Cardiomyocyte-Specific Deletion of GCN5L1 Limits Cardiac Contractile Decline in Obese Pre-Diabetic Mice

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ABSTRACT

Healthy hearts use several fuel substrates for energy metabolism, with fatty acid oxidation (FAO) accounting for ~70% of total ATP production. Reduced substrate flexibility, and a shift towards increased fatty acid utilization, is a key feature of obesity and diabetes. We previously reported that increased acetylation of several mitochondrial FAO enzymes, regulated in part by increased abundance of the mitochondrial acetyltransferase GCN5L1, correlated with increased FAO enzyme activity in the heart. The focus of the current study was to investigate whether decreased acetylation, via cardiomyocyte-specific deletion of GCN5L1 (GCN5L1 cKO), regulates cardiac energy metabolism following exposure to a high fat diet (HFD). Excess dietary fat led to similar cardiac hypertrophy in wildtype and GCN5L1 cKO mice, however animals lacking cardiac GCN5L1 expression were protected from a reduction in cardiac workload. We show that GCN5L1 deletion led to decreased acetylation and FAO activity following a HFD. Furthermore, we show that acetylation of pyruvate dehydrogenase (PDH) was significantly reduced in HFD GCN5L1 cKO animals, which resulted in its increased enzymatic activity relative to HFD wildtype controls. Finally, the acetylation of both electron transport chain Complex I protein NDUFB8 and manganese superoxide dismutase 2 (SOD2) was significantly reduced by cardiac GCN5L1 deletion in HFD animals, resulting in decreased lipid peroxidation. In summary, we show that GCN5L1 deletion limits cardiac contractile decline observed in HFD mice through increased glucose oxidation potential and decreased reactive oxygen species damage.

INTRODUCTION

A healthy heart uses several fuel substrates to power contractile function [1]. Fatty acid oxidation (FAO) accounts for ~70% of total ATP production [2-4], with remaining ATP produced from other sources like glucose, lactose and ketones [5]. The ability of the heart to use various fuel substrates is lost in disease states such as diabetes, which features a shift towards increased fatty acid utilization and decreased glucose oxidation [6]. While controversy exists about the possible protective role of cardiomyocyte insulin resistance in the failing heart [7], the over reliance on fatty acids during diabetes can result in increased myocardial lipid accumulation and decreased cardiac efficiency [8-11]. The mechanisms underlying these metabolic changes remain to be fully uncovered.

Growing evidence points to a crucial role played by lysine acetylation, a reversible post-translational modification capable of regulating mitochondrial protein activity. Pougovkina et al. demonstrated that circulating free fatty acids, prevalent in obesity and diabetes, are the main source of acetyl-coA used for lysine acetylation [12]. Furthermore, we and others have shown that mitochondrial protein acetylation is significantly increased in various tissues during obesity [13-16]. Specifically in the heart, we have shown that an increase in acetylation results in increased activity of several FAO enzymes, which is regulated in part by the mitochondrial acetyltransferase GCN5L1 [16]. These findings place nutritional inputs, lysine acetylation, substrate utilization, and GCN5L1 at a key regulatory node in cardiac energy metabolism.

GCN5L1 is a mitochondrial matrix protein that has been shown to counter the activity of the mitochondrial deacetylase SIRT3. Specifically, it has been shown to regulate the acetylation of several mitochondrial FAO, glucose oxidation, and electron transport chain (ETC) proteins [15-17]. Loss of GCN5L1 has been shown to limit mitochondrial respiratory capacity [18], while increasing cardiac glucose utilization and reducing fatty acid utilization [16, 19]. While the role of GCN5L1 in regulating FAO and glucose oxidation enzyme acetylation has been studied *in vitro*, it is not known whether

cardiomyocyte-specific deletion of GCN5L1 results in decreased acetylation of mitochondrial proteins in response to a high fat diet (HFD) *in vivo*.

MATERIALS AND METHODS

Animal Care and Use

Animals were housed in the University of Pittsburgh animal facility under standard conditions with ad libitum access to water and food, and maintained on a constant 12h light/12h dark cycle. Male GCN5L1 WT and cKO animals were fed either a standard low fat chow diet (70% carbohydrate, 20% protein, 10% fat; Research Diets D12450B) or a high fat diet (HFD; 20% carbohydrate, 20% protein, 60% fat; Research Diets D12492) for 24 weeks. Experiments were conducted in compliance procedures approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Protein Isolation

For whole heart protein lysate, tissues were minced and lysed in CHAPS buffer on ice for ~2 hours. Homogenates were spun at 10,000 *g*, and supernatants collected for western blotting or co-immunoprecipitation experiments. For activity assays, muscle homogenates were prepared in a modified Chappell-Perry Medium A buffer (120 mM KCl, 20mM Hepes, 5 mM $MgCl_2$, 1 mM EGTA, ~pH 7.2) supplemented with 5 mg/ml fat-free bovine serum albumin. Mitochondria isolation was performed using the Qproteome Mitochondria isolation kit (Qiagen).

Western Blotting

Protein lysates were prepared in LDS sample buffer, separated using Bolt SDS/PAGE 4-12% or 12% Bis-Tris gels, and transferred to nitrocellulose membranes (All Life Technologies). Protein expression was analyzed using the following primary antibodies: rabbit LCAD, rabbit SCAD, and rabbit HADHA from Proteintech; rabbit acetyl-lysine (Ac-

K), rabbit glutamate dehydrogenase (GDH), and rabbit PDH from Cell Signaling Technologies; rabbit acetylated SOD2 (K122), rabbit SOD2, and rabbit OXPHOS cocktail to analyze NDUFB8, SDHB and UQCR2 protein content from Abcam. Fluorescent anti-mouse or anti-rabbit secondary antibodies (red, 700 nm; green, 800 nm) from LiCor were used to detect expression levels. Protein densitometry was measured using Image J software (National Institutes of Health, Bethesda, MD).

Co-Immunoprecipitation

For co-immunoprecipitation experiments, protein lysates were harvested in CHAPS buffer, and equal amounts of protein were incubated overnight at 4 ºC with rabbit acetyl-lysine antibody (AcK; Cell Signaling). Immunocaptured proteins were isolated using Protein-G agarose beads (Cell Signaling Technology), washed multiple times with CHAPS buffer and then eluted in LDS sample buffer at 95 ºC.

Isolated Working Heart

Cardiac contraction, relaxation and workload were calculated using the isolated working heart system as previously described [20].

Biochemical Assays

PDH activity and lipid peroxidation were measured using commercial kits (Sigma-Aldrich) according to manufacturer's instructions. Palmitoyl-CoA Dehydrogenase activity assay was carried out as previously described [14].

Statistics

Graphpad Prism software was used to perform statistical analyses. Means ± SE were calculated for all data sets. Data were analyzed using two-tailed Student's t-tests or

one-way ANOVA with Dunnett's post hoc tests as appropriate. $P < 0.05$ was considered significant.

RESULTS

Loss of GCN5L1 maintains cardiac contractility and workload under high fat conditions

HFD feeding resulted in a significant increase of body weight (Fig 1A), heart weight (Fig 1B) and heart weight:tibia length ratio (Fig 1C) in both WT and GCN5L1 cKO animals. Measurement of cardiac function *ex vivo* using an isolated working heart system showed that there were no significant changes in cardiac contractility (Fig 1D) and relaxation rate (Fig 1E) in WT and cKO animals under chow or HFD conditions. However, a significant decrease in normalized cardiac workload was observed in WT HFD animals compared to chow controls, which was not present in GCN5L1 cKO animals (Fig 1F). In conclusion, cardiac specific deletion of GCN5L1 abrogated the decrease in cardiac workload observed during HFD feeding.

Loss of GCN5L1 decreases acetylation and enzymatic activities of FAO proteins

We next examined the total level of mitochondrial protein acetylation in chow and HFD WT and GCN5L1 cKO animals. While significantly elevated relative to chow-fed WT mice, there was a ~67% decrease in mitochondrial acetylation in GCN5L1 cKO HFD mice relative to their WT HFD controls (Fig 2A,B). Immunoprecipitation pull down with an acetylated lysine antibody revealed a significant increase in the acetylation of FAO enzymes HADHA and LCAD, and a trend towards increased acetylation of SCAD ($P = 0.06$) levels in WT HFD animals (Fig 2C-E) as previously reported [13,15]. As anticipated, this increase in acetylation levels was attenuated in GCN5L1 cKO HFD animals (Fig 2C-E). We examined whether this decreased acetylation would affect FAO enzyme activity, and found that a significant increase in palmitoyl-CoA dehydrogenase activity in WT HFD mice was absent in animals lacking cardiac GCN5L1 expression (Fig

2F). In conclusion, cardiac-specific deletion of GCN5L1 resulted in decreased acetylation of FAO enzymes with high fat feeding, concomitantly reducing FAO activity *in vitro*.

GCN5L1 regulates acetylation and enzymatic activity of PDH

We next examined whether deletion of GCN5L1 had any effect on pyruvate dehydrogenase (PDH) acetylation and activity. Acetylation of PDH was significantly decreased in HFD GCN5L1 cKO animals when compared to their WT HFD controls (Fig 3A, B). Acetylation of PDH has been shown to inhibit its activity in the heart [21], and we observed a significant increase in PDH activity in GCN5L1 cKO HFD animals when compared to WT HFD controls (Fig 3C). Furthermore, regression analyses between PDH acetylation status and enzyme activity showed that there was a significant negative correlation between PDH activity and acetylation ($r^2 = 0.3352$, $P = 0.03$; Fig 3D). Based on these data, we conclude that loss of GCN5L1 in the heart may allow glucose oxidation activity to continue in obese, pre-diabetic mice by maintaining PDH activity.

Role of GCN5L1 in electron transport chain (ETC) complex acetylation and redox status

We next examined the role of GCN5L1 in the acetylation of ETC complex proteins, and found a significant increase in the acetylation of Complex I subunit NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8) in WT HFD animals (Fig 4A). This was completely attenuated in GCN5L1 cKO mice (Fig 4A). No significant changes were observed in acetylation of Complex II and III subunits SDHB and UQCR2, respectively (Fig 4B,C).

ETC Complex I is a major source of reactive oxygen species production [22]. We next investigated whether changes in the acetylation Complex I proteins impacted the redox milieu in GCN5L1 cKO mice. Deacetylation of mitochondrial superoxide dismutase 2

(SOD2) at lysine 122 (K122) has been shown to increase its activity [23]. With HFD, we observed a significant increase in acetylation (K122) of SOD2 in WT mice, which was reduced in GCN5L1 cKO animals (Fig 4D,E). Furthermore, we observed that an increase in lipid peroxidation in WT HFD animals was significantly attenuated in GCN5L1 cKO mice. Based on these findings, we conclude that acetylation of SOD2 is in part regulated by GCN5L1, and loss of this modification in GCN5L1 cKO animals results in decreased lipid peroxidation in response to HFD exposure.

DISCUSSION

We have previously shown that prolonged high fat feeding leads to increased acetylation of mitochondrial proteins, which was in part regulated by GCN5L1 *in vitro* [16]. In order to further substantiate the role played by GCN5L1 in regulating this acetylation profile following exposure to a HFD, we utilized a newly-described GCN5L1 cKO animal model [19]. As anticipated, we observed a significant decrease in mitochondrial protein acetylation in GCN5L1 cKO animals on a HFD. A number of studies have extensively reported the role of SIRT3-mediated mitochondrial protein deacetylation [14, 24, 25], however the role of GCN5L1 in this context is much less known. Upon further investigation of specific proteins targeted by GCN5L1, we found that increased acetylation of SCAD, LCAD, HADHA and PDH is greatly attenuated with deletion of GCN5L1 under HFD conditions. The relative decrease in acetylation following GCN5L1 increased PDH activity and decreased LCAD function, the latter result matching what we had previously found in HFD wildtype mice and *in vitro* cell culture studies [16].

Interestingly, increased acetylation of FAO enzymes in the heart has been found to increase their activity [13,16]. This is the opposite of what occurs in the liver, where FAO protein acetylation decreases enzymatic activity [14,15]. The cause of this discrepancy is currently unknown, and may reflect the different metabolic activities of the two organs, or different acetylation sites on the same protein leading to opposing

effects on enzyme activity. Further proteomic analyses of modified sites in these organs will be required to elucidate this effect further.

It was previously reported that ETC Complex I is one of the major sites of ROS production in the mitochondria [26-28]. Complex I deficiency has also been shown to increase mitochondrial protein acetylation and accelerate heart failure via SIRT3-mediated hyperacetylation [29]. Furthermore, in a streptozotocin induced type 1 diabetic model, enhanced mitochondrial protein lysine acetylation has been reported as a common consequence of increased FAO and Complex I defects, which could ultimately be responsible for metabolic inflexibility of the diabetic heart. These authors reported no change in mitochondrial deacetylase SIRT3, while the role of GCN5L1 was not examined [30]. We report here for the first time that mitochondrial GCN5L1 partly regulates the acetylation of the Complex I protein subunit NDUFB8, which may contribute to complex activity defects under HFD conditions.

Superoxide dismutase 2 (SOD2) is a major mitochondrial ROS scavenger that reduces superoxide levels [31]. It has been reported that SIRT3-mediated deacetylation of SOD2 at K122 results in its increased enzymatic activity [23]. As such, we examined if the mitochondrial acetyltransferase activity of GCN5L1 regulated this acetylation site in our study, and found that deletion of GCN5L1 resulted in significantly decreased acetylation of SOD2 K122 in HFD cKO animals (Fig 4D-E). This may contribute to the significantly decreased lipid peroxidation in GCN5L1 cKO HFD hearts (Fig 4F), and work is currently underway to elucidate the functional significance of GCN5L1 regulation of SOD2 activity in the heart.

Based upon our findings, we conclude that cardiomyocyte-specific deletion of GCN5L1 impacts the acetylation and activities of fuel substrate metabolism enzymes and ROS regulators, and loss of GCN5L1 expression in the heart protects from cardiac functional decline following exposure to nutrient excess. These findings further our growing appreciation of GCN5L1 as a mitochondrial regulator, and its subsequent role in controlling cardiac bioenergetics.

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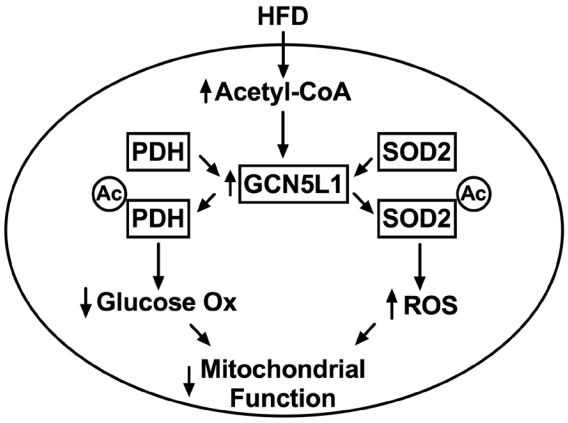
Graphical Abstract



GCN5L1 regulates mitochondrial function under high fat diet (HFD) conditions by acetylating pyruvate dehydrogenase (PDH) and manganese superoxide dismutase 2 (SOD2).

Figure 1

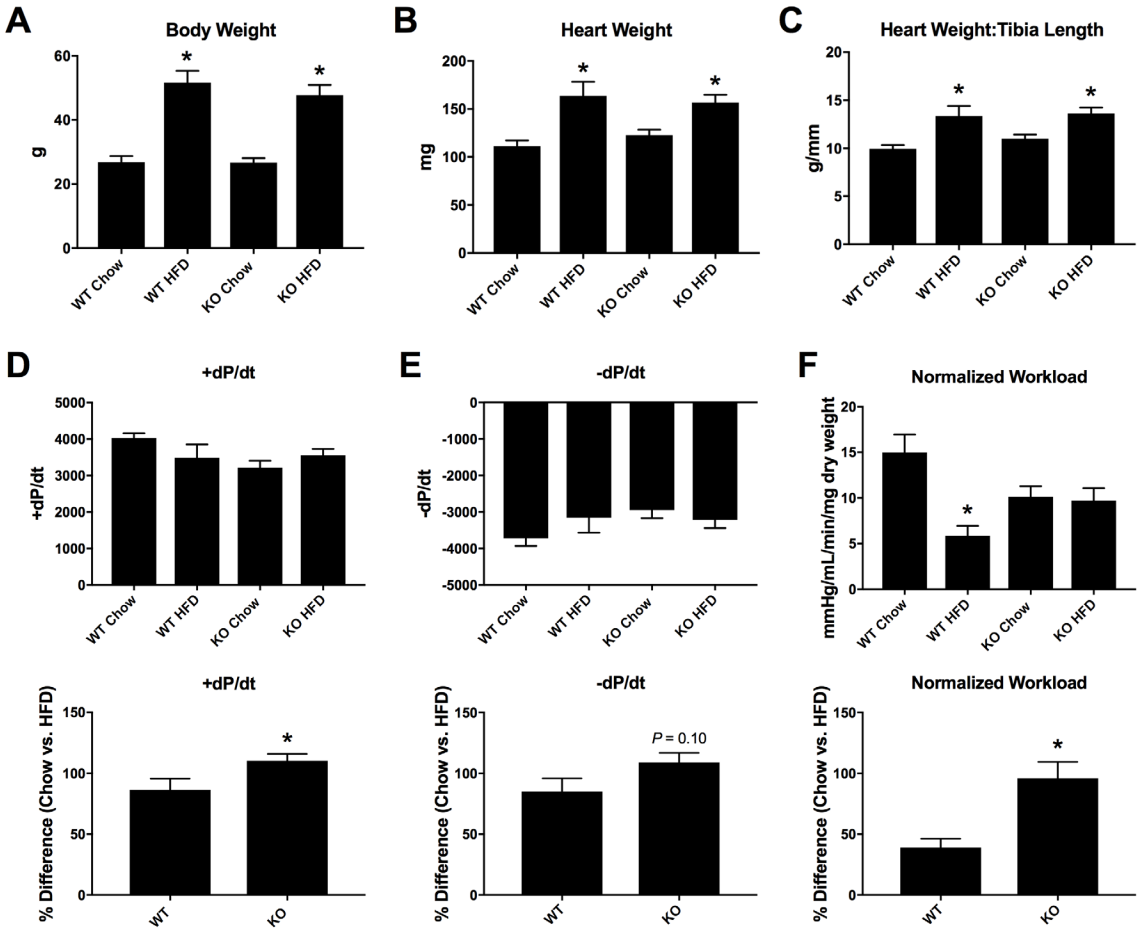
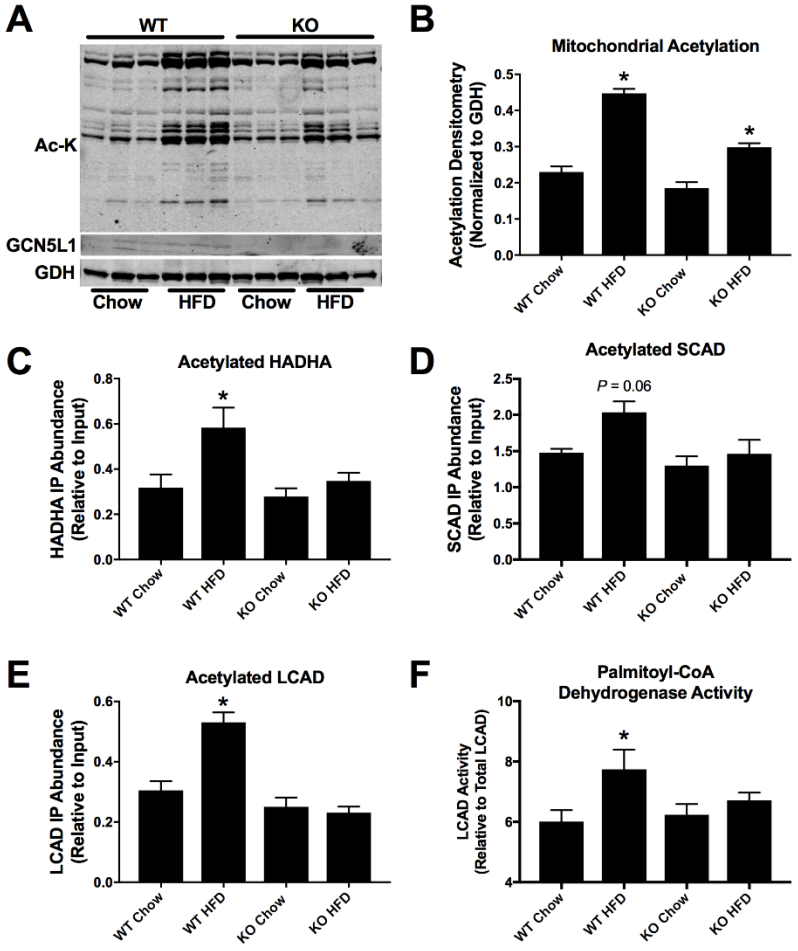

A
Body Weight
g
60
40
20
0
*
*
WT Chow
WT HFD
KO Chow
KO HFD
B
Heart Weight
mg
200
150
100
50
0
*
*
WT Chow
WT HFD
KO Chow
KO HFD
C
Heart Weight:Tibia Length
g/mm
20
15
10
5
0
*
*
WT Chow
WT HFD
KO Chow
KO HFD
D
+dP/dt
+dP/dt
5000
4000
3000
2000
1000
0
WT Chow
WT HFD
KO Chow
KO HFD
E
-dP/dt
-dP/dt
0
-1000
-2000
-3000
-4000
-5000
WT Chow
WT HFD
KO Chow
KO HFD
F
Normalized Workload
mmHg/mL/min/mg dry weight
20
15
10
5
0
*
WT Chow
WT HFD
KO Chow
KO HFD
+dP/dt
% Difference (Chow vs. HFD)
150
100
50
0
*
WT
KO
-dP/dt
% Difference (Chow vs. HFD)
150
100
50
0
P = 0.10
WT
KO
Normalized Workload
% Difference (Chow vs. HFD)
150
100
50
0
*
WT
KO

Figure 2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WT
KO
Ac-K
GCN5L1
GDH
Chow
HFD
Chow
HFD
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Mitochondrial Acetylation
Acetylation Densitometry (Normalized to GDH)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
WT Chow
WT HFD
KO Chow
KO HFD
C
Acetylated HADHA
HADHA IP Abundance (Relative to Input)
0.8
0.6
0.4
0.2
0.0
*
WT Chow
WT HFD
KO Chow
KO HFD
D
Acetylated SCAD
P = 0.06
SCAD IP Abundance (Relative to Input)
2.5
2.0
1.5
1.0
0.5
0.0
WT Chow
WT HFD
KO Chow
KO HFD
E
Acetylated LCAD
LCAD IP Abundance (Relative to Input)
0.6
0.4
0.2
0.0
*
WT Chow
WT HFD
KO Chow
KO HFD
F
Palmitoyl-CoA Dehydrogenase Activity
LCAD Activity (Relative to Total LCAD)
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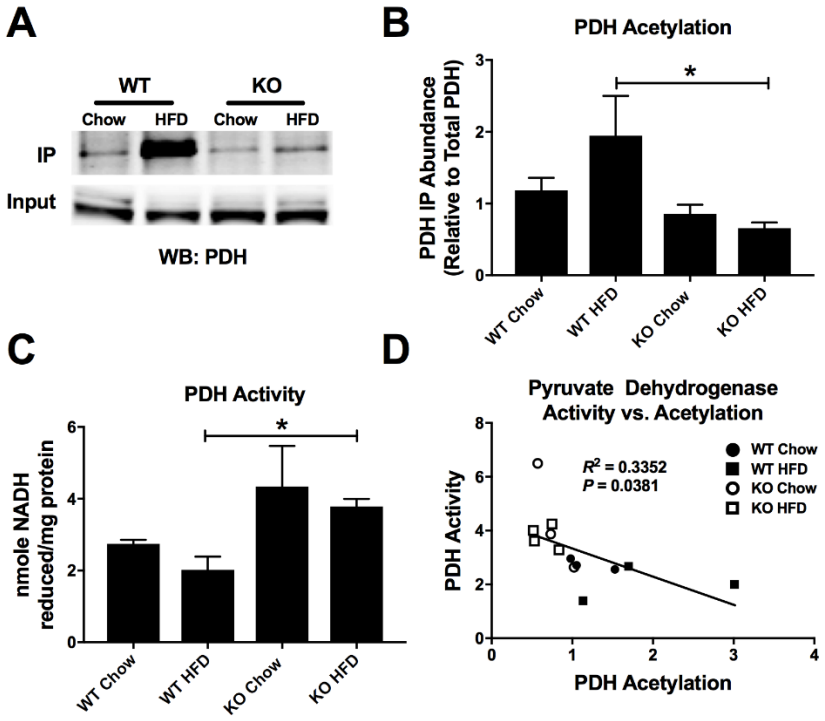
8
6
4
*
WT Chow
WT HFD
KO Chow
KO HFD

Figure 3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WT
KO
Chow
HFD
Chow
HFD
IP
Input
WB: PDH
B
PDH Acetylation
PDH IP Abundance
(Relative to Total PDH)
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WT Chow
WT HFD
KO Chow
KO HFD
C
PDH Activity
nmole NADH
reduced/mg protein
*
WT Chow
WT HFD
KO Chow
KO HFD
D
Pyruvate Dehydrogenase
Activity vs. Acetylation
PDH Activity
PDH Acetylation
R^2 = 0.3352
P = 0.0381
WT Chow
WT HFD
KO Chow
KO HFD

Figure 4

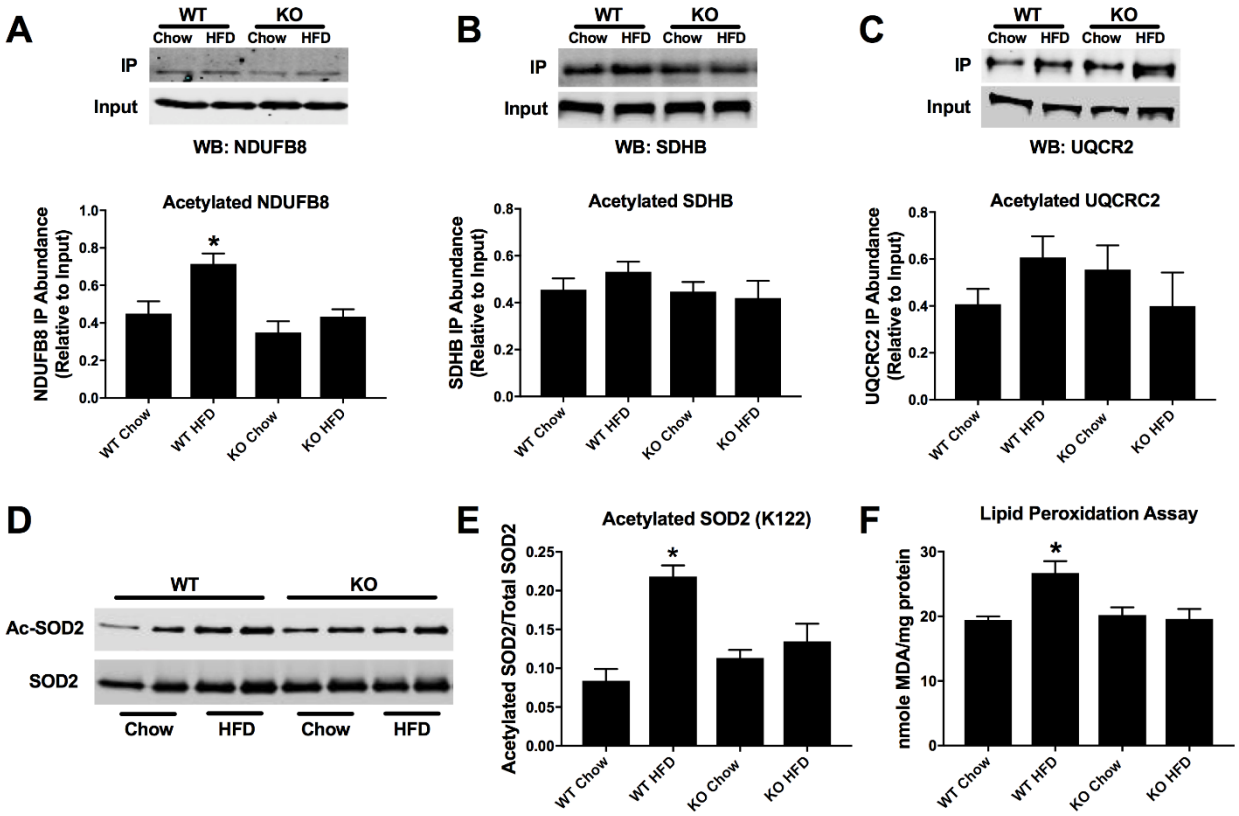
A
WT
KO
Chow HFD Chow HFD
IP
Input
WB: NDUFB8
Acetylated NDUFB8
NDUFB8 IP Abundance (Relative to Input)
WT Chow
WT HFD
KO Chow
KO HFD
B
WB: SDHB
Acetylated SDHB
SDHB IP Abundance (Relative to Input)
C
WB: UQCR2
Acetylated UQCRC2
UQCRC2 IP Abundance (Relative to Input)
D
Ac-SOD2
SOD2
Chow
HFD
E
Acetylated SOD2 (K122)
Acetylated SOD2/Total SOD2
F
Lipid Peroxidation Assay
nmole MDA/mg protein

FIGURE LEGENDS

Figure 1: Characterization of morphology and cardiac contractile function in GCN5L1 WT and cKO animals. Significant increases in (A) body weight, (B) heart weight, and (C) Heart weight:tibia length were observed in both WT and cKO HFD animals. Although no changes were observed in (D) contractility and (E) relaxation rate between the groups, there was a significant increase in (D) % change in contractility between chow and HFD cKO animals. (F) Normalized workload was significantly reduced in WT HFD animals, with no change observed in cKO mice. Values are expressed as means ± SEM, n = 5-6, * $P < 0.05$ vs WT Chow.

Figure 2: Mitochondrial protein acetylation and FAO activity in GCN5L1 WT and cKO animals. (A-B) Overall cardiac mitochondrial protein acetylation was significantly increased in HFD WT animals, which was greatly attenuated in HFD cKO mice. Immunoprecipitation pull down with acetylated lysine showed an increase in acetylation of (C) HADHA, (D) SCAD and, (E) LCAD in WT HFD animals, which was attenuated in HFD cKO mice. (F) Increased palmitoyl-CoA dehydrogenase activity in HFD WT mice was absent in cKO animals under the same conditions. Values are expressed as means ± SEM, n = 5-6, * $P < 0.05$ vs WT Chow.

Figure 3: Impact of GCN5L1 deletion on acetylation and activity of PDH in chow and HFD animals. (A-B) Increased PDH acetylation levels observed in HFD WT animals were absent in HFD cKO mice. Furthermore, (B) decreased acetylation of PDH correlated with (C) significantly increased activity of PDH. (D) Regression analyses showed a significant negative correlation between PDH acetylation status and enzymatic activity from all tested animals. Values are expressed as means ± SEM, n = 4-5, * $P < 0.05$.

Figure 4: Acetylation status of electron transport chain complex I-III proteins, SOD2, and lipid peroxidation. Acetylated lysine pull down showed an increase in the acetylation of (A) NDUFB8 in HFD WT animals relative to cKO mice under the same

conditions. There were no changes observed in the acetylation of (B) SDHB and (C) UQCR2. (D-E) Significant increases in acetylated SOD2 (K122) observed in WT HFD animals were absent in cKO mouse hearts. (F) Significant increases in lipid peroxidation observed with WT HFD mice were absent in cKO HFD mice. Values are expressed as means ± SEM, n = 5-6, * $P < 0.05$ vs WT Chow.