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Increased fatty acid oxidation enzyme activity in the hearts of mice fed a high fat diet does not correlate with improved cardiac contractile function

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ABSTRACT

Changes in the acetylation status of mitochondrial proteins have been linked to the development of metabolic dysfunction in a number of tissues. Increased lysine acetylation has been reported in the hearts of obese mice, and is associated with changes in fuel metabolism, redox status, and mitochondrial oxidative phosphorylation. In this study, we examined whether diet-induced changes in the acetylation of mitochondrial acyl-CoA dehydrogenases affected fatty acid oxidation enzyme activity and contractile function in the obese mouse heart. Exposure to a long-term high fat diet in wildtype mice led to the hyperacetylation of short- and long-chain acyl-CoA dehydrogenases SCAD and LCAD, which correlated with their increased enzymatic activity *in vitro*. Cardiomyocyte-specific deletion of the mitochondrial acetyltransferase-related protein GCN5L1 prevented both the hyperacetylation and increased activity of these enzymes under the same conditions of dietary excess. Despite the potential for increased cardiac fatty acid oxidation activity, wildtype mice did not display any increase in cardiac contractility following exposure to a high fat diet. We conclude that the potential energetic benefits of elevated fatty acid oxidation activity are not sufficient to counter the various deleterious effects of a high fat diet on cardiac function.

1. Introduction

Lysine acetylation, a reversible post-translational modification, has been detected on a wide range of proteins in several cellular compartments. Acetylation uses free acetyl groups from the metabolic breakdown of acetyl-CoA to modify the ε-position of the lysine side chain, and changes in protein acetylation status have been linked to the regulation of protein localization, enzymatic function, and turnover/stability (Carrico et al., 2018). In mitochondria, acetylation has been shown to regulate the function of fuel metabolism enzymes, redox regulatory components, and the electron transport chain (Scott, 2012; Trefely et al., 2020). While the removal of acetyl groups from mitochondrial protein lysine residues is dependent on the sirtuin deacetylase enzyme SIRT3, debate continues about whether lysine acetylation in the mitochondria is a non-enzymatic (Wagner and Payne, 2013) or enzymatic event (Scott et al., 2012). Using conditions that match the chemical composition of the mitochondrial matrix (a basic pH and high acetyl-CoA concentration), it was previously demonstrated that mitochondrial proteins can be chemically acetylated *in vitro* (Wagner and Payne, 2013). In contrast, work from our group and others has shown that GCN5L1, a mitochondria-localized acetyltransferase related protein, is required for the efficient acetylation of several mitochondrial metabolic proteins (Scott et al., 2012; Alrob et al., 2014; Fukushima et al., 2016; Thapa et al., 2017, 2018, 2020). While growing evidence suggests that GCN5L1 plays a role in the regulation of mitochondrial protein acetylation, its function in the heart under pathophysiological conditions remains poorly understood.

In the present study, we examined how exposure to a long-term high fat diet (HFD) affected the acetylation status and activity of fatty acid oxidation enzymes in the hearts of wildtype (WT) and cardiomyocyte-specific GCN5L1 knockout (GCN5L1 KO) mice. In addition, we examined whether changes induced by a HFD in these mice had an effect on cardiac contractility and relaxation using an *ex vivo* isolated working heart model. Our results suggest that GCN5L1-dependent acetylation of cardiac fatty acid oxidation enzymes improves their activity, but that this has little effect on overall cardiac contractile function.

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2. Materials and methods

2.1. Transgenic mice

Cardiomyocyte-specific inducible GCN5L1 knockout (GCN5L1 KO) mice used in the studies were generated as previously reported (Manning et al., 2019). Cardiomyocyte-specific GCN5L1 deletion was induced via tamoxifen injection (single 40 mg/kg IP injection), and confirmed by Western blot (see Supplemental Fig. 1).

2.2. Animal care and experimental diets

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 WT and GCN5L1 KO animals aged 10 weeks were fed either a standard low fat diet (LFD; 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. At the end of 24 weeks, animals were euthanized and heart tissues excised for analysis. Experiments were conducted in compliance with National Institutes of Health guidelines, and followed procedures approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

For tissue/biochemical studies, the group sizes were: WT LFD = 6, WT HFD = 5, KO LFD = 7, KO HFD = 10. For isolated working heart studies, the group sizes were: WT LFD = 5, WT HFD = 4, KO LFD = 6, KO HFD = 8.

2.3. Isolated working heart

Cardiac *ex vivo* contractility and relaxation were measured using a Harvard Apparatus ISHR isolated working heart system as previously described (Manning et al., 2019). Hearts from anesthetized mice were rapidly excised and cannulated via the aorta in warm oxygenated Krebs-Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 0.5 mM Na-EDTA [disodium salt dihydrate], 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 11 mM glucose). Retrograde (i.e. Langendorff) perfusion was initiated to blanch the heart, maintained at a constant aortic pressure of 50 mmHg with a peristaltic pump through a Starling resistor. A small incision was next made in the pulmonary artery to allow perfusate to drain, and the heart was paced at a rate slightly higher than endogenous (~360–500 bpm). The left atrium was then cannulated via the pulmonary vein, and anterograde perfusion was initiated with a constant atrial pressure of 11 mmHg against an aortic workload of 50 mmHg. Left ventricle pressure was measured via Mikro-tip pressure catheter (Millar) carefully inserted into the left ventricle through the aorta. The work-performing heart was permitted to equilibrate for 30 min to establish baseline functional parameters.

2.4. Protein isolation, western blotting, and immunoprecipitation

Tissues were minced and lysed in CHAPS buffer (1% CHAPS, 150 mM NaCl, 10 mM HEPES, pH 7.4) on ice for ~2 h. Homogenates were spun at 10,000 *g*, and supernatants were collected.

For 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 acetyl-lysine (Ac–K, #9441) from Cell Signaling Technology; rabbit long-chain acyl-CoA dehydrogenase (LCAD, #17526-1-AP) and rabbit short-chain acyl-CoA dehydrogenase (SCAD, #16623-1-AP) from Proteintech. GCN5L1 antibody was made by Covance and previously validated (Scott et al., 2012; Wang et al., 2017). Fluorescent anti-mouse or anti-rabbit secondary antibodies (red, 700 nm; green, 800 nm) from Li-Cor were used to detect expression levels. Protein densitometry was measured using Image J software (National Institutes of Health, Bethesda, MD).

For immunoprecipitation experiments, protein lysates were incubated overnight at 4 °C with rabbit Ac–K antibody. Immunocaptured proteins were isolated using Protein-G agarose beads (Cell Signaling Technology, #9007), washed multiple times with CHAPS buffer, and then eluted in LDS sample buffer (Life Technologies) at 95 °C. Samples were separated on 12% Bis-Tris Bolt gels and probed with appropriate antibodies. Protein densitometry was measured using Image J software (National Institutes of Health, Bethesda, MD).

2.5. Biochemical assays

To assess the activity of acyl-CoA dehydrogenase enzymes (SCAD and LCAD), homogenized protein samples were incubated with butyryl-CoA or palmitoyl-CoA as described previously (Thapa et al., 2017, 2018). Briefly, 25 μg of protein was incubated with 0.1 M potassium phosphate, 50 μM 2,6-dichlorophenolindophenol, 2 mM phenazine ethosulfate, 0.2 mM N-ethylmaleimide, 0.4 mM potassium cyanide, and 0.1% Triton X-100 at 37 °C for 4 min. The reaction was initiated with 60 μM acyl-CoA, and the rate of absorbance change was measured at 600 nm over 5 min. Activities were converted to moles of substrate oxidized/min/mass of protein against a standard curve.

2.6. Statistics

Graphpad Prism software was used to perform statistical analyses. Means ± SEM were calculated for all data sets. Data were analyzed using two-way ANOVA with Sidak's post-hoc testing to determine differences between genotypes and feeding groups. Data were analyzed with two-tailed Student's T-Tests to determine differences between single variable groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cardiomyocyte-specific deletion of GCN5L1 prevents high fat diet-induced hyperacetylation of mitochondrial fatty acid oxidation enzymes

After 24 weeks of HFD, both WT and GCN5L1 KO mice displayed significant increases in body weight and heart weight relative to mice on a LFD (Fig. 1A and B). However, there were no differences in either weight parameter between the two genotypes under the same dietary conditions (Fig. 1A and B). There was no difference between the two genotypes in heart weight:body weight ratios (Fig. 1C). Immunoprecipitation of cardiac lysates with an acetylated lysine antibody revealed that there was a significant increase in the acetylation status of the acyl-CoA dehydrogenase enzymes LCAD and SCAD in WT mice on a HFD relative to LFD controls (Fig. 2A–D). In contrast, there was no detectable increase in LCAD or SCAD acetylation in HFD-fed GCN5L1 KO mice compared to LFD controls (Fig. 2A–D). From these data, we conclude that excess dietary fat intake results in the GCN5L1-dependent hyperacetylation of mitochondrial fatty acid oxidation enzymes.

3.2. GCN5L1-dependent hyperacetylation of fatty acid oxidation enzymes correlates with their increased activity in vitro

Our group and others have previously demonstrated that increased acetylation of cardiac fatty acid oxidation enzymes is correlated with an increase in their enzymatic activity (Alrob et al., 2014; Thapa et al., 2017). However, the role of GCN5L1 in this context has not been investigated beyond cell culture models. To address this, we examined whether cardiomyocyte-specific deletion of GCN5L1 counters the effects of a HFD on fatty acid oxidation enzyme activity. In isolated cardiac lysates, the activity of LCAD and SCAD was significantly increased in HFD-fed WT mice relative to LFD controls (Fig. 3A and B). However, this

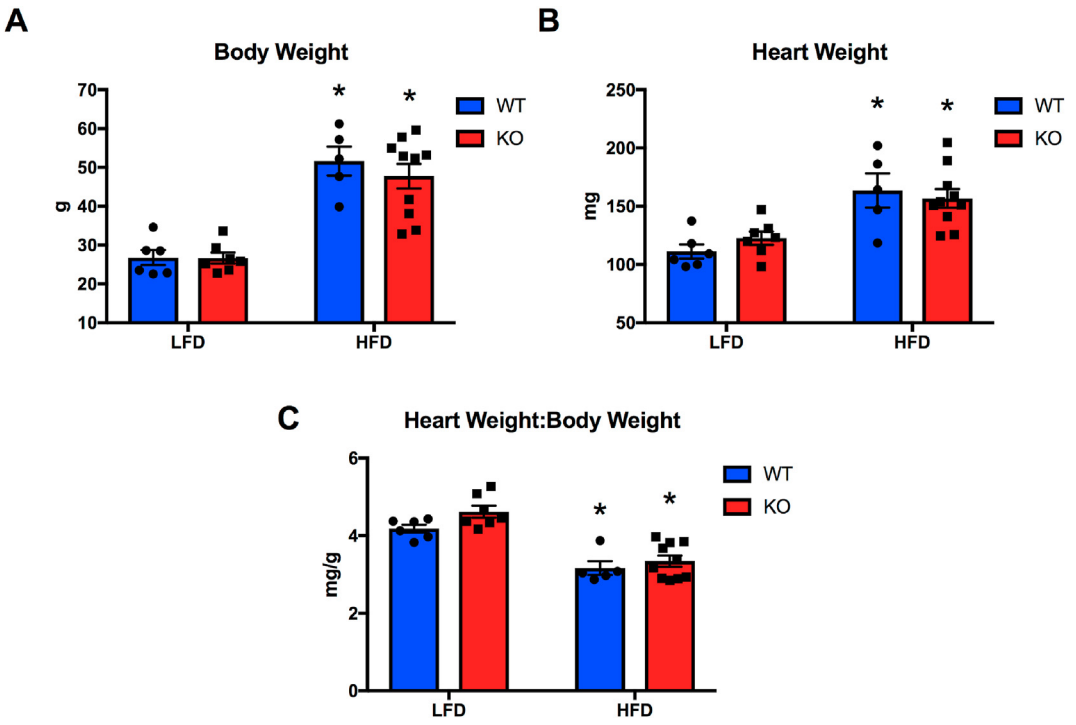

Fig. 1. Physical characteristics of WT and cardiac-specific GCN5L1 KO mice. A 24 week HFD led to significant increases in (**A**) body weight and (**B**) heart weight in both WT and GCN5L1 KO mice. There was no change between genotypes in heart weight relative to body weight (**C**), suggesting that cardiac size changes are independent of GCN5l1 expression levels. N = 5–10, * = $P < 0.05$ vs. WT LFD.

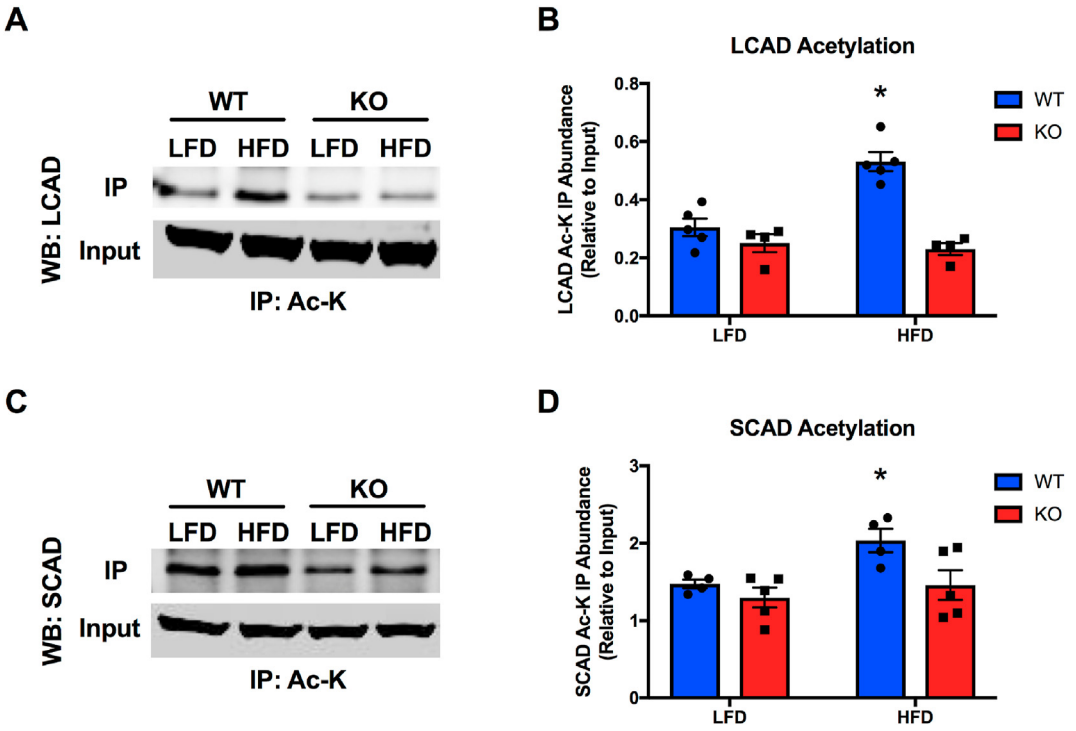

Fig. 2. Loss of GCN5L1 expression prevents HFD-induced fatty acid oxidation enzyme hyperacetylation. The acetylation status of (**A, B**) LCAD and (**C, D**) SCAD was significantly increased in the hearts of WT mice, but not GCN5L1 KO mice, after a 24 week HFD. N = 4–5, * = $P < 0.05$ vs. WT LFD.

relative increase was absent in mice lacking cardiac GCN5L1 expression, and the activity of LCAD and SCAD in these mice was not significantly different between LFD and HFD states (Fig. 3A and B). From these data, we conclude that GCN5L1-dependent hyperacetylation is necessary to promote the increased fatty acid oxidation enzyme activity observed in HFD-exposed mouse hearts.

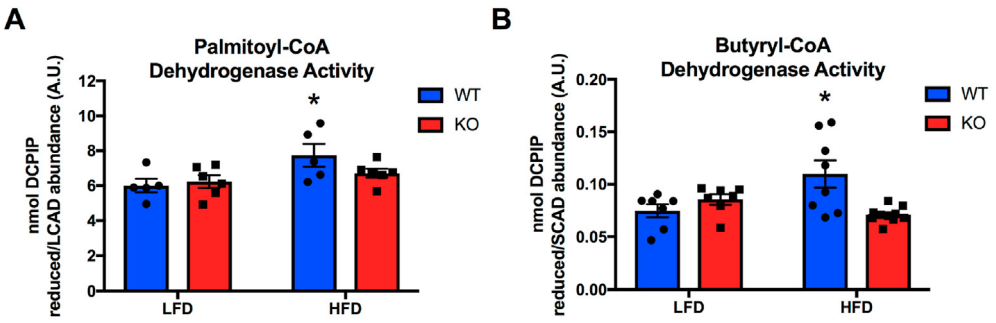

Fig. 3. Fatty acid oxidation activity is increased in isolated cardiac lysates from HFD-fed WT mice. The enzymatic activity of (**A**) LCAD and (**B**) SCAD was significantly increased in cardiac lysates from WT mice, but not GCN5L1 KO mice, after a 24 week HFD. N = 5–10, * = $P < 0.05$ vs. WT LFD.

3.3. Increased cardiac fatty acid oxidation enzyme activity does not result in improved cardiac contractility after a high fat diet

Finally, we examined whether potential increases in the enzymatic activity of cardiac fatty acid oxidation enzymes would benefit cardiac contractile function. Using an *ex vivo* isolated working heart apparatus, we measured the contractility and relaxation capacity of WT and GCN5L1 KO hearts under LFD and HFD conditions. Despite an increased capacity to oxidize fatty acids under HFD conditions, WT mice did not display any improvement in cardiac functional parameters relative to LFD controls (Fig. 4A and B). Furthermore, we did not detect any significant difference in cardiac contractility between WT and GCN5L1 KO mice under LFD or HFD conditions. From these data, we conclude that an increase in the activity of mitochondrial fatty acid oxidation enzymes does not confer a functional benefit to hearts exposed to a HFD.

4. Discussion

4.1. General discussion

The role of lysine acetylation in the regulation of metabolism in different tissues remains subject to continued debate. In the liver, hyperacetylation of fatty acid oxidation enzymes has been linked to a decrease in their enzymatic function, leading to metabolic dysfunction and the development of hepatic steatosis (Bao et al., 2010; Hirschey et al., 2011; Kendrick et al., 2011). In the heart, our group and others have demonstrated that hyperacetylation of beta oxidation proteins improves their enzymatic function *in vitro*, and increases rates of fatty acid oxidation *ex vivo* (Alrob et al., 2014; Thapa et al., 2017). Furthermore, these studies have shown that acetylation of the same enzymes (e.g. LCAD, HADHA) in different tissues have opposite effects on enzyme function in different tissues (Alrob et al., 2014; Thapa et al., 2017, 2018). Work is currently ongoing to understand whether the acetylation of different lysine residues in each tissue is responsible for these conflicting results, or whether the metabolic differences of each tissue result in a context-dependent effect of lysine acetylation.

In this study, we report that the cardiac fatty acid oxidation enzymes LCAD and SCAD are subject to GCN5L1-dependent hyperacetylation in response to a HFD, which correlates with an increase in their enzymatic activity *in vitro*. These findings from mouse cardiac tissues match previous work performed in cardiac cell culture models (Fukushima et al., 2016; Thapa et al., 2017). However, increased fatty acid oxidation in WT hearts does not improve contractile function *ex vivo* relative to GCN5L1 KO hearts, suggesting that the other deleterious effects of a HFD may negate the potential energetic benefits. These include an increase in reactive oxygen species damage, and the potential bioenergetic defects that result from hyperacetylation of mitochondrial electron transport chain complex proteins, both of which are reduced in the absence of cardiac GCN5L1 expression (Thapa et al., 2020).

The lack of a difference in *ex vivo* contractile function between hearts containing hyperacetylated mitochondria (HFD-fed WT mice) and hypoacetylated mitochondria (LFD-fed WT mice and GCN5L1 KO mice on either diet) mirrors recent findings in the failing heart, which showed that mice with genetically-elevated levels of mitochondrial protein acetylation displayed no bioenergetic or functional deficits relative to WT mice (Davidson et al., 2020). In this study, mice with striated muscle-specific deletion of both SIRT3 (the major mitochondrial deacetylase) and CrAT (a carnitine acetyltransferase that buffers the mitochondria acetyl-CoA pool) displayed similar mitochondrial function relative to WT, and these deletions did not exacerbate the effects of pressure overload on cardiac function *in vivo* (Davidson et al., 2020).

Interestingly, at the early stages of pressure overload, SIRT3/CrAT double knockout mice displayed improved survival rates relative to WT mice (Davidson et al., 2020). SIRT3 KO mice have previously been shown to have increased fatty acid oxidation rates (Alrob et al., 2014), and it was recently shown that increased beta oxidation protects the heart from hypertrophy by blocking the use of glucose for anaplerosis (Ritterhoff et al., 2020). As such, while elevated fatty acid oxidation resulting from

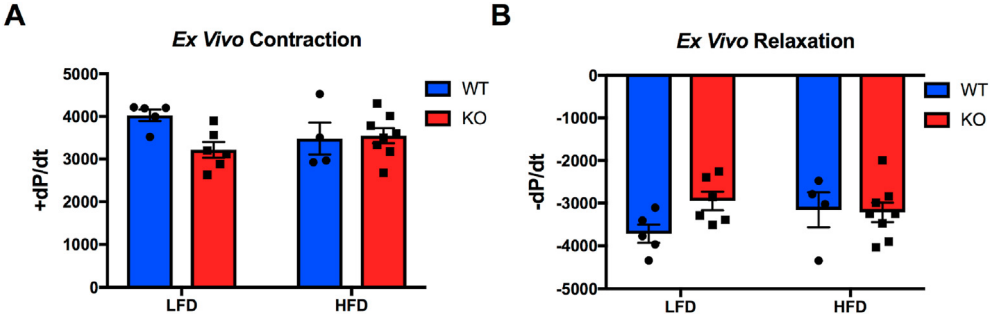

Fig. 4. Increased fatty acid oxidation activity in HFD-fed WT mice does not result in improved cardiac function *ex vivo*. WT mice fed a 24 week HFD did not demonstrate improved cardiac (**A**) contractility or (**B**) relaxation relative to GCN5L1 KO mice under the same conditions. N = 4–8.

enzyme hyperacetylation may not have beneficial effects on cardiac contractile function, improved use of fats via this mechanism may have a protective effect against some forms of cardiac insult. Further work will be required to determine whether changes in mitochondrial protein acetylation have a protective or deleterious effect on other aspects of cardiac health in response to different etiological stressors. This will be particularly important to understand the physiological differences caused by accumulation of acetyl-CoA (the co-factor for acetylation) resulting from nutritional excess, and changes in acetylation status caused by mechanical or hemodynamic stress. These two pressures may result in similar outcomes in terms of gross changes in acetylation, but may affect metabolism in different ways at the molecular level.

4.2. Study limitations

Our study contained three principal limitations that should be noted: (1) The perfusion buffer used in the functional studies contained only glucose, and not a physiological mix of fats/carbohydrates/proteins. While the buffer used did not contain fat, previous studies have shown that isolated working rodent hearts can use endogenous triglyceride-derived fats for several hours, and are not limited in their fuel substrate choice when perfused with glucose alone (Saddik and Lopaschuk, 1991). (2) The biochemical studies used whole heart tissue, as opposed to isolated cardiomyocytes. As our GCN5L1 knockout is cardiomyocyte specific, its continued expression in other cardiac cell types may provide a confounding effect. Future studies using isolated adult cardiomyocytes may be useful to address this issue. (3) Our studies used only male mice, and therefore these results may not be applicable to female animals. These limitations will be addressed in future studies.

4.3. Conclusions

We conclude that GCN5L1 regulates fuel metabolism enzyme acetylation in the mouse heart in response to a HFD, but that *in vitro* FAO capacity does not necessarily translate into improved cardiac function. More *in vivo* studies to address this discrepancy are warranted.

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Author contributions

DT and IS conceived the study; DT, JRM, MWS, and MZ obtained data; DT analyzed data; DT, BASM, and IS wrote or edited the manuscript.

CRediT authorship contribution statement

Dharendra Thapa: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **Janet R. Manning:** Funding acquisition, Investigation, Methodology. **Bellina A.S. Mushala:** Writing - review & editing. **Michael W. Stoner:** Investigation, Methodology. **Manling Zhang:** Investigation, Methodology. **Iain Scott:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Roles, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2020.11.001.

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