled

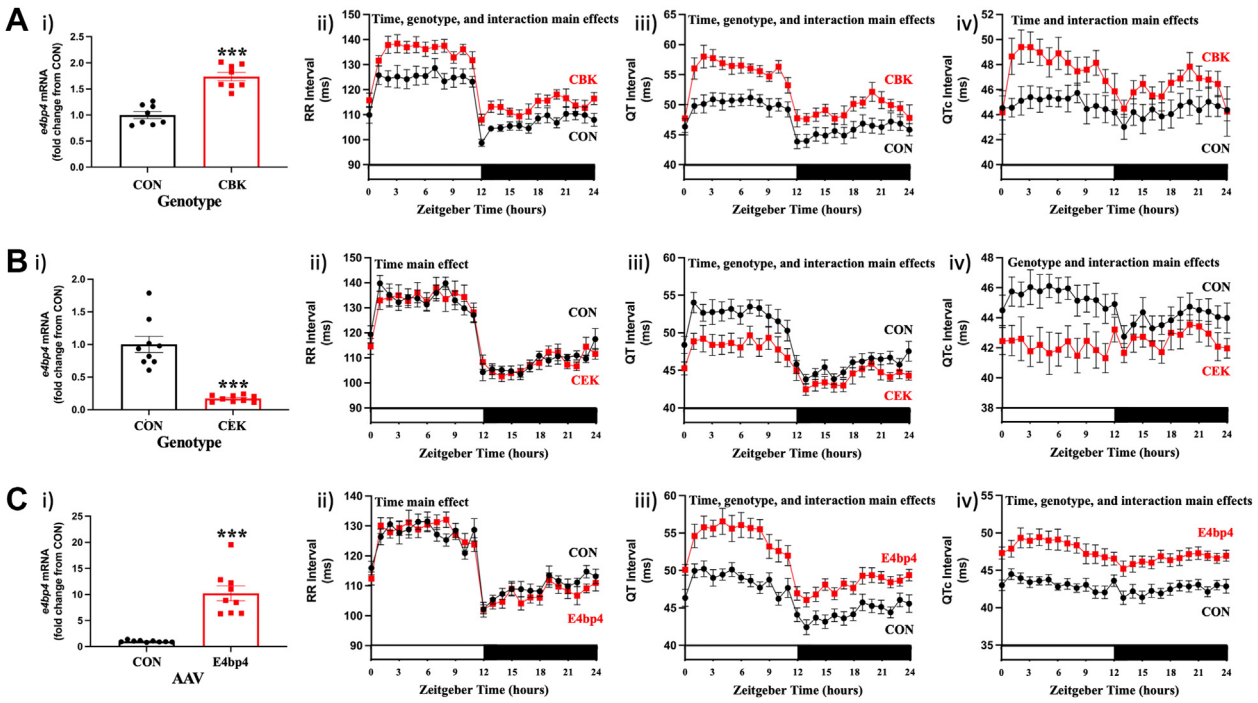
FIGURE 4 E4BP4 Impacts Diurnal Variations in Cardiac Fatty Acid Metabolism



(A) Volcano plot showing metabolites with large and significant differences between CEK and littermate CON hearts, independent of the time of day (n = 48). The x-axis shows log10 of fold change (CEK/CON), whereas the y-axis shows log10 of false discovery rate-corrected *P* values following 2-way analysis of variance (OMU package in R software). **Shaded boxes** behind graph show cutoffs for $P < 0.05$, and for 1.25-fold change. Metabolites meeting both thresholds are labeled; additional highly significant metabolites that did not meet the fold change threshold are also labeled. **(B)** Day (ZT3-ZT9; **white box**) vs night (ZT15-ZT21; **black box**) differences in oleate oxidation **(i)**, cardiac power **(ii)**, and heart rate **(iii)** between isolated working hearts from CEK and CON mice (n = 5-8). Main effects of genotype, time-of-day, and/or interaction are reported at the **top** of the figure panels. **(C)** Diurnal variations in palmitoylcarnitine **(i)**, 3-hydroxypalmitate **(ii)**, and 3-hydroxyoleate **(iii)** for hearts collected from CEK and littermate CON mice (n = 6). Data are presented as mean ± SEM. Cosinor analysis outcomes are reported at the **top** of the figure panels. *$P < 0.05$ for CON vs CEK hearts (at the same time of day); †$P < 0.05$ for day vs night (within the same genotype). Abbreviations as in Figure 1.

tracers in isolated working mouse heart perfusions. Consistent with previous studies,[12,15,47,48] day-night differences in oleate oxidation are not observed in CON hearts (Figure 4B). By contrast, CEK hearts exhibited day-night differences in oleate oxidation in CEK hearts (in the absence of significant alterations in cardiac function) (Figure 4B). Moreover, higher rates of oleate oxidation observed in CEK hearts during the

FIGURE 5 E4BP4 Regulates the QT Interval



(A) Cardiac *e4bp4* mRNA at ZTO **(i)**, as well as diurnal variations in RR intervals **(ii)**, QT intervals **(iii)**, and QTc intervals **(iv)** in CBK and littermate CON mice (n =8). **(B)** Cardiac *e4bp4* mRNA at ZTO **(i)**, as well as diurnal variations in RR intervals **(ii)**, QT intervals **(iii)**, and QTc intervals **(iv)** in CEK and littermate CON mice (n = 9). **(C)** Cardiac *e4bp4* mRNA at ZTO **(i)**, as well as diurnal variations in RR intervals **(ii)**, QT intervals **(iii)**, and QTc intervals **(iv)** in mice 6 weeks after retro-orbital injection of E4bp4-AAV or CON-AAV (n = 9). Data are presented as mean ± SEM. Main effects of genotype/AAV, time of day, and/or interaction are reported at the **top** of the figure panels. ***$P < 0.001$ for CON vs CBK/CEK/E4bp4-AAV hearts (at the same time of day). Abbreviations as in **Figure 1**.

light phase (**Figure 4B**) were associated with increased levels of fatty acyl intermediates in CEK hearts at this time (**Figure 4C**, Supplemental Figure 5B). Alterations in diurnal variations of cardiac lipid metabolism observed in CEK mice was in the absence of perturbations in daily behavior (physical activity, food intake) (Supplemental Figure 6A), whole-body metabolism (energy expenditure, respiratory exchange ratio) (Supplemental Figure 6B), or body weight (Supplemental Figure 6C). These observations suggest that one function of E4BP4 may be to attenuate cardiac fatty acid oxidation specifically during the light (sleep) phase.

CARDIOMYOCYTE E4BP4 REGULATES QT INTERVALS IN THE MURINE HEART. During the ex vivo tracer studies, a 2-way ANOVA revealed a trend for higher heart rates in isolated working CEK hearts, independent of the time of day (ie, $P = 0.077$ for a genotype main effect) (**Figure 4B**). Moreover, using nonadjusted *P* values (as opposed to BHq-corrected *P* values) for the RNA-seq pathway analysis, ion channels were identified as a class of genes that were altered in CEK hearts (Supplemental Figure 7A). Various cardiac electrophysiology parameters fluctuate over the course of the day, including heart rate and QT intervals; these rhythms appear to be secondary to a combination of extracardiac (eg, autonomic tone) and intracardiac (ie, the cardiomyocyte circadian clock) influences.[49] To gain further insight regarding whether E4BP4 influences cardiac electrophysiology, telemetry studies were performed in 12-week-old mice harboring different cardiac E4BP4 levels. These studies were initially performed in CBK mice; consistent with previously published studies,[50] CBK mice exhibit increased cardiac *e4bp4* mRNA levels, associated with increased R-R ($P < 0.05$), QT ($P < 0.05$), and heart rate corrected QT (QTc; $P = 0.075$) intervals, relative to littermate controls (**Figure 5A**, Supplemental Figure 7B). Interrogation of CEK mice revealed lower cardiac *e4bp4* mRNA levels, associated with decreased QT and QTc intervals (without effect on R-R intervals) (**Figure 5B**, Supplemental Figure 7B). Further analysis identified a positive correlation between individual

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cardiac *e4bp4* mRNA levels with both QT and QTc intervals (Supplemental Figure 7C). AAVs were next employed to establish whether E4BP4 overexpression was sufficient to affect QT and/or QTc intervals. Six weeks after retro-orbital E4bp4-AAV injection, cardiac *e4bp4* mRNA increased by ~10-fold, associated with increased QT and QTc intervals (relative to mice receiving a control AAV); R-R intervals were not affected (**Figure 5C**, Supplemental Figure 7D). Importantly, physical activity did not differ between the experimental groups investigated (Supplemental Figure 7E). These observations highlight E4BP4 as a novel regulator of QT intervals.

DISCUSSION

The purpose of the present study was to identify novel roles for the transcriptional repressor E4BP4 in the heart. We initially hypothesized that chronic induction of E4BP4 following genetic disruption of the cardiomyocyte circadian clock causes age-onset cardiomyopathy. Consistent with this hypothesis, genetic deletion of E4BP4 in CBK hearts completely prevented age-onset adverse cardiac remodeling (cardiomyocyte hypertrophy and interstitial fibrosis) and heart failure. Mechanistically, we observed partial/full normalization of 55 putative E4BP4 target genes in CBK hearts following concomitant deletion of E4BP4; many of these transcripts have established roles in processes that are critical for maintenance of normal cardiac function (ie, cellular signaling, metabolism, and ion transport). Moreover, we report that approximately 46.6% of the diurnal transcriptome in the heart is dependent on E4BP4; many of these transcripts cluster in metabolic pathways. Consistent with the latter, metabolomics revealed striking perturbations in cardiac metabolism following E4BP4 deletion; tracer studies confirmed that E4BP4 represses cardiac fatty acid oxidation during the sleep period. In addition, telemetry studies uncovered a novel role for E4BP4 in regulation of QT intervals. Collectively, these data highlight critical roles for E4BP4 in both cardiac physiology and pathophysiology.

Circadian disruption is invariably associated with pathology. For example, shift workers exhibit increased risk of cancer, neurocognitive dysfunction, obesity, diabetes, and numerous cardiovascular diseases (including stroke and ischemic heart disease).[51] Evidence exists supporting the concept that impairment of intrinsic circadian clocks precipitates disease. In humans, polymorphisms in genes encoding for circadian clock components (eg, CLOCK and BMAL1) are associated with increased risk for obesity, diabetes, and hypertension.[52,53] Moreover, genetic disruption of clocks in animal models lead to numerous age-onset diseases.[54,55] This includes severe adverse cardiac remodeling and contractile dysfunction in both germline and cardiomyocyte-specific BMAL1 deletion mice.[13,18,56] In the case of CBK mice, we have previously reported histological alterations in 8- to 16-week-old mouse hearts (ie, moderate cardiomyocyte hypertrophy and interstitial fibrosis, in the absence of systolic dysfunction), which transitions to dilated cardiomyopathy after 30 weeks of age (characterized by exacerbated hypertrophy and fibrosis, as well as reduced ejection fraction).[13] Here, we confirm this chronology of events, and further report that the heart failure observed in CBK mice at 36 weeks of age is prevented at functional, gravimetric, histologic, and molecular levels when E4BP4 is deleted (**Figure 1**, Supplemental Figure 1, **Table 1**). These data suggest that heart failure development following genetic disruption of the cardiomyocyte circadian clock is dependent on the transcriptional repressor E4BP4 (which is chronically induced in CBK hearts). Given that E4BP4 appears to be directly responsible for only 55 repressed transcripts in the heart following BMAL1 deletion (Supplemental Table 1), these transcripts serve as candidate mediators for cardiomyopathy development. Although great care should be taken to not overly speculate which of these E4BP4 targets causatively impact cardiomyopathy development in CBK hearts, several previously published observations are worthy of discussion. Many of these transcripts encode for proteins that influence critical cardiac processes, such as signaling, metabolism, and electrophysiology (Supplemental Table 1). Moreover, genetic deletion of several candidates has been reported to result in adverse remodeling and cardiac dysfunction (eg, Cacna1c, Herpud1, Nr3c1, Oplah) and/or worsen outcomes in response to stresses (such as pressure overload; eg, Gpr22, Nampt, Steap3).[57-63] Extensive additional studies are undoubtedly required to define the relative contribution of individual E4BP4 target genes toward adverse cardiac remodeling during circadian disruption.

Dramatic time-of-day-dependent fluctuations are observed in the myocardium at molecular, cellular, and functional levels.[6] This includes oscillations in heart rate, contractile performance, coronary flow, oxidative metabolism, calcium homeostasis, sensitivity to neurohumoral stimulation, and propensity for hypertrophic growth.[12,14,16,35,64,65] Daily fluctuations in many of these parameters appear to be orchestrated (to differing extents) by the cardiomyocyte circadian clock.[1] What remains less clear

is the nature of specific mechanistic links between the cardiomyocyte circadian clock and clock-controlled cardiac processes. This is in part because the circadian clock mechanism is composed of a collection of interdependent transcription factors, such that disruption of one clock component often alters them all. Identification and interrogation of immediate downstream targets of the clock is therefore considered an important knowledge gap. E4BP4 was recently suggested to be a candidate mechanistic link.[19] This transcriptional repressor was initially characterized in immune cells, where it regulates T-cell, B-cell, and natural killer cell function.[26] More recently, E4BP4 has emerged as a modulator of metabolic processes that are critical for nutrient intake responses.[24,25] Consistent with the concept that E4BP4 potentially regulates cardiac metabolism, Dierickx et al[19] reported that Nampt and Ces1d are direct E4BP4 target genes in the heart. Here, we reveal that E4BP4 deletion in cardiomyocytes attenuates/abolishes 24-hour oscillations in 705 transcripts in the heart (Supplemental Table 7), some of which have established functions as metabolic regulators (eg, *acot1*) (**Figure 3F**). Somewhat surprisingly, 423 transcripts exhibit augmented 24-hour oscillations in CEK hearts (Supplemental Table 8). Consistent with identification of fatty acid metabolism as the most highly impacted biological process following E4BP4 deletion (through Pathway Analysis) (**Figure 3D**), both metabolomics and tracer studies reveal enhanced day-night differences in fatty acid metabolism in CEK hearts, characterized by higher oleate oxidation rates during the light (sleep) phase (**Figure 4**). Given that *e4bp4* mRNA levels peak in the mouse heart toward the beginning of the light phase (**Figure 3A**), these data suggest that one function of E4BP4 is likely the repression of fatty acid oxidation specifically during the sleep period. It is similarly noteworthy that our studies indicate that E4BP4 is potentially an important regulator of QT intervals (**Figure 5**). QT intervals increase during the sleep period, which is associated with increased risk of arrhythmias (eg, Brugada syndrome).[66,67] Given that *e4bp4* mRNA peaks in the heart toward the onset of the sleep period (**Figure 3A**), and that E4BP4 increases QT intervals (**Figure 5**), our studies lead to the hypothesis that E4BP4 may promote arrhythmogenesis. Indirect evidence for this hypothesis includes observations that cardiomyocyte-specific BMAL1 deletion (which increases cardiac E4BP4 levels) leads to a proarrhythmogenic phenotype.[50,68]

STUDY LIMITATIONS. Although the current study reveals that heart failure development following cardiomyocyte BMAL1 deletion is dependent on E4BP4, the precise mechanisms by which this occurs have not been established. More specifically, despite identifying 55 direct E4BP4 target genes as candidate mediators of heart failure development following disruption of the cardiomyocyte circadian clock, whether one of these target genes plays a dominant role was not investigated. Similarly, although the current study reports that E4BP4 modulates 24-hour patterns in the cardiac transcriptome, metabolism (eg, fatty acid oxidation), and electrophysiology (eg, QT intervals), the precise molecular links by which these perturbations occur were not established. This is likely through a combination of direct (ie, E4BP4 binding to *cis*-regulatory elements of target genes) and secondary indirect mechanisms. In the latter case, *acot1* expression serves as an example. More specifically, E4BP4 is known to transcriptionally repress target genes, yet *acot1* mRNA levels are decreased in CEK hearts (**Figure 3F**). Moreover, neither ChIP-seq[19] nor Hi-C[69] in wild-type hearts suggests that E4BP4 binds to DNA within the vicinity of the *Acot1* promoter. Future studies are required to elucidate fully the mechanisms by which E4BP4 impacts cardiac diurnal variations.

CONCLUSIONS

In summary, the present study indicates that cardiac pathology secondary to disruption of the cardiomyocyte circadian clock is dependent on E4BP4, likely through direct repression of 55 transcripts. Moreover, E4BP4 has emerged as a novel regulator of cardiac metabolism (eg, fatty acid oxidation) and electrophysiology (eg, QT intervals). Collectively, these studies highlight the importance of E4BP4 in both cardiac physiology and pathophysiology.

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ADDRESS FOR CORRESPONDENCE: Dr Martin E. Young, Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, 703 19th Street South, ZRB 308, Birmingham, Alabama 35294, USA. E-mail: meyoung@uab.edu.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The preclinical studies described herein indicate that cardiac disease following genetic circadian disruption is dependent on E4BP4, and that this transcriptional modulator controls processes that are critical to cardiac function, including metabolism and electrophysiology. In doing so, these studies highlight the importance for maintaining normal circadian rhythms, for the prevention of cardiac disease.

TRANSLATIONAL OUTLOOK: Circadian perturbations, secondary to either genetics (eg, single nucleotide polymorphisms in core circadian clock genes), the environment (eg, acute changes in the light-dark cycle during daylight savings, time-zone travel, etc.), or behaviors (eg, sleep deprivation), are associated with various pathologies in humans, including cardiometabolic and cardiovascular diseases. This is exemplified by nightshift workers, who have increased risk of obesity, diabetes mellitus, hypertension, and adverse ischemic events. However, what remains less well understood are the mechanisms by which circadian disruption predisposes individuals toward pathogenesis. The studies described within this paper highlight E4BP4 as one such mechanistic link. Translation of these findings toward human health may be through a number of potential avenues. First, multiple genome-wide association studies have previously revealed an association between E4BP4 single nucleotide polymorphisms and body mass index (consistent with a role in lipid metabolism). Second, preclinical studies in numerous models of cardiometabolic and cardiovascular diseases (eg, atherosclerosis, myocardial infarction) consistently reveal benefit of REV-ERBα/β agonists; given that these agonists decrease E4BP4 levels in the heart (secondary to REV-ERBα/β activation), and that E4BP4 deletion is beneficial in the setting of circadian clock disruption, the possibility exists that REV-ERBα/β agonists may exert benefit through E4BP4 repression.

REFERENCES

1. Rana S, Prabhu SD, Young ME. Chronobiological influence over cardiovascular function: the good, the bad, and the ugly. *Circ Res*. 2020;126(2):258–279.

2. Degaute JP, van de Borne P, Linkowski P, Van Cauter E. Quantitative analysis of the 24-hour blood pressure and heart rate patterns in young men. *Hypertension*. 1991;18(2):199–210.

3. Muller J, Tofler G, P. Stone P. Circadian variation and triggers of onset of acute cardiovascular disease. *Circulation*. 1989;79:733–743.

4. Solocinski K, Gumz ML. The circadian clock in the regulation of renal rhythms. *J Biol Rhythms*. 2015;30(6):470–486.

5. Rudic RD, Fulton DJ. Pressed for time: the circadian clock and hypertension. *J Appl Physiol*. 2009;107(4):1328–1338.

6. Young ME. Anticipating anticipation: pursuing identification of cardiomyocyte circadian clock function. *J Appl Physiol*. 2009;107(4):1339–1347.

7. Scheer FA, Hu K, Evoniuk H, et al. Impact of the human circadian system, exercise, and their interaction on cardiovascular function. *Proc Natl Acad Sci U S A*. 2010;107(47):20541–20546.

8. Edery I. Circadian rhythms in a nutshell. *Physiol Genomics*. 2000;3:59–74.

9. Takahashi JS, Hong HK, Ko CK, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet*. 2008;9(10):764–775.

10. Curtis AM, Cheng Y, Kapoor S, Reilly D, Price TS, Fitzgerald GS. Circadian variation of blood pressure and the vascular response to asynchronous stress. *Proc Natl Acad Sci U S A*. 2007;104(9):3450–3455.

11. Delisle BP, George AL Jr, Nerbonne JM, et al. Understanding circadian mechanisms of sudden cardiac death: a report from the National Heart, Lung, and Blood Institute Workshop, part 1: basic and translational aspects. *Circ Arrhythm Electrophysiol*. 2021;14(11):e010181.

12. Bray M, Shaw C, Moore M, et al. Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function; metabolism; and gene expression. *Am J Physiol Heart Circ Physiol*. 2008;294:H1036–H1047.

13. Young ME, Brewer RA, Peliciari-Garcia RA, et al. Cardiomyocyte-specific BMAL1 plays critical roles in metabolism, signaling, and maintenance of contractile function of the heart. *J Biol Rhythms*. 2014;29(4):257–276.

14. Durgan DJ, Pat BM, Laczy B, et al. O-GlcNAcylation, novel post-translational modification linking myocardial metabolism and cardiomyocyte circadian clock. *J Biol Chem*. 2011;286(52):44606–44619.

15. Tsai JY, Kienesberger PC, Pulinilkunnil T, et al. Direct regulation of myocardial triglyceride metabolism by the cardiomyocyte circadian clock. *J Biol Chem*. 2010;285(5):2918–2929.

16. McGinnis GR, Tang Y, Brewer RA, et al. Genetic disruption of the cardiomyocyte circadian clock differentially influences insulin-mediated processes in the heart. *J Mol Cell Cardiol*. 2017;110:80–95.

17. Schroder EA, Burgess DE, Zhang X, et al. The cardiomyocyte molecular clock regulates the circadian expression of Kcnh2 and contributes to ventricular repolarization. *Heart Rhythm*. 2015;12(6):1306–1314.

18. Ingle KA, Kain V, Goel M, Prabhu SD, Young ME, Halade GV. Cardiomyocyte-specific Bmal1 deletion in mice triggers diastolic dysfunction, extracellular matrix response, and impaired resolution of inflammation. *Am J Physiol Heart Circ Physiol*. 2015;309(11):H1827–H1836.

19. Dierickx P, Zhu K, Jiang C, et al. Circadian REV-ERBs repress E4bp4 to activate NAMPT-dependent NAD+ biosynthesis and sustain cardiac function. *Nat Cardiovasc Res*. 2022;1(1):45–58. https://doi.org/10.1038/s44161-021-00001-9

20. Preitner N, Damiola F, Lopez-Molina L, et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*. 2002;110:251–260.

21. Zhang Y, Fang B, Emmett MJ, et al. Discrete functions of nuclear receptor Rev-erbalpha couple

metabolism to the clock. *Science*. 2015;348(6242):1488–1492.

22. Duez H, Staels B. The nuclear receptors Rev-erbs and RORs integrate circadian rhythms and metabolism. *Diab Vasc Dis Res*. 2008;5(2):82–88.

23. Mitsui S, Yamaguchi S, Matsuo T, Ishida Y, Okamura H. Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. *Genes Dev*. 2001;15:995–1006.

24. Wang Y, Kuang Z, Yu X, Ruhn KA, Kubo M, Hooper LV. The intestinal microbiota regulates body composition through NFIL3 and the circadian clock. *Science*. 2017;357(6354):912–916.

25. Tong X, Muchnik M, Chen Z, et al. Transcriptional repressor E4-binding protein 4 (E4BP4) regulates metabolic hormone fibroblast growth factor 21 (FGF21) during circadian cycles and feeding. *J Biol Chem*. 2010;285(47):36401–36409.

26. Male V, Nisoli I, Gascoyne DM, Brady HJ. E4BP4: an unexpected player in the immune response. *Trends Immunol*. 2012;33(2):98–102.

27. Storch KF, Paz C, Signorovitch J, et al. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. *Cell*. 2007;130(4):730–741.

28. Motomura Y, Kitamura H, Hijikata A, et al. The transcription factor E4BP4 regulates the production of IL-10 and IL-13 in CD4+ T cells. *Nat Immunol*. 2011;12(5):450–459.

29. Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest*. 1997;100(1):169–179.

30. Bray MS, Ratcliffe WF, Grenett MH, Brewer RA, Gamble KL, Young ME. Quantitative analysis of light-phase restricted feeding reveals metabolic dyssynchrony in mice. *Int J Obes (Lond)*. 2013;37(6):843–852.

31. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Res*. 1996;6(10):995–1001.

32. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res*. 1996;6(10):986–994.

33. Stavinoha M, RaySpellicy J, Hart-Sailors M, Mersmann H, Bray M, Young M. Diurnal variations in the responsiveness of cardiac and skeletal muscle to fatty acids. *Am J Physiol Endocrinol Metab*. 2004;287(5):E878–E887.

34. Durgan D, Smith J, Hotze M, et al. Distinct transcriptional regulation of long-chain acyl-CoA synthetase isoforms and cytosolic thioesterase 1 in the rodent heart by fatty acids and insulin. *Am J Physiol Heart Circ Physiol*. 2006;290:H2480–H2497.

35. Durgan DJ, Tsai JY, Grenett MH, et al. Evidence suggesting that the cardiomyocyte circadian clock modulates responsiveness of the heart to hypertrophic stimuli in mice. *Chronobiol Int*. 2011;28(3):187–203.

36. Tsai JY, Villegas-Montoya C, Boland BB, et al. Influence of dark phase restricted high fat feeding on myocardial adaptation in mice. *J Mol Cell Cardiol*. 2013;55:147–155.

37. Speerschneider T, Thomsen MB. Physiology and analysis of the electrocardiographic T wave in mice. *Acta Physiol (Oxf)*. 2013;209(4):262–271.

38. Speerschneider T, Grubb S, Metoska A, Olesen SP, Calloe K, Thomsen MB. Development of heart failure is independent of K+ channel-interacting protein 2 expression. *J Physiol*. 2013;591(23):5923–5937.

39. Mitchell GF, Jeron A, Koren G. Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol*. 1998;274(3):H747–H751.

40. Aittokallio T. Dealing with missing values in large-scale studies: microarray data imputation and beyond. *Brief Bioinform*. 2010;11(2):253–264.

41. Peliciari-Garcia RA, Bargi-Souza P, Young ME, Nunes MT. Repercussions of hypo and hyperthyroidism on the heart circadian clock. *Chronobiol Int*. 2018;35(2):147–159.

42. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.

43. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics*. 2016;32(21):3351–3353.

44. Fabregat A, Jupe S, Matthews L, et al. The Reactome pathway knowledgebase. *Nucleic Acids Res*. 2018;46(D1):D649–D655.

45. Kuleshov MV, Jones MR, Rouillard AD, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res*. 2016;44(W1):W90–W97.

46. Mia S, Kane MS, Latimer MN, et al. Differential effects of REV-ERBalpha/beta agonism on cardiac gene expression, metabolism, and contractile function in a mouse model of circadian disruption. *Am J Physiol Heart Circ Physiol*. 2020;318(6):H1487–H1508.

47. Young M, Razeghi P, Cedars A, Guthrie P, Taegtmeyer H. Intrinsic diurnal variations in cardiac metabolism and contractile function. *Circ Res*. 2001;89:1199–1208.

48. Mia S, Sonkar R, Williams L, et al. Impact of obesity on day-night differences in cardiac metabolism. *FASEB J*. 2021;35(3):e21298.

49. Delisle BP, Stumpf JL, Wayland JL, et al. Circadian clocks regulate cardiac arrhythmia susceptibility, repolarization, and ion channels. *Curr Opin Pharmacol*. 2021;57:13–20.

50. Gottlieb LA, Larsen K, Halade GV, Young ME, Thomsen MB. Prolonged QT intervals in mice with cardiomyocyte-specific deficiency of the molecular clock. *Acta Physiol (Oxf)*. 2021;233(1):e13707.

51. Pallesen S, Bjorvatn B, Waage S, Harris A, Sagoe D. Prevalence of shift work disorder: a systematic review and meta-analysis. *Front Psychol*. 2021;12(652):638252. https://doi.org/10.3389/fpsyg.2021.638252

52. Woon PY, Kaisaki PJ, Braganca J, et al. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc Natl Acad Sci U S A*. 2007;104(36):14412–14417.

53. Scott EM, Carter AM, Grant PJ. Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. *Int J Obes (Lond)*. 2008;32(4):658–662.

54. Bunger MK, Walisser JA, Sullivan R, et al. Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis*. 2005;41(3):122–132.

55. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev*. 2006;20(14):1868–1873.

56. Lefta M, Campbell KS, Feng HZ, Jin JP, Esser KA. Development of dilated cardiomyopathy in Bmal1-deficient mice. *Am J Physiol Heart Circ Physiol*. 2012;303(4):H475–H485.

57. Goonasekera SA, Hammer K, Auger-Messier M, et al. Decreased cardiac L-type Ca^{2+} channel activity induces hypertrophy and heart failure in mice. *J Clin Invest*. 2012;122(1):280–290.

58. Torrealba N, Navarro-Marquez M, Garrido V, et al. Herpud1 negatively regulates pathological cardiac hypertrophy by inducing IP3 receptor degradation. *Sci Rep*. 2017;7(1):13402.

59. Oakley RH, Ren R, Cruz-Topete D, et al. Essential role of stress hormone signaling in cardiomyocytes for the prevention of heart disease. *Proc Natl Acad Sci U S A*. 2013;110(42):17035–17040.

60. van der Pol A, Gil A, Tromp J, et al. OPLAH ablation leads to accumulation of 5-oxoproline, oxidative stress, fibrosis, and elevated fillings pressures: a murine model for heart failure with a preserved ejection fraction. *Cardiovasc Res*. 2018;114(14):1871–1882.

61. Adams JW, Wang J, Davis JR, et al. Myocardial expression, signaling, and function of GPR22: a protective role for an orphan G protein-coupled receptor. *Am J Physiol Heart Circ Physiol*. 2008;295(2):H509–H521.

62. Byun J, Oka SI, Imai N, et al. Both gain and loss of Nampt function promote pressure overload-induced heart failure. *Am J Physiol Heart Circ Physiol*. 2019;317(4):H711–H725.

63. Li PL, Liu H, Chen GP, et al. STEAP3 (six-transmembrane epithelial antigen of prostate 3) inhibits pathological cardiac hypertrophy. *Hypertension*. 2020;76(4):1219–1230.

64. Collins HE, Rodrigo GC. Inotropic response of cardiac ventricular myocytes to beta-adrenergic stimulation with isoproterenol exhibits diurnal variation: involvement of nitric oxide. *Circ Res*. 2010;106(7):1244–1252.

65. Latimer MN, Sonkar R, Mia S, et al. Branched chain amino acids selectively promote cardiac

growth at the end of the awake period. *J Mol Cell Cardiol*. 2021;157:31–44.

66. Matsuo K, Kurita T, Inagaki M, et al. The circadian pattern of the development of ventricular fibrillation in patients with Brugada syndrome. *Eur Heart J*. 1999;20(6):465–470.

67. Schwartz PJ, Wolf S. QT interval prolongation as predictor of sudden death in patients with myocardial infarction. *Circulation*. 1978;57(6):1074–1077.

68. Schroder EA, Lefta M, Zhang X, et al. The cardiomyocyte molecular clock, regulation of Scn5a, and arrhythmia susceptibility. *Am J Physiol Cell Physiol*. 2013;304(10):C954–C965.

69. Rosa-Garrido M, Chapski DJ, Schmitt AD, et al. High-resolution mapping of chromatin conformation in cardiac myocytes reveals structural remodeling of the epigenome in heart failure. *Circulation*. 2017;136(17):1613–1625.

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APPENDIX For supplemental figures and tables, please see the online version of this paper.