Circadian Mutant Mice with Obesity and Metabolic Syndrome are Resilient to Cardiovascular Disease

Cristine J. Reitz¹, Faisal J. Alibhai¹, Bruna Gazzi de Lima Seolin², Ashley Nemec-Bakk², Neelam Khaper², and Tami A. Martino¹*

¹Centre for Cardiovascular Investigations, Department of Biomedical Sciences, University of Guelph, Ontario, Canada, N1G2W1
²Medical Sciences Division, Northern Ontario School of Medicine, Lakehead University, Thunder Bay, Ontario, Canada, P7B5E1

*Corresponding author at: Centre for Cardiovascular Investigations, Biomedical Sciences/OVC Room 1646B, University of Guelph, Guelph, Ontario N1G2W1, Canada.
E-mail address: tmartino@uoguelph.ca

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ABSTRACT

Obesity and metabolic syndrome commonly underly cardiovascular disease. Clock$^{Δ19/Δ19}$ mice fed a normal diet develop obesity and metabolic syndrome; however, it is not known whether they develop or are resilient to cardiovascular disease. We found that Clock$^{Δ19/Δ19}$ mice do not develop cardiac dysfunction, despite their underlying conditions. Moreover, in contrast to wildtype controls fed a high-fat diet (HFD), Clock$^{Δ19/Δ19}$ HFD mice still do not develop cardiovascular disease. Indeed, Clock$^{Δ19/Δ19}$ HFD mice have preserved heart weight despite their obesity, no cardiomyocyte hypertrophy, and preserved heart structure and function, even after 24-weeks of a HFD. To determine why Clock$^{Δ19/Δ19}$ mice are resilient to cardiac dysfunction despite their underlying obesity and metabolic conditions, we examined global cardiac gene expression profiles by microarray and bioinformatics analyses, revealing that oxidative stress pathways were involved. We examined the pathways in further detail and found 1) SIRT-dependant oxidative stress pathways were not directly involved in resilience. 2) Increased 4-hydroxynonenal (4-HNE) in wildtype HFD but not Clock$^{Δ19/Δ19}$ mice, suggesting less reactive oxygen species in Clock$^{Δ19/Δ19}$ mice. 3) Increased cardiac catalase (CAT) and glutathione peroxidase (GPx) suggesting strong antioxidant defences in Clock$^{Δ19/Δ19}$ hearts. 4) Upregulation of Pparγ in Clock$^{Δ19/Δ19}$ hearts; this circadian-regulated gene drives transcription of CAT and GPx, providing a molecular basis for resilience in the Clock$^{Δ19/Δ19}$ mice. These findings shed new light on the circadian regulation of oxidative stress, and demonstrate an important role for the circadian mechanism in resilience to cardiovascular disease.

KEYWORDS

Circadian, Cardiovascular, Resilience, Obesity, High-fat diet, Oxidative stress
NEW & NOTEWORTHY

We examined whether obesity and metabolic syndrome underlie the development of cardiac
dysfunction in circadian mutant $Clock^{Δ19/Δ19}$ mice. Surprisingly, we demonstrate that although
$Clock^{Δ19/Δ19}$ mice develop metabolic dysfunction, they are protected from cardiac hypertrophy, left
ventricular remodeling, and diastolic dysfunction, in contrast to wild type controls, even when
challenged with a chronic high-fat diet. These findings shed new light on the circadian regulation
of oxidative stress pathways which can mediate resilience to cardiovascular disease.
INTRODUCTION

Adverse dietary choices can lead to chronic long-term health conditions such as cardiovascular disease. One of the most commonly cited examples is the diet-heart hypothesis proposed by Dr. Ancel Keys in the 1950s, which causally relates high dietary saturated fat to increased risk of cardiovascular disease (26). This hypothesis subsequently led to the creation of numerous nutritional guidelines and practices over many decades, to reduce fat and increase carbohydrate intake. However, this approach has generated considerable controversy, including possibly inadvertently leading to our current epidemic of obesity and metabolic syndrome, and it seems no longer tenable (66). In addition, decades of promoting a low-fat diet have had negative implications for other clinical cohorts as well, for example in obscuring the benefits of isocaloric substitution of fats for carbohydrates to better control blood sugar levels in patients with type 2 diabetes (7). Moreover, despite meaningful dietary interventions aimed at reducing fat intake to improve heart health, cardiovascular disease remains a leading cause of morbidity and mortality worldwide (41). New understanding of the interplay between diet and obesity and metabolic syndrome is clearly warranted, and especially identifying key factors that contribute to the development of, or resilience to, cardiovascular disease.

The discovery of the circadian mechanism was recognized by the Nobel Prize in Physiology or Medicine in 2017 (57). Research is now focussed on translation of circadian biology to clinical medicine (e.g. (10)), and we and others are especially interested in how circadian biology can benefit patients with cardiovascular disease (4, 19, 35, 37, 38, 48, 50, 53, 58). The circadian mechanism is a molecular transcription/translation feedback loop driven by complex 24 h oscillations between CLOCK and BMAL1 (positive arm), PERIOD and CRYPTOCHROME (negative arm), and others (as reviewed in (30, 51)). CLOCK is a key component of this molecular mechanism (63) and has been demonstrated to regulate rhythms in cardiac metabolism (8), gene and protein expression (45), and play a role in heart disease outcomes, including age-related cardiovascular dysfunction (1, 2) and healing responses following myocardial infarction (3).
Interestingly, previous studies have suggested that the circadian mechanism gates diet related cardiometabolic outcomes. For example, time-of-day restricted feeding or intermittent fasting (e.g. circadian strategies) can improve cardiac metabolic homeostasis (28, 55). Conversely, food intake at the wrong time of day can exacerbate cardiometabolic dysfunction (9, 59). The latter findings are especially a caveat for shift workers, who frequently eat meals during the night shift (11), and for whom there is an increased risk of obesity, metabolic disorders, and cardiovascular disease (25, 56, 64). Intriguingly, CLOCK mutant mice (Clock^Δ19/Δ19) develop obesity and metabolic syndrome (61), which are underlying risk factors for cardiovascular disease. However, it’s not known whether or not the Clock^Δ19/Δ19 mice actually develop or are resilient to obesity induced cardiovascular disease.

To investigate, we used circadian mutant Clock^Δ19/Δ19 mice, which have profoundly disrupted metabolic energy balance and significant body weight gain (61). We found that despite this underlying phenotype, the Clock^Δ19/Δ19 mice did not develop cardiac dysfunction. We next used a high-fat diet (HFD) to see if this in combination would precipitate the development of heart disease. As expected in wild type (WT) controls, the HFD led to obesity, metabolic syndrome, and cardiac dysfunction with cardiac hypertrophy and compensatory adverse structural and functional remodeling. However, in contrast, the Clock^Δ19/Δ19 mice fed a HFD remained resilient to cardiovascular disease. At a molecular level, we found that the Clock^Δ19/Δ19 mice had reduced oxidative stress and increased antioxidant responses under circadian regulatory control, which help explain their better cardiac outcomes. Thus, these findings reveal that Clock^Δ19/Δ19 mice do not develop cardiac remodeling and contractile dysfunction despite the underlying obesity and metabolic disorder, and they continue to be resilient even when fed a HFD. These studies shed new light on CLOCK, identifying it as a target that mediates resilience to obesity or HFD induced cardiovascular disease.
MATERIALS AND METHODS

Mice

Male Clock\textsuperscript{Δ19/Δ19} mice (63) on a C57Bl/6 background, bred at the University of Guelph Central Animal Facilities, and wild type (WT) controls were housed in a 12 h light (L): 12 h dark (D) environment, with food and water provided ad libitum. Clock\textsuperscript{Δ19/Δ19} mice are homozygous for the CLOCK point mutation, an A-T transversion mutation resulting in the deletion of exon 19 and a 51 amino acid deletion in the CLOCK protein (29). Clock\textsuperscript{Δ19/Δ19} mice were genotyped by allele-specific polymerase chain reaction (PCR) and phenotyped for circadian locomotor activity using running wheel actigraphy, as described previously (1, 49, 61). Starting at 8 weeks of age, the mice were fed either a HFD (45% fat, 20% protein, and 35% carbohydrate, TD.06415, Envigo Teklad Diets), or a normal standard chow (SC) diet (10% fat, 20% protein, and 70% carbohydrates, TD.08806, Envigo Teklad Diets), as described (61). All animals were weighed weekly, for up to 24 weeks on the HFD or SC diet. All experiments were performed in accordance with the Canadian Council on Animal Care, and were approved by the University of Guelph Institutional Animal Care and Use Committee.

Comprehensive lab animal monitoring system (CLAMS)

The non-invasive CLAMS (Columbus Instruments) was used to monitor food intake and metabolic parameters in Clock\textsuperscript{Δ19/Δ19} and WT mice after 24 weeks on either a HFD or SC diet, using methods previously described (1, 2, 6). Animals were individually housed and acclimatized for 48 h in the CLAMS unit under normal L:D conditions. Activity, food intake, oxygen consumption (VO\textsubscript{2}), whole body substrate utilization (respiratory exchange ratio; RER), and energy expenditure were measured every 15 min over a 24 h period. 24 mice were used, n=6 mice/group.

Metabolic measurements
After 24 weeks of HFD or SC, mice were fasted for 6 h, and blood was collected at zeitgeber time (ZT) 0. For glucose, cholesterol, and triglyceride measurements, nonterminal blood collection was performed from manually restrained, non-anesthetized mice via the saphenous vein. Fasted blood glucose levels were measured from a drop of blood from the saphenous vein using a hand-held glucometer (Freestyle Lite, Abbott). Cholesterol and triglyceride levels were assessed from ~200 µl of blood collected from the saphenous vein into a microvette capillary tube with clotting activator (Sarstedt), clotted on ice for 1 h, centrifuged at 10,000xg for 5 min at room temperature, and serum was aliquoted and stored at -80°C until use. From these serum samples, triglyceride levels were determined using the IDEXX Rodent Lipid Panel (IDEXX BioAnalytics). Serum cholesterol levels were measured using a commercially available kit, according to the manufacturer's instructions (Cholesterol E kit, Wako Diagnostic). 27 mice were used, \( n = 6-7 \) mice/group. For non-fasted insulin measurements, another set of \( \text{Clock}^{419/419} \) and WT mice on a standard \textit{ad libitum} diet were used. The mice were anesthetized with 4% isoflurane and euthanized at 4 h intervals over one 24 h period (ZT03, 07, 11, 15, 19, 23). Approximately 1 mL of blood was collected at each timepoint, via cardiac puncture into EDTA-treated microcentrifuge tubes (Sarstedt), and centrifuged at 1,500xg for 10 min at 4°C, and plasma was pooled and stored at -80°C until use. Insulin levels were determined by ELISA (Crystal Chem), according to the manufacturer's instructions, as described (61). 36 mice were used, \( n = 18 \) mice per genotype, \( n = 3 \) mice/timepoint.

**Morphometry and Histology**

\( \text{Clock}^{419/419} \) and WT mice fed a HFD or SC for 24 weeks were euthanized with 4% isoflurane and cervical dislocation at ZT07. Upon sacrifice, body weight (BW), heart weight (HW), epididymal white adipose tissue weight (eWAT), and tibia length (TL), were collected from each mouse. A total of 27 mice were used, with \( n = 6-7 \) mice/group. Hearts were collected for histopathology, as previously described (1, 3, 49). Briefly, hearts were removed, perfused with 1 M KCl to arrest in
diastole, and fixed in 10% neutral buffered formalin for 24 h. Formalin fixed hearts were processed, embedded, and 5 μm sections were collected at the mid-papillary level. Sections were stained with Masson’s trichrome for quantification of myocyte cross sectional area (MCSA) from at least 100 cardiomyocytes/heart, over at least 3 sections, with \( n = 3 \) hearts/group. Images were taken using Q-Capture (QImaging) and analyzed in Image J 1.46 (NIH).

**Echocardiography**

At baseline (8 weeks of age, SC) and after 4, 8, 12, 16, and 24 weeks of HFD or SC, cardiac structure and function were assessed under light anesthesia (1.5% isoflurane) using a GE Vivid 7 Dimension ultrasound machine (GE Medical Systems) with a i13L 14MHz linear-array transducer, as described previously (3, 17, 49). All echocardiography assessments were performed between ZT07 - ZT09. Measurements were taken at the mid-papillary level from at least 5 images per mouse. End diastolic (EDV) and systolic (ESV) volumes were calculated using the cube formula, stroke volume (SV) was calculated as EDV-ESV, and cardiac output (CO) was calculated as SV x heart rate (HR). A total of 24 mice were used, \( n = 6 \) mice/group.

**In vivo hemodynamics**

At the 24 week endpoint, *in vivo* hemodynamics measurements were collected in animals anesthetized with 4% isoflurane, intubated, and ventilated (Harvard Apparatus model 687), using our described methods (1-3, 5, 49). The right carotid artery was isolated and a 1.2Fr pressure catheter (Transonic) was advanced through the ascending aorta into the left ventricle (LV). *In vivo* LV and aortic pressure measurements were recorded with ADInstruments PowerLab and analyzed using Lab Chart 7 (Colorado Creeks). Following hemodynamic recordings, mice were euthanized with 4% isoflurane and cervical dislocation. 24 mice were used, \( n = 6 \) mice/group.

**RNA isolation, microarray, and bioinformatics analyses**
Clock\textsuperscript{Δ19/Δ19} and WT mice fed a HFD or SC for 24 weeks were euthanized with 4% isoflurane and cervical dislocation at ZT07. Hearts were collected, snap frozen in liquid nitrogen, and stored at -80°C until use. RNA isolation, microarray, and bioinformatics analyses were performed as described previously (6, 33, 36, 60). Briefly, total RNA was isolated from hearts using TRIZOL (Invitrogen). RNA quantity and quality were assessed by Nanodrop (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). Whole genome microarray experiments were performed using the Affymetrix GeneChip Mouse Gene 2.0 ST array (GEO Accession: GSE110245). Bioinformatics analyses were performed using GeneSpring GX v14.8 (Agilent Technologies Inc.). Data were normalized from raw fluorescence values using the exon robust multiarray algorithm and significant differences were determined for genes with a \textgreater 1.35-fold change in expression. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the Database for Annotation, Visualization, and Integrated Discovery Functional Annotation Tool (DAVID Bioinformatics 6.8, NIAID/NIH) (22). Circos plots were generated using Circos v.0.69-9 (32). A total of 16 mice were used, with \( n = 4 \) mice/group.

**Real time polymerase chain reaction (RT-PCR)**

To validate selected microarray expression profiles, RT-PCR was performed using the Power SYBR Green RNA-to-Ct one step kit (Life Technologies) on a ViiA7 real time PCR system (Applied Biosystems), as previously described (3, 6, 17, 49). The primers used were: \textit{peroxisome proliferator activated receptor gamma (Ppar)} forward 5\textquoteleft -ccagtttcgatcgtagaag-3' and reverse 5\textquoteleft -cttgagcagagtcacttgg-3', and \textit{histone} forward 5\textquoteleft -gcaagagtgcgccctctactg-3' and reverse 5\textquoteleft -ggcctcacttgctcctgcaa-3'. Relative gene expression was normalized to \textit{histone} using the \( \Delta \Delta CT \) method. 16 mice were used, \( n = 4 \) mice/group.

**Protein isolation and immunoblotting**
Proteins in oxidative stress or antioxidant pathways were investigated by Western blotting, as described previously (1, 2, 46). Tissues were homogenized at 20 Hz for 2 min (TissueLyser; Qiagen) with PathScan Lysis Buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 20 mM sodium fluoride, 1 mM Na3VO4) containing protease inhibitor cocktail (Sigma-Aldrich). Sedimentation was pelleted by centrifugation at 8,000xg for 10 min at 4°C, and the supernatant collected for subsequent experiments. Protein from liver (15 µg) or heart (30 µg) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (containing 0.1% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: rabbit anti-mouse manganese-dependent superoxide dismutase (Mn-SOD; Millipore) (1:2000), rabbit anti-mouse 4-hydroxynonenal (4-HNE; Abcam) (1:1000), rabbit anti-mouse catalase (CAT; Millipore) (1:2000), rabbit anti-mouse glutathione peroxidase (GPx; Abcam) (1:1000), and mouse anti-mouse monoclonal to GADPH (1:2000; Santa Cruz Biotechnology) was used as a loading control. Blots were then incubated with anti-rabbit (1:1000) or anti-mouse (1:5000) horseradish peroxidase-conjugated secondary antibodies for 2 hours, washed with tris-buffered saline, and proteins were visualized by chemiluminescence and densitometry was analyzed using ImageJ software (NIH). A total of 16 mice were used, with n=4 mice/group.

Statistics

All values are mean ± SEM. Statistical comparisons were made using an unpaired Student’s t-test or two-way analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple comparisons, as applicable. All analyses were performed using GraphPad Prism 8 (GraphPad Software) or Excel (Microsoft). Results of P≤0.05 are considered statistically significant. Statistical parameters, including n-values, are noted in the figure legends.
RESULTS

ClockΔ19/Δ19 mice develop obesity and metabolic dysfunction.

First, we confirmed the adverse metabolic effects of circadian disruption in ClockΔ19/Δ19 mice, so we could next investigate the effects on cardiac structure and function. To characterize the phenotype of the ClockΔ19/Δ19 mice, we first examined 24 h locomotor running wheel activity under standard L:D conditions (Fig. 1A). The ClockΔ19/Δ19 mice actigraphy was as expected, with less activity in the dark phase as compared to WT controls (Fig. 1B). We also analyzed whole-body metabolism by indirect calorimetry using CLAMS metabolic cages. Both genotypes exhibited the anticipated diurnal rhythm in locomotor activity, however, the ClockΔ19/Δ19 mice showed significantly blunted (P<0.001) wake time activity, along with increased (P<0.001) activity in the light (sleep time) phase, as compared to WT controls (Fig. 1C). Moreover, ClockΔ19/Δ19 mice showed a loss of diurnal feeding rhythms, with an average of 47% of food intake occurring in the light phase, as compared to only 29% for WT mice during this time (P<0.001) (Fig. 1D). Similarly, the ClockΔ19/Δ19 mice exhibited a significantly attenuated (P<0.05) daily rhythm in oxygen consumption as compared to WT controls (Fig. 1E). The ClockΔ19/Δ19 mice also showed a loss of rhythmic substrate utilization, as measured by respiratory exchange ratio (RER) (Fig. 1F). Finally, ClockΔ19/19 mice develop hypercholesterolemia, hyperglycemia, and hypoinsulinemia, as anticipated (Table 1). Together these results confirm that disrupted ClockΔ19/Δ19 mice have underlying metabolic syndrome.

Next, we characterized the development of obesity in ClockΔ19/Δ19 and WT mice, in groups of animals fed SC, and also in groups fed a HFD for 24 weeks. We found greater body weight in the WT mice on the HFD (Fig. 2A), increasing significantly (P<0.0001) over the 24-week period, as compared to WT SC controls (Fig. 2B, C, Table 2). Increased body weight occurred more rapidly in the ClockΔ19/Δ19 mice on the HFD (Fig. 2D), and persisted (P<0.0001) over 24 weeks, as compared to ClockΔ19/Δ19 SC controls (Fig. 2E, F, Table 2). Moreover, the HFD fed mice had increased (P<0.001) epididymal white adipose tissue (eWAT) weight, suggesting that the body
weight gain was likely due in part to an increase in visceral fat (Fig. 2G, Table 3). Interestingly, overall daily caloric intake was similar for both genotypes (Fig. 2H), despite greater weight gain in the ClockΔ19/Δ19 mice. We also found that HFD fed mice had a similar rise in serum cholesterol levels regardless of genotype (Fig. 2I), however, only the WT mice showed elevated fasting glucose levels under the HFD conditions (Fig. 2J). Together these findings confirm that at baseline the ClockΔ19/Δ19 mice have obesity and metabolic dysfunction, but not WT mice, as anticipated. Moreover, they show that both genotypes respond to a HFD with obesity and metabolic dysfunction.

ClockΔ19/Δ19 mice with obesity and metabolic dysfunction do not develop cardiac hypertrophy.

We next looked at whether the obesity and metabolic dysfunction in the ClockΔ19/Δ19 mice was associated with the development of heart disease. We found that even though the ClockΔ19/Δ19 mice had greater heart weight (HW) than the WT mice at baseline (Fig. 3A), their HW:BW (Fig. 3B) and HW:TL (Fig. 3C) ratios were proportionate, suggesting that any increase in HW was a normal physiological response to increased BW. Furthermore, as shown in Figure 3A-C and Table 3, even when fed a HFD, the ClockΔ19/Δ19 mice showed no significant increase in HW as compared to ClockΔ19/Δ19 SC controls, despite the HFD induced BW gain. In contrast, the WT HFD mice had a significant increase in HW disproportionate to their BW gain, suggestive of pathological remodeling in this group. The adverse cardiac remodeling was also evident on histological analyses, as the WT HFD mice exhibited cardiomyocyte hypertrophy (Fig. 3D, left) and increased myocyte cross-sectional area (Fig. 3D, right), as compared to WT SC controls. In contrast the ClockΔ19/Δ19 hearts showed no change in cardiomyocyte hypertrophy under SC or HFD conditions (Fig. 3E), consistent with these animals being resistant to obesity induced cardiac remodeling.
Clock\textsuperscript{Δ19/Δ19} mice with obesity and metabolic dysfunction are resilient to cardiovascular disease.

Given that Clock\textsuperscript{Δ19/Δ19} mice showed resilience to cardiomyocyte hypertrophy, we next examined whether this correlated with cardiac structure and function, by echocardiography. Representative M-mode echocardiography images after 24 weeks of diet are shown in Figure 4A. The time-series data are illustrated in Figure 4B. We found that the Clock\textsuperscript{Δ19/Δ19} mice maintained normal cardiac structure and function, consistent with the lack of pathological findings in the heart (Fig. 4B, Table 2). Moreover, the Clock\textsuperscript{Δ19/Δ19} mice showed normal physiological cardiac adaptations to a HFD, with increased end diastolic volume (EDV), end systolic volume (ESV), stroke volume (SV), and cardiac output (CO), while maintaining normal cardiac function, by 24 weeks (Fig. 4B, Table 2). In contrast, the WT HFD mice developed significant pathophysiologic changes in structure and function by echocardiography, consistent with the earlier findings of cardiac hypertrophy in these animals (Fig. 4B, Table 2). The WT HFD hearts also exhibited significantly impaired contractility by in vivo hemodynamics (Fig. 5A). Moreover, although systolic function was preserved in the WT HFD mice (Fig. 5B), there was significant (P<0.005) diastolic dysfunction indicated by an impaired relaxation rate (Fig. 5A), increased LVEDP (Fig. 5C), and an increase (P<0.005) in the LV diastolic time constant tau (Fig. 5D). Together these data show that WT mice exhibit a number of cardiometabolic risk responses to a HFD, which are associated with left ventricular remodeling and diastolic dysfunction. The Clock\textsuperscript{Δ19/Δ19} mice also develop cardiometabolic risk profiles, on either SC or HFD, however surprisingly, they are resilient to cardiac dysfunction.

Cardiac transcriptional analyses and oxidative stress pathways.

To investigate the underlying gene expression patterns driving adverse cardiac remodeling in WT mice and, in parallel, the resilience observed in Clock\textsuperscript{Δ19/Δ19} mice, we performed genome-wide microarray analysis. First, we examined transcriptional changes driven by HFD in WT hearts. A
total of 174 transcripts (≥1.35-fold change) showed differential expression in WT HFD versus WT
SC hearts (Fig. 6A, Supplemental Table 1; see https://doi.org/10.6084/m9.figshare.12855530). Further Gene Ontology (GO) analysis revealed that the differentially regulated genes in WT HFD hearts mapped to functional biological categories of stress, growth/remodeling, transcription, and metabolism (Fig. 6B). In contrast, differential expression of these same cardiac remodeling genes were not found in the hearts of the ClockΔ19/Δ19 mice, consistent with their resilience to cardiovascular disease (Table 4). Ontological mapping further revealed a role for the circadian mechanism in driving these transcriptional responses (Fig. 6C, Supplemental Table 1; available at https://doi.org/10.6084/m9.figshare.12855530), as the ClockΔ19/Δ19 hearts showed the anticipated blunted expression of core circadian mechanism genes (Fig. 6D), and KEGG analysis revealed a link with the oxidative stress and antioxidant pathways (Supplemental Table 2; available at https://doi.org/10.6084/m9.figshare.12855530).

SIRT-dependent oxidative stress pathways.

In terms of mechanism, we interrogated the oxidative stress gene data in more detail. Our microarray data showed significantly increased nicotinamide phosphoribosyltransferase (Nampt) expression only in WT HFD hearts, but not in WT SC, nor ClockΔ19/Δ19 SC nor ClockΔ19/Δ19 HFD mice (Fig. 7A). Since NAMPT enhances susceptibility to oxidative stress via the sirtuin (SIRT) dependent oxidative stress pathway, we next investigated this pathway. However, we found no differences for any groups in the expression of downstream Sirt1, Sirt2, Sirt3, Sirt4, and Sirt6 genes (Fig. 7B), nor for downstream Foxo1 and Foxo3 genes (Fig. 7C), nor did we observe differences in the abundance of the downstream antioxidant protein manganese-dependent superoxide dismutase (MnSOD) which mitigates oxidative stress (Fig. 7D). Thus, while WT HFD are susceptible to cardiac dysfunction, and ClockΔ19/Δ19 mice are resilient, that protection does not appear to be mediated by changes in the overall gene and protein expression of the SIRT-dependent oxidative stress pathways.
**H₂O₂-dependent antioxidant signaling.**

Next, we investigated the H₂O₂-dependent antioxidant signaling pathways. First, we found that 4-hydroxynonenal (4-HNE) was increased only in the WT HFD mice (Fig. 8A), but not in the ClockΔΙ9/ΔΙ9 mice (Fig. 8B), suggesting increased activation of oxidative stress driving cardiovascular disease in the WT mice. Second, we found that catalase (CAT) protein levels were increased in both the WT HFD heart (Fig. 8C) and ClockΔΙ9/ΔΙ9 HFD heart (Fig. 8D), consistent with there being activation of antioxidant pathways in response to HFD in both genotypes. Third, we found that glutathione peroxidase (GPx) protein levels were increased only in the ClockΔΙ9/ΔΙ9 HFD mice (Fig. 8E), suggesting better antioxidant protection in the hearts of these mice. Finally, in order to better understand the molecular drivers, we next evaluated Pparγ mRNA levels, a transcription factor that underlies GPx production. We found that that peroxisome proliferator-activated receptor gamma (Pparγ) was significantly (P<0.005) upregulated in ClockΔΙ9/ΔΙ9 hearts (Fig. 8F), consistent with the finding that GPx is upregulated and protective. Taken together, these data demonstrate that ClockΔΙ9/ΔΙ9 mice are resilient to cardiovascular disease, even though they have underlying obesity and metabolic syndrome, concurrent with reduced oxidative stress and increased antioxidant protection in the heart (Fig. 9).
DISCUSSION

In this study, we demonstrate that ClockΔ19/Δ19 mice have obesity and metabolic syndrome – well known risk factors for cardiovascular disease – yet they do not develop cardiac dysfunction. Moreover, even when fed a HFD that normally precipitates obesity, metabolic syndrome and cardiovascular disease (as in WT mice) the ClockΔ19/Δ19 mice continue to be resilient to heart disease. That is, WT mice fed a HFD develop cardiac hypertrophy with compensatory cardiac remodeling and diastolic dysfunction, but in contrast the ClockΔ19/Δ19 HFD mice have cardiac physiology similar to healthy controls. We used microarrays and bioinformatics analyses to investigate underlying mechanisms for resilience. We found that SIRT-dependant oxidative stress pathways did not appear to be directly involved in resilience. However, the H2O2-dependant pathways involving 4-HNE, CAT, and GPx revealed that the ClockΔ19/Δ19 hearts had reduced oxidative stress and better antioxidant responses. Both genetics and lifestyle contribute to serious chronic health conditions such as obesity, metabolic syndrome and the subsequent development of cardiovascular disease. Notably, these findings shed new light on how the circadian mechanism is an important player in mediating resilience to the cardiovascular disease outcomes.

One of the important foundations of our study is that ClockΔ19/Δ19 mice have underlying obesity and metabolic syndrome. This is consistent with earlier studies that showed that the circadian clock is an important regulator of mammalian energy balance, and that disruptions to the circadian mechanism can impair metabolic homeostasis (61). However, even though the ClockΔ19/Δ19 mice have obesity and metabolic syndrome, which are risk factors for cardiovascular disease, they do not develop heart disease. Moreover, even when challenged with a HFD the ClockΔ19/Δ19 mice exhibit normal physiologic cardiac adaptations associated with obesity, yet are resilient to pathological cardiac remodeling and contractile dysfunction, in contrast to their WT littermates. Thus, these studies shed new light on a role for the circadian mechanism, that is, as a factor that mediates resilience to cardiovascular disease.
An intriguing outcome of this study, however, is that by revealing a cardioprotective role in Clock mutant mice, our findings appear counter-intuitive to earlier reports that an intact circadian mechanism is needed to benefit the heart (e.g. (1, 3, 8, 17-19, 36, 39, 68)). Collectively, the message has long been that maintaining circadian rhythms promotes heart health, and disruption causes or exacerbates disease. Why then are the circadian mutant mice resilient to heart disease? In this study, the protective outcomes relate to the underlying condition. That is, diet, obesity, and metabolic dysfunction trigger adverse oxidative stress pathways that are modifiable by products of the circadian mechanism. Previous studies also strongly support this notion, and our findings, that cardiac remodeling can be improved on even in the absence of an intact circadian mechanism; the circadian mechanism remains fundamentally important because the genes and proteins that drive the outcome are under circadian mechanism transcriptional control.

For example, 1) homozygote tau/tau (casein kinase-1ε mutant) hamsters are unable to synchronize with the 24 h period, yet they do not develop cardiovascular dysfunction like their heterozygote tau/+ littermates do (34). 2) Ex-vivo hearts from cardiomyocyte specific CLOCK mutant mice (CCM) fed a HFD exhibit normal contractility on Langendorff perfusion, maintaining baseline levels of cardiac power and efficiency as compared to WT (59). 3) Cardiac specific circadian mechanism transcriptional outputs can change with aging, and sex hormones, and thus resilience may change to susceptibility in old ClockΔ19/Δ19 mice (1, 2). 4) Disruption of CLOCK is cardioprotective in the myocardial infarction ischemia reperfusion (mi/R) model, with reduced infarct size in CCM mice versus WT controls (18). 5) Also, pharmacologically targeting the circadian mechanism with the repressive REV-ERB agonist SR9009, temporarily holding back the clock, improves outcomes post-mi/R in mice (49). Importantly suspending the circadian mechanism in a manner in which it can mitigate outcomes is driven by the changes in output genes and proteins under its regulatory control, including outputs involved in cardiac remodeling. That is, perhaps what we think of as desynchrony should not be so much about the circadian clock being “broken” – but rather we should consider how it works differently, and how those
changes in controlled output genes can improve outcome. Thus, the message remains that the circadian mechanism is important for cardiovascular health, but one must also consider the circadian mechanism regulated outputs that are specific to the disease process, and that those can directly influence outcome.

Mechanistically, in this study, resilience of the \( \text{Clock}^{\Delta 19/\Delta 19} \) mice was mediated through oxidative stress pathways. We found that \( \text{Clock}^{\Delta 19/\Delta 19} \) mice have less 4-HNE response to HFD, as compared to the WT mice, and consistent with their better outcomes. 4-HNE is a product of lipid peroxidation with well-known adverse oxidative stress responses \textit{in vitro} \cite{13, 62}, and elevated 4-HNE corresponds with adverse cardiac remodeling in human heart failure \cite{42}. We also found greater antioxidant (CAT, GPx) responses in the \( \text{Clock}^{\Delta 19/\Delta 19} \) HFD hearts, as compared to WTs, and consistent with their resilience to HFD induced cardiovascular disease. These antioxidant enzymes are involved in detoxification of \( \text{H}_2\text{O}_2 \), with cardioprotective benefits as shown in experimental heart failure models \textit{in vivo} \cite{24, 47}. Importantly, the increased antioxidant protection may be driven by its transcription regulator PPAR\( \gamma \) \cite{12, 14, 21}, which is a circadian mechanism regulated product \cite{67}. The \( \text{Clock}^{\Delta 19/\Delta 19} \) hearts have increased \( \text{Ppar}_\gamma \) expression, thus providing a molecular explanation for the improved outcomes in these animals.

It has long been known that oxidative stress pathways underlie cardiac dysfunction, and that strategies aimed at reducing damage could be beneficial \cite{43}. However it is only recently that these oxidative stress pathways have been linked to the circadian mechanism in the cardiovascular system \cite{16, 23, 27, 44, 52}. Together, our findings demonstrate that the circadian mechanism drives oxidative stress and antioxidant pathways and in doing so it acts as a driver modulating resilience to cardiovascular disease.

\section*{Conclusions}

In this study we show that \( \text{Clock}^{\Delta 19/\Delta 19} \) mice develop obesity and metabolic syndrome, yet remarkably they are protected from cardiac dysfunction, even when fed a HFD. Resilience
appears to be mediated by down regulating adverse oxidative stress pathways and up regulating beneficial antioxidant pathways that are under circadian regulatory control. It is worth noting that these studies were done in mice, which raises some caveats with regards to translation to humans. Interestingly though, our findings reflect the common observation that although many individuals in contemporary society have obesity and metabolic syndrome, only some will develop cardiovascular disease. Additional studies in humans also support the notion that the circadian mechanism mediates resilience to cardiovascular disease. For example, minor allele carriers for the CLOCK polymorphism rs4580704 have a significantly lower risk of developing hypertension versus non-carriers (20). Also, circadian manipulations through time-restricted-feeding can improve cardiometabolic health in humans (40, 65). Pharmacologic targeting of the circadian mechanism might also be useful in mitigating cardiovascular disease outcomes; indeed, small molecule inhibitors are currently being developed for a wide variety of human clinical uses (15, 31, 49, 54). In conclusion, cardiovascular disease remains a leading cause of morbidity and mortality worldwide. New insights into protection against cardiovascular disease are clearly warranted. These findings highlight the important role of the circadian mechanism, in promoting resilience to cardiovascular disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS

C.J.R. and T.A.M. conceptualized the study and designed the experiments. C.J.R., F.J.A., B.G.D.L.S., A.N.B., N.K., and T.A.M. conducted the experiments. All authors analyzed and/or interpreted the experimental results. C.J.R. and T.A.M. drafted the paper. All authors have read and give permission to the paper.

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FIGURE LEGENDS

Fig. 1. Metabolic dysfunction and obesity in Clock$^{Δ19/Δ19}$ mice at baseline. A) Representative running wheel actigraphy of WT and Clock$^{Δ19/Δ19}$ mice under diurnal (12 h light: 12 h dark) conditions, and B) quantification of 24 h locomotor activity, n=14 days of analyses per genotype. C) Diurnal rhythms in activity; D) food intake; E) oxygen consumption (VO$_2$); and F) respiratory exchange ratio (RER) in WT vs. Clock$^{Δ19/Δ19}$ mice, quantified over the light (L) versus dark (D) phase by CLAMS, *$P<0.05$, n=6 mice/group. Data are presented as mean ± SEM.

Fig. 2. Metabolic dysfunction and obesity in Clock$^{Δ19/Δ19}$ mice in response to HFD. A) WT mice have increased body size in response to HFD, representative images; B) body weight tracking over the 24 week period; and C) quantification at the 24 week endpoint, and D) Clock$^{Δ19/Δ19}$ mice; E) tracking over 24 weeks; and F) quantification at the 24 week endpoint, *$P<0.01$ SC vs. HFD, #$P<0.05$ Clock$^{Δ19/Δ19}$ HFD vs. WT HFD, n$≥6$ mice/group. G) Increased epidydimal white adipose tissue (eWAT) in response to HFD, H) no change in daily caloric intake, and I) increased serum cholesterol and J) fasting blood glucose in response to 24 weeks HFD, serum samples taken at ZT0, *$P<0.05$, n$≥6$ mice/group. Data are presented as mean ± SEM. See Tables 2 and 3 for all morphometry values.

Fig. 3. Clock$^{Δ19/Δ19}$ mice are protected from cardiac hypertrophy. A) Clock$^{Δ19/Δ19}$ mice have larger hearts than WT mice at baseline, yet B) even after 24 weeks of HFD they maintain proportionate HW:BW, and C) HW:TL, suggesting physiologic growth but not pathologic hypertrophy, *$P<0.05$, n$≥6$ mice/group. See Table 3 for all morphometry values. D) Cardiomyocyte hypertrophy in WT HFD but not in E) Clock$^{Δ19/Δ19}$ mice, Masson’s trichrome stain (left), and quantification of cardiomyocyte cross sectional area (MCSA; right), *$P<0.05$, n=3 hearts/group with 100 cardiomyocytes quantified per heart. Data are presented as mean ± SEM.
**Fig. 4.** *Clock*Δ19/Δ19* mice are resilient to cardiac dysfunction, by echocardiography. A) Representative M-mode echocardiography images, and B) time series data, showing that *Clock*Δ19/Δ19* mice maintain normal left ventricular internal diastolic (LVIDd) and systolic (LVIDs) dimensions, and conserved % ejection fraction (EF) and % fractional shortening (FS) at baseline, 4, 8, 12, 16 and 24 weeks of study. *P*<0.05 WT HFD vs. WT SC, #*P*<0.05 *Clock*Δ19/Δ19* HFD vs. *Clock*Δ19/Δ19* SC, n=6 mice/group. Data are presented as mean ± SEM. See Table 2 for all echocardiography values.

**Fig. 5.** *Clock*Δ19/Δ19* mice are resilient to cardiac dysfunction, by *in vivo* hemodynamics. A) The *Clock*Δ19/Δ19* mice maintain normal contractility parameters (dP/dt<sub>max</sub>, dP/dt<sub>min</sub>), and maintain B) left ventricular end systolic (LVESP), C) diastolic (LVEDP) pressure, and D) LV diastolic time constant tau, whereas the WT HFD mice develop diastolic dysfunction. *P*<0.05, n=6 mice per group except n=5 for WT SC controls. Data are presented as mean ± SEM. See Table 3 for all hemodynamics values.

**Fig. 6.** Cardiac transcriptomics identifies oxidative stress pathways involved in resilience to cardiovascular disease. A) Heat map illustrating the 174 genes that changed in WT HFD versus *Clock*Δ19/Δ19* hearts (fold change ≥ 1.35; ZT07; see Table 4 and Supplemental Table S1, available at [https://doi.org/10.6084/m9.figshare.12855530](https://doi.org/10.6084/m9.figshare.12855530) for data values); B) gene ontology (GO) biological analysis; C) Circos mapping of links between the differentially regulated genes in the WT vs. *Clock*Δ19/Δ19* hearts in response to HFD, where red = up regulated and blue = down regulated with HFD in both genotypes; and D) *Clock*Δ19/Δ19* hearts show blunted expression of core circadian clock mechanism genes, as expected. *P*<0.001 genotype effect by two-way ANOVA, n=4 hearts/group. Data are presented as mean ± SEM.
**Fig. 7.** SIRT-dependent oxidative stress pathways and cardiac remodeling. **A)** Analysis of the SIRT-dependent oxidative stress pathway reveals increased cardiac *Nampt* expression in WT HFD mice, but no change in downstream pathway **B)** sirtuin (*Sirt*) or **C)** forkhead box (*Foxo*) genes or in **D)** manganese-dependent superoxide dismutase (*MnSOD*) protein abundance, ZT07, *P*<0.005, *n*=4 hearts/group. Data are presented as mean ± SEM.

**Fig. 8.** H$_2$O$_2$-dependent oxidative stress pathways and cardiac remodeling. **A)** 4-HNE shows increased abundance in WT HFD heart and liver by 24 weeks, but **B)** not in *Clock*$_{Δ19/Δ19}$ mice, whereas **C)** CAT is increased in both WT and **D)** *Clock*$_{Δ19/Δ19}$ hearts in response to HFD, but **E)** GPx is increased only in the *Clock*$_{Δ19/Δ19}$ HFD hearts, by Western blot analyses, ZT07, *P*<0.05, *n*=4 hearts/group. **F)** *Pparγ* mRNA expression is increased in *Clock*$_{Δ19/Δ19}$ hearts, by RT-PCR, ZT07, *P*<0.005, *n*=4 hearts/group. Data are presented as mean ± SEM.

**Fig. 9.** Schematic illustration showing that *Clock*$_{Δ19/Δ19}$ mice have obesity and metabolic syndrome, but reduced activation of adverse free radical pathways and increased antioxidant protection underlying their resilience to cardiovascular disease.
Table 1. Metabolic parameters in WT and ClockΔ19/Δ19 mice

| Metabolic Parameter       | WT        | ClockΔ19/Δ19 | P value |
|--------------------------|-----------|-------------|---------|
| Triglyceride (mg/dl)     | 76.83±4.46| 77.33±3.59  | n.s.    |
| Cholesterol (mg/dl)      | 135.25±10.31| 171.29±7.98*| <0.05   |
| Fasting glucose (mg/dl)  | 76.96±5.42| 97.30±3.09* | <0.01   |
| Insulin (ng/mL) – sleep time | 0.75±0.11 | 1.18±0.38  | n.s.    |
| Insulin (ng/mL) – wake time | 4.63±0.72 | 1.05±0.23* | <0.005  |

WT and ClockΔ19/Δ19 mice on an ad libitum standard chow diet (n=6-7/group), by Student’s t-test.
Fasting blood glucose was measured at ZT0. Triglycerides and cholesterol were determined from blood serum samples collected at ZT0. Insulin was measured from blood plasma samples collected at 4 h intervals over one 24 h period. Values are mean ± SEM.
Table 2. Clock\textsuperscript{Δ19/Δ19} mice are protected from cardiac remodeling following 24 weeks of HFD, by echocardiography analyses

|                      | Wild type SC | Wild type HFD | Clock\textsuperscript{Δ19/Δ19} SC | Clock\textsuperscript{Δ19/Δ19} HFD |
|----------------------|--------------|---------------|----------------------------------|----------------------------------|
| **Echocardiography - Baseline (8 weeks of age)** |              |               |                                  |                                  |
| LVIDd (mm)           | 3.88±0.04    | 3.96±0.04     | 3.91±0.03                        | 3.88±0.05                        |
| LVIDs (mm)           | 2.30±0.03    | 2.38±0.03     | 2.23±0.06                        | 2.25±0.05                        |
| EF (%)               | 77.84±0.63   | 76.90±0.28    | 79.92±1.07                       | 79.05±1.22                       |
| FS (%)               | 40.75±0.58   | 39.89±0.25    | 43.13±1.04                       | 42.22±1.37                       |
| IVSd (mm)            | 0.78±0.01    | 0.78±0.01     | 0.77±0.01                        | 0.77±0.02                        |
| LVPWd (mm)           | 0.74±0.02    | 0.74±0.01     | 0.73±0.02                        | 0.74±0.01                        |
| LV mass (mg)         | 83.22±2.84   | 86.25±2.28    | 82.63±1.61                       | 82.21±3.00                       |
| BW (g)               | 22.07±0.36   | 23.32±0.80    | 24.33±0.40                       | 24.72±0.56                       |
| HR (bpm)             | 470±10       | 484±10        | 467±5                            | 454±3                            |
| **4 weeks on diet (3 months of age)**     |              |               |                                  |                                  |
| LVIDd (mm)           | 3.97±0.03    | 3.94±0.04     | 3.94±0.04                        | 3.89±0.02                        |
| LVIDs (mm)           | 2.35±0.03    | 2.39±0.05     | 2.25±0.04                        | 2.25±0.03                        |
| EF (%)               | 77.84±0.38   | 76.47±0.58    | 79.96±0.75                       | 79.19±0.54                       |
| FS (%)               | 40.78±0.32   | 39.56±0.50    | 42.82±0.73                       | 42.06±0.54                       |
| IVSd (mm)            | 0.78±0.01    | 0.79±0.01     | 0.78±0.01                        | 0.79±0.01                        |
| LVPWd (mm)           | 0.76±0.01    | 0.76±0.01     | 0.76±0.01                        | 0.76±0.01                        |
| LV mass (mg)         | 87.71±1.07   | 87.61±1.42    | 87.11±2.79                       | 85.49±1.03                       |
| BW (g)               | 24.17±0.36   | 26.43±0.56**  | 26.78±0.42                       | 31.60±0.99##                     |
| HR (bpm)             | 472±13       | 505±11        | 460±14                           | 455±12                           |
| **8 weeks on diet (4 months of age)**      |              |               |                                  |                                  |
| LVIDd (mm)           | 3.98±0.01    | 4.08±0.03*    | 4.00±0.02                        | 3.99±0.03                        |
| LVIDs (mm)           | 2.37±0.02    | 2.56±0.03*    | 2.36±0.03                        | 2.37±0.05                        |
| EF (%)               | 77.33±0.57   | 73.57±0.79*   | 78.26±0.40                       | 77.47±1.06                       |
| FS (%)               | 40.44±0.45   | 37.09±0.63*   | 41.14±0.37                       | 40.50±0.96                       |
| IVSd (mm)            | 0.78±0.01    | 0.80±0.01     | 0.79±0.01                        | 0.80±0.01                        |
| LVPWd (mm)           | 0.76±0.01    | 0.77±0.01     | 0.76±0.01                        | 0.77±0.01                        |
| LV mass (mg)         | 88.10±0.59   | 93.71±1.73*   | 89.29±1.14                       | 90.57±1.24                       |
| BW (g)               | 26.43±0.71   | 31.62±0.83*** | 29.55±0.17                       | 36.15±1.48##                     |
| HR (bpm)             | 479±8        | 497±7         | 456±6                            | 478±8                            |
| **12 weeks on diet (5 months of age)**     |              |               |                                  |                                  |
| LVIDd (mm)           | 4.00±0.02    | 4.12±0.06*    | 4.04±0.02                        | 4.06±0.04                        |
| LVIDs (mm)           | 2.39±0.04    | 2.62±0.08*    | 2.40±0.03                        | 2.41±0.05                        |
| EF (%)               | 77.22±0.89   | 72.63±1.33*   | 77.49±0.44                       | 77.57±0.89                       |
| FS (%)               | 40.29±0.81   | 36.56±1.05*   | 40.49±0.39                       | 40.16±0.62                       |
| IVSd (mm)            | 0.79±0.01    | 0.80±0.01     | 0.79±0.01                        | 0.80±0.01                        |
| LVPWd (mm)           | 0.76±0.01    | 0.77±0.01     | 0.76±0.01                        | 0.78±0.01                        |
| LV mass (mg)         | 89.66±0.87   | 95.68±2.37*   | 91.19±0.76                       | 93.54±1.36                       |
| BW (g)               | 28.03±0.89   | 34.97±1.04*** | 31.67±0.62                       | 40.93±1.65###                    |
| HR (bpm)             | 484±9        | 487±7         | 472±7                            | 464±12                           |
16 weeks on diet (6 months of age)

| Variable          | WT HFD | WT SC | ClockΔ19/Δ19 HFD | ClockΔ19/Δ19 SC |
|-------------------|--------|-------|------------------|-----------------|
| LVIDd (mm)        | 4.05±0.03 | 4.34±0.08** | 4.13±0.02 | 4.17±0.02 |
| LVIDs (mm)        | 2.45±0.04 | 2.86±0.09** | 2.51±0.02 | 2.57±0.02 |
| EF (%)            | 76.09±0.84 | 69.46±1.49** | 75.82±0.52 | 74.89±0.62 |
| FS (%)            | 39.36±0.73 | 34.16±1.12** | 39.16±0.43 | 38.42±0.53 |
| IVSd (mm)         | 0.78±0.01 | 0.80±0.01   | 0.79±0.01 | 0.80±0.01 |
| LVPWd (mm)        | 0.76±0.01 | 0.78±0.01   | 0.78±0.01 | 0.78±0.01 |
| LV mass (mg)      | 91.21±1.22 | 104.60±3.26** | 95.64±0.57 | 98.10±1.32 |
| BW (g)            | 29.45±0.89 | 38.45±0.81*** | 35.35±0.79 | 44.78±1.79### |
| HR (bpm)          | 495±7    | 499±3   | 478±9 | 488±8 |

24 weeks on diet (8 months of age)

| Variable          | WT HFD | WT SC | ClockΔ19/Δ19 HFD | ClockΔ19/Δ19 SC |
|-------------------|--------|-------|------------------|-----------------|
| LVIDd (mm)        | 4.09±0.03 | 4.57±0.05*** | 4.17±0.04 | 4.35±0.04# |
| LVIDs (mm)        | 2.48±0.04 | 3.12±0.05*** | 2.53±0.05 | 2.74±0.04# |
| EF (%)            | 76.40±0.82 | 66.27±0.99*** | 76.09±0.87 | 73.12±1.04 |
| FS (%)            | 39.53±0.72 | 31.76±0.69*** | 39.32±0.75 | 36.92±0.83 |
| IVSd (mm)         | 0.78±0.01 | 0.81±0.01*** | 0.81±0.01 | 0.82±0.01 |
| LVPWd (mm)        | 0.76±0.01 | 0.79±0.01*** | 0.78±0.01 | 0.80±0.01 |
| LV mass (mg)      | 92.38±0.98 | 116.65±1.90*** | 100.00±1.41 | 108.88±1.85# |
| EDV (µl)          | 68.63±1.26 | 95.32±3.00*** | 72.43±1.87 | 82.35±2.55# |
| ESV (µl)          | 15.22±0.69 | 30.47±1.47*** | 16.26±0.98 | 21.15±1.06# |
| SV (µl)           | 53.41±0.91 | 64.85±2.04**  | 56.17±1.07 | 61.20±1.79# |
| CO (mL/min)       | 26.11±0.89 | 31.14±1.32**  | 26.12±0.55 | 29.08±1.13# |
| BW (g)            | 32.57±1.36 | 45.25±1.32*** | 37.13±0.97 | 51.38±1.67### |
| HR (bpm)          | 488±9    | 480±8   | 465±10 | 477±11 |

LVIDd, left ventricle (LV) internal diastolic dimension; LVIDs, LV systolic dimension; % EF, % ejection fraction; % FS, % fractional shortening; IVSd, interventricular septal wall at diastole; LVPWd, left ventricular posterior wall at diastole; BW, body weight; HR, heart rate; EDV, end diastolic volume; ESV, end systolic volume; SV, stroke volume; CO, cardiac output. n=6/group.
P<0.05, **P<0.01, ***P<0.001 WT HFD vs. WT SC, #P<0.05, ##P<0.01, ###P<0.001 ClockΔ19/Δ19 HFD vs. ClockΔ19/Δ19 SC by Student’s t-test. Values are mean±SEM.
Table 3. ClockΔ^{19/Δ^{19}} mice are protected from cardiac remodeling following 24 weeks of HFD, by hemodynamics and morphometry analyses

|                        | Wild type SC | Wild type HFD | ClockΔ^{19/Δ^{19}} SC | ClockΔ^{19/Δ^{19}} HFD |
|------------------------|--------------|---------------|------------------------|------------------------|
| **In vivo hemodynamics (24 weeks on diet)** |              |               |                        |                        |
| HR (bpm)               | 567±17       | 521±20        | 571±10                 | 543±27                 |
| SBP (mmHg)             | 97.70±1.19   | 97.76±0.80    | 96.60±0.78             | 98.05±0.61             |
| DBP (mmHg)             | 66.56±0.94   | 63.78±1.13    | 65.00±0.66             | 65.54±1.43             |
| MAP (mmHg)             | 76.17±0.78   | 74.36±0.79    | 74.78±0.50             | 75.62±1.11             |
| LVESP (mmHg)           | 98.27±0.87   | 98.45±1.33    | 100.84±0.73            | 100.27±1.60            |
| LVEDP (mmHg)           | -0.42±0.44   | 2.59±0.56**   | 0.01±0.37              | 1.36±0.63              |
| +dP/dt_{max} (mmHg/s)  | 10626±380    | 7756±287***   | 10042±527              | 9135±353               |
| -dP/dt_{min} (mmHg/s)  | -9789±231    | -7911±221***  | -9184±253              | -9234±500              |
| Tau (ms)               | 7.13±0.32    | 10.94±0.88**  | 7.33±0.41              | 8.54±0.50              |
| **Morphometry (24 weeks on diet)** |              |               |                        |                        |
| BW (g)                 | 32.57±1.36   | 45.25±1.32*** | 37.13±0.97             | 51.38±1.67###          |
| HW (mg)                | 132.71±3.50  | 145.57±2.66*  | 150.83±2.12            | 156.14±3.73            |
| HW:BW (mg/g)           | 4.12±0.13    | 3.36±0.15**   | 4.23±0.10              | 3.17±0.07###           |
| HW:TL (mg/mm)          | 6.63±0.16    | 7.26±0.13**   | 7.46±0.11              | 7.73±0.18              |
| eWAT (g)               | 1.10±0.08    | 1.93±0.15***  | 1.63±0.07              | 2.39±0.10###           |

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; LVESP, LV end systolic pressure; LVEDP, LV end diastolic pressure; dP/dt_{max} and dP/dt_{min}, maximum and minimum first derivative of LV pressure; Tau, LV diastolic time constant; HW, heart weight; TL, tibia length; eWAT, epididymal white adipose tissue weight. n=5-7/group. *P<0.05, **P<0.01, ***P<0.001 WT HFD vs. WT SC, #P<0.05, ##P<0.01, ###P<0.001 ClockΔ^{19/Δ^{19}} HFD vs. ClockΔ^{19/Δ^{19}} SC by Student’s t-test. Values are mean±SEM.
Table 4. ClockΔ19/Δ19 mice are resilient to HFD-induced changes in cardiac gene expression

| Gene Symbol | Gene Description | WT HFD vs. SC (fold change, mean ± SEM) | P Value | ClockΔ19/Δ19 HFD vs. SC (fold change, mean ± SEM) | P Value |
|-------------|------------------|----------------------------------------|--------|-----------------------------------------------|--------|
| **Circadian clock mechanism** | | | | | |
| Per3 | Period circadian clock 3 | 1.57 ± 0.10 | 0.017 | 1.07 ± 0.09 | 0.42 |
| Nr1d2 | Nuclear receptor subfamily 1, group D, member 2 | 1.47 ± 0.08 | 0.070 | 1.17 ± 0.04 | 0.027 |
| Per2 | Period circadian clock 2 | 1.44 ± 0.11 | 0.041 | 1.06 ± 0.07 | 0.75 |
| Arntl | Aryl hydrocarbon receptor nuclear translocator-like | -2.70 ± 0.29 | 0.030 | -1.18 ± 0.04 | 0.27 |
| Npas2 | Neuronal PAS domain protein 2 | -1.40 ± 0.05 | 0.040 | -1.23 ± 0.07 | 0.12 |
| **Cardiac genes** | | | | | |
| Ces1d | Carboxylesterase 1D | 1.76 ± 0.10 | 0.030 | -1.08 ± 0.05 | 0.74 |
| Wee1 | WEE1 homolog 1 (S. pombe) | 1.73 ± 0.18 | 0.0092 | 1.11 ± 0.05 | 0.24 |
| Egln3 | Egl-9 family hypoxia-inducible factor 3 | 1.45 ± 0.24 | 0.030 | 1.00 ± 0.09 | 0.61 |
| Bmp4 | Bone morphogenetic protein 4 | 1.45 ± 0.05 | 2.58E-04 | -1.11 ± 0.13 | 0.91 |
| Gpcpd1 | Glycero-phosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae) | 1.42 ± 0.12 | 0.012 | 1.13 ± 0.01 | 0.029 |
| Hdac4 | Histone deacetylase 4 | 1.39 ± 0.08 | 0.033 | 1.06 ± 0.05 | 0.59 |
| Xdh | Xanthine dehydrogenase | 1.39 ± 0.17 | 0.050 | 1.23 ± 0.10 | 0.024 |
| Me1 | Malic enzyme 1, NADP(+) dependent, cytosolic | 1.38 ± 0.10 | 1.55E-04 | 1.16 ± 0.03 | 0.037 |
| Fmo2 | Flavin containing monooxygenase 2 | 1.37 ± 0.15 | 0.022 | 1.15 ± 0.08 | 0.13 |
| Aldh1a1 | Aldehyde dehydrogenase family 1, subfamily A1 | 1.35 ± 0.13 | 0.016 | 1.16 ± 0.11 | 0.10 |
| Cdkn1a | Cyclin-dependent kinase inhibitor 1A (P21) | -2.46 ± 0.20 | 0.0015 | 1.04 ± 0.05 | 0.63 |
| Thbs1 | Thrombospondin 1 | -2.34 ± 0.36 | 0.0035 | -1.03 ± 0.23 | 0.99 |
| Nppb | Natriuretic peptide type B | -2.18 ± 0.24 | 2.87E-04 | -1.19 ± 0.07 | 0.73 |
| Sik1 | Salt inducible kinase 1 | -2.03 ± 0.23 | 0.011 | -1.02 ± 0.18 | 0.92 |
| Apold1 | Apolipoprotein L domain containing 1 | -1.99 ± 0.31 | 0.021 | -1.06 ± 0.24 | 0.91 |
| Errfi1 | ERBB receptor feedback inhibitor 1 | -1.89 ± 0.35 | 0.030 | -1.01 ± 0.11 | 0.93 |
| Nr4a2 | Nuclear receptor subfamily 4, group A, member 2 | -1.85 ± 0.18 | 0.0070 | -1.23 ± 0.29 | 0.45 |
| Nppa | Natriuretic peptide type A | -1.84 ± 0.21 | 0.013 | -1.08 ± 0.08 | 0.93 |
| Ugdh | UDP-glucose dehydrogenase | -1.82 ± 0.09 | 0.0023 | 1.05 ± 0.14 | 0.67 |
Microarray data of genes with ≥1.35-fold change in expression in WT HFD vs. WT SC hearts, yet showed conserved expression (<1.35-fold) in ClockΔ19/Δ19 HFD vs. SC.

| Gene ID | Gene Name | Expression in WT HFD vs. WT SC | P-value HFD vs. SC | Expression in ClockΔ19/Δ19 HFD vs. SC | P-value ClockΔ19/Δ19 HFD vs. SC |
|---------|-----------|--------------------------------|-------------------|----------------------------------------|-------------------------------|
| Ccl7    | Chemokine (C-C motif) ligand 7 | -1.81 ± 0.08 | 0.018 | -1.21 ± 0.20 | 0.31 |
| Fosl2   | Fos-like antigen 2 | -1.80 ± 0.17 | 0.011 | -1.09 ± 0.13 | 0.72 |
| Bcl6b   | B cell CLL / lymphoma 6, member B | -1.77 ± 0.04 | 0.0019 | -1.22 ± 0.07 | 0.22 |
| Ccl2    | Chemokine (C-C motif) ligand 2 | -1.75 ± 0.24 | 0.024 | -1.13 ± 0.30 | 0.76 |
| Cldn5   | Claudin 5 | -1.75 ± 0.14 | 1.51E-04 | 1.10 ± 0.14 | 0.34 |
| Tnrsf10b| Tumor necrosis factor receptor superfamily, member 10b | -1.67 ± 0.09 | 0.0014 | -1.07 ± 0.13 | 0.73 |
| Wsb1    | WD repeat and SOCS box-containing 1 | -1.58 ± 0.13 | 0.015 | -1.17 ± 0.06 | 0.091 |
| Rcan1   | Regulator of calcineurin 1 | -1.54 ± 0.11 | 0.025 | -1.18 ± 0.05 | 0.40 |
| Il2rg   | Interleukin 2 receptor, gamma chain | -1.53 ± 0.15 | 0.0056 | -1.23 ± 0.05 | 0.032 |
| Rhoj    | Ras homolog gene family, member J | -1.50 ± 0.08 | 4.70E-04 | -1.01 ± 0.04 | 0.73 |
| Phlda1  | Pleckstrin homology-like domain, family A, member 1 | -1.48 ± 0.04 | 0.0030 | -1.16 ± 0.10 | 0.34 |
| Adamts9 | A disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 9 | -1.48 ± 0.12 | 0.0077 | 1.04 ± 0.04 | 0.29 |
| Lgals3bp| Lectin, galactoside-binding, soluble, 3 binding protein | -1.47 ± 0.12 | 0.048 | -1.11 ± 0.08 | 0.33 |
| Tuba4a  | Tubulin, alpha 4A | -1.46 ± 0.14 | 0.0018 | -1.22 ± 0.12 | 0.29 |
| Cpxm2   | Carboxypeptidase X2 (M14 family) | -1.46 ± 0.28 | 0.011 | -1.05 ± 0.05 | 0.75 |
| Dusp1   | Dual specificity phosphatase 1 | -1.45 ± 0.12 | 0.033 | -1.06 ± 0.12 | 0.85 |
| Ctxla2b | Cytotoxic T lymphocyte-associated protein 2 | -1.44 ± 0.07 | 0.029 | 1.12 ± 0.15 | 0.61 |
| Lirr4b  | Leukocyte immunoglobulin-like receptor, subfamily B, member 4B | -1.44 ± 0.08 | 0.0028 | -1.21 ± 0.05 | 0.18 |
| Slc16a13| Solute carrier family 16 (monocarboxylic acid transporters), member 13 | -1.43 ± 0.08 | 0.0016 | -1.25 ± 0.07 | 0.015 |
| Fgf18   | Fibroblast growth factor 18 | -1.42 ± 0.12 | 0.0055 | -1.23 ± 0.08 | 0.17 |
| Has2    | Hyaluronan synthase 2 | -1.42 ± 0.07 | 0.0041 | 1.00 ± 0.08 | 0.76 |
| Egr2    | Early growth response 2 | -1.41 ± 0.03 | 0.0045 | -1.11 ± 0.04 | 0.20 |
| Gstp1   | Glutathione S-transferase, pi1 | -1.41 ± 0.10 | 5.65E-04 | -1.21 ± 0.09 | 0.21 |
Reitz et al – Fig. 1

A

Wild type

ClockΔ19/Δ19

B

WT

ClockΔ19/Δ19

Activity, counts/h (x10^2)

ZT0 12 24

C

% Activity

L D L D

WT ClockΔ19/Δ19

D

% Food Intake

L D L D

WT ClockΔ19/Δ19

E

% VO2

L D L D

WT ClockΔ19/Δ19

F

RER

L D L D

WT ClockΔ19/Δ19
Reitz et al – Fig. 2

A

WT SC  WT HFD
24 wks on Diet

B

![Graph showing body weight (g) vs diet (wks) and age (wks).](image)

C

WT

Body Weight (g) at 24 weeks on diet

D

ClockΔ19/Δ19

SC  HFD
24 wks on Diet

E

ClockΔ19/Δ19

SC  HFD
Body Weight (g) at 24 weeks on diet

F

ClockΔ19/Δ19

Body Weight (g) at 24 weeks on diet

G

eWAT (g)

|    | SC | HFD |
|----|----|-----|
| WT |    |     |
| ClockΔ19/Δ19 |    |     |

H

Energy Intake (kcal/day)

|    | SC | HFD |
|----|----|-----|
| WT |    |     |
| ClockΔ19/Δ19 |    |     |

I

Cholesterol (mg/dl)

|    | SC | HFD |
|----|----|-----|
| WT |    |     |
| ClockΔ19/Δ19 |    |     |

J

Fasting glucose (mg/dl)

|    | SC | HFD |
|----|----|-----|
| WT |    |     |
| ClockΔ19/Δ19 |    |     |

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Reitz et al – Fig. 3

A

B

C

D

Wild type

E

ClockΔ19Δ19

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A  

Weeks

WT SC  WT HFD  Clock\textsuperscript{Δ19/Δ19} SC  Clock\textsuperscript{Δ19/Δ19} HFD

LVID\textsubscript{d}  LVID\textsubscript{s}

B  

LVID\textsubscript{d} (mm)  LVID\textsubscript{s} (mm)  EF (%)  FS (%)

Clock\textsuperscript{Δ19/Δ19} HFD  Clock\textsuperscript{Δ19/Δ19} SC  WT HFD  WT SC

Weeks
Reitz et al – Fig. 5

A

\[
\frac{dP}{dt}_{\text{max}} \text{ (mmHg/s)} \times 10^3
\]

B

\[
\text{LVEDP (mmHg)}
\]

C

\[
\text{LVESP (mmHg)}
\]

D

\[
\text{Tau (msec)}
\]
Reitz et al – Fig. 6

A

|       | WT SC | ClockΔ19/Δ19 SC | HFD SC | HFD HFD |
|-------|-------|----------------|--------|--------|
| WT    |       |                |        |        |
| HFD   |       |                |        |        |

B

GO Biological Function

| Category          | # of entities |
|-------------------|---------------|
| Stress Remodeling | 180           |
| Transcription     | 90            |
| Metabolism        | 0             |

C

WT HFD vs. SC (174 genes)

ClockΔ19/Δ19

HFD vs. SC (41 genes)

D

Normalized Fluorescence Intensity

| Gene    | Normalized Fluorescence Intensity |
|---------|-----------------------------------|
| Arnt1   |                                  |
| Npas2   |                                  |
| Per2    |                                  |
| Per3    |                                  |
| Nr1d2   |                                  |

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Reitz et al – Fig. 9

Wild type

- Normal cardiovascular structure/function
- Metabolic dysfunction
- Obesity
- Cardiovascular Disease
  - ↑ Cardiac hypertrophy
  - ↓ Cardiac function

Clock$^{Δ19/Δ19}$

- Metabolic dysfunction
- Obesity
- HFD
- No cardiovascular disease

- ↑ Antioxidant activity
- ↓ Oxidative stress

O$_2^-$ → H$_2$O$_2$ →CAT→ H$_2$O

↑ Ppara → PPRE → Catalase

↑ Ppara → PPRE → GPx

= 4-HNE

O$_2^-$ → SOD→ H$_2$O$_2$ → CAT→ H$_2$O

GPx → H$_2$O