Cardiac Myocyte KLF5 Regulates Ppara Expression and Cardiac Function

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ABSTRACT

**Rationale:** Fatty acid oxidation is transcriptionally regulated by peroxisome proliferator-activated receptor (PPAR)α and under normal conditions accounts for 70% of cardiac ATP content. Reduced Ppara expression during sepsis and heart failure leads to reduced fatty acid oxidation and myocardial energy deficiency. Many of the transcriptional regulators of Ppara are unknown.

**Objective:** To determine the role of Krüppel-like factor 5 (KLF5) in transcriptional regulation of Ppara.

**Methods and Results:** We discovered that KLF5 activates Ppara gene expression via direct promoter binding. This is blocked in hearts of septic mice by c-Jun, which binds an overlapping site on the Ppara promoter and reduces transcription. We generated cardiac myocyte-specific Klf5 knockout mice that showed reduced expression of cardiac Ppara and its downstream fatty acid metabolism-related targets. These changes were associated with reduced cardiac fatty acid oxidation, ATP levels, increased triglyceride accumulation and cardiac dysfunction. Diabetic mice showed parallel changes in cardiac Klf5 and Ppara expression levels.

**Conclusions:** Cardiac myocyte KLF5 is a transcriptional regulator of Ppara and cardiac energetics.

**Keywords:** KLF5, PPARα, fatty acids, heart, diabetic cardiomyopathy.
### Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>FAO</td>
<td>Fatty acid oxidation</td>
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<td>FA</td>
<td>fatty acid</td>
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<td>TG</td>
<td>triglycerides</td>
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<td>PPARα</td>
<td>peroxisome proliferator-activated receptor α</td>
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<td>KLF</td>
<td>Krüppel-like factor</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>LPS</td>
<td>lipopolysaccharides</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>SGLT</td>
<td>sodium/glucose cotransporter</td>
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<td>ASO</td>
<td>anti-sense oligonucleotides</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>αMHC</td>
<td>alpha myosin heavy chain</td>
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<tr>
<td>Acot</td>
<td>acyl-CoA thioesterase</td>
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<tr>
<td>Serpina</td>
<td>serine (or cysteine) peptidase inhibitor, clade A</td>
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<tr>
<td>Phka1</td>
<td>phosphorylase kinase α1</td>
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<td>PGC</td>
<td>PPARγ coactivator</td>
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<tr>
<td>Acox</td>
<td>acyl-CoA oxidase</td>
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<tr>
<td>Cpt1b</td>
<td>carnitine palmitoyl-transferase</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>Lpl</td>
<td>lipoprotein lipase</td>
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<tr>
<td>Dgat1</td>
<td>diacylglycerol acyltransferase</td>
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<tr>
<td>Plin</td>
<td>perilipin</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>Lcad</td>
<td>long chain acyl-CoA dehydrogenase</td>
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<tr>
<td>Vlcad</td>
<td>very long chain acyl-CoA dehydrogenase</td>
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<tr>
<td>Glut</td>
<td>glucose transporter</td>
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<tr>
<td>PDK</td>
<td>pyruvate dehydrogenase kinase</td>
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<td>UCP</td>
<td>uncoupling protein</td>
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INTRODUCTION

Fatty acid oxidation (FAO) accounts for the production of approximately 70% of the ATP that the heart utilizes\(^1\). Some forms of heart failure are due to perturbations in heart energetics and severe heart failure is associated with energy starvation and reprogramming of cardiac energetics\(^2\). These metabolic changes occur regardless of whether the primary cause of cardiac dysfunction is metabolic disease, pressure overload or ischemia\(^3,4\). A dramatic example of cardiac dysfunction due to reduction in FAO\(^5\) and energy depletion occurs in sepsis\(^5,6\). The transcriptional mechanisms that underlie inhibition of cardiac FAO and cardiac dysfunction during sepsis and other types of cardiac dysfunction are incompletely understood.

Cardiac FAO is regulated at several stages: FA uptake, triglyceride (TG) formation and storage in lipid droplets, TG lipolysis leading to release of unesterified fatty acids, and transfer of fatty acids into the mitochondria for FAO and ATP production. Most of the proteins that participate in this cascade are transcriptionally regulated by peroxisome proliferator-activated receptor \(\alpha\) (PPAR\(\alpha\))\(^8\). Although it is known that PPAR\(\alpha\) is activated by FAs that are released via lipolysis from the intracellular triglyceride pool\(^9,10\), the transcriptional regulation of \(Ppara\) is not fully elucidated. Various gain or loss of PPAR\(\alpha\) function animal models resulted in mixed outcome with either protective or aggravating roles of PPAR\(\alpha\) in cardiac function. A variety of metabolic and pathological stress conditions influence cardiac PPAR\(\alpha\) expression in multiple ways, which are not fully defined.

Metabolism in several tissues is regulated by members of the Krüppel-like factor (KLF) protein family, which regulate proliferation, differentiation, development, and cell death\(^11\). Thus, 17 KLF isoforms have been identified in humans and mice, while several homologs were described in other species\(^11\). Adipocyte KLF2\(^12\), KLF3\(^13\) and KLF7\(^14\) inhibit adipose tissue development. On the other hand, KLF4\(^15\), KLF6\(^16\) and KLF15\(^17\) have the opposite effect in adipocytes, as they induce \(Pparg\) and adipogenesis. Hepatic KLF11 induces \(Ppara\) and FAO genes and prevents hepatic TG accumulation\(^18\). KLF15 promotes lipid utilization in the heart\(^19\) and skeletal muscle\(^20\). Thus, several KLF isoforms have been implicated in the regulation of metabolic pathways in several organs including the heart.

KLF5 is involved in pressure overload-mediated cardiac hypertrophy, but its role in cardiac metabolism remains unknown. Heterozygote \(Klf5\)^\text{+/-}\ mice are protected from pressure overload cardiac hypertrophy\(^21\), due to reduced transforming growth factor (TGF)\(\beta\) production in cardiac fibroblasts and not because of changes in cardiac myocytes\(^22\). Heterozygote \(Klf5\)^\text{+/-}\ mice showed increased skeletal muscle FA consumption due to activation of PPAR\(\delta\)\(^23\), suggesting that KLF5 is an inhibitor of lipid catabolism. Conversely, \(Klf5\) deletion inhibited lipid production in lung surfactant\(^24\), indicating that KLF5 is a positive regulator of lipid homeostasis in lungs. Thus, the actions of KLF5 in lipid metabolism vary depending on its site of expression.

We focused on the role of KLF5 in the regulation of cardiac metabolic gene expression. Unexpectedly, we first discovered that \(Klf5\) gene expression was induced in energy-depleted hearts of mice treated with \(E. coli\) lipopolysaccharides (LPS) that had lower \(Ppara\) expression. Although this observation implicated cardiac KLF5 in \(Ppara\) and FAO inhibition, our subsequent studies showed the opposite. We created a cardiac myocyte-specific \(Klf5\)^\text{-/-} mouse and conducted gain-of-function experiments in cardiac myocytes that revealed KLF5 to be a transcriptional activator of \(Ppara\). Klf5 ablation in cardiac myocytes reduced cardiac FAO and ATP content, increased TG accumulation and caused cardiac dysfunction. Furthermore, cardiac KLF5 was reduced in the early stages of Type 1 and in Type 2 diabetes mouse models along with \(Ppara\) gene expression. Thus, KLF5 is a novel regulator of \(Ppara\) and cardiac lipid utilization.
METHODS

Expanded Methods are presented in the Online Data Supplement. All animal studies were approved by the institutional animal care and use committees. Data are expressed as the mean ± SEM. Statistical significance was assessed with t-test or 1-way ANOVA followed by Bonferroni post hoc tests, performing all pairwise comparisons. A p-value of less than 0.05 was considered statistically significant.

Wild type C57BL/6 mice were treated with E. coli LPS to mimic sepsis. Microarrays for cardiac mRNA of LPS-treated mice was performed by Ocean Ridge Biosciences. The data are deposited in the Gene Expression Omnibus database (GSE63920). HL-1 cells were infected with adenoviruses expressing constitutively active c-Jun or KLF5 and were harvested 48h post-infection for gene expression and chromatin immunoprecipitation (ChIP).

We generated mice with cardiac myocyte-specific Klf5 gene deletion (aMHC-Klf5−/−). Cardiac function was assessed by 2D echocardiography. The microarray analysis for cardiac mRNA obtained from αMHC-Klf5−/− mice was performed by Arraystar (data deposited in GSE63839).

C57BL/6 mice were injected IP with streptozotocin (STZ) to mimic Type 1 diabetes (insulin dependent). Inhibition of sodium/glucose cotransporter (SGLT)2 in diabetic mice was performed either via treatment with dapagliflozin (drinking water) or with SGLT2 anti-sense oligonucleotides (ASO, ISIS Pharmaceuticals).

RESULTS

In silico Ppara promoter analysis identified two overlapping potential AP-1 and KLF binding sites.

We showed previously that LPS-mediated activation of the c-Jun N-terminal kinase (JNK) signaling pathway reduces cardiac Ppara gene expression. Aiming to identify potential binding sites for the substrate of JNK, c-Jun (AP-1 sites), on the mouse Ppara gene promoter we performed in silico promoter analysis (Genomatix software). This analysis identified two potential AP-1 sites in the anti-sense strand of the region -792/-772 bp (region A) and in the sense strand of the region -719/-698 bp (region B) (Figure 1A). Interestingly, both predicted AP-1 sites overlapped with potential KLF binding sites (Figure 1A).

KLF5 expression is induced by LPS treatment.

We next evaluated the expression profile of the 17 existing KLF isoforms in hearts from LPS-treated mice. We performed whole genome microarray analysis followed by qRT-PCR gene expression analysis. Among the 10 KLF isoforms that were detected in microarrays, Klf5 was the most profoundly upregulated (8-fold; Figure 1B). KLF5 was also increased at the protein level (Figure 1C & Online Figure 1). Although KLF6 mRNA levels increased 3-fold (Figure 1B), KLF6 protein was not increased significantly in LPS-treated hearts (Online Figure 1).

In order to assess whether LPS-mediated downregulation of Ppara gene expression involved KLF5 or KLF6, we treated a mouse cardiac myocyte cell line (HL-1) with LPS. Ppara expression was downregulated by 55% in LPS-treated cells (Figure 1D). This was associated with a Klf5 mRNA increase by 75%, while Klf6 gene expression was not altered (Figure 1D). These changes in Klf5 expression could be induced either directly by LPS-triggered signaling or indirectly due to cardiac dysfunction, which alters KLFs. In order to test this, we utilized mice with constitutive PPARG expression in cardiac myocytes (aMHC-Pparg) that are resistant to LPS-mediated cardiac dysfunction. Treatment of aMHC-Pparg mice with LPS reduced Ppara gene expression by 75% and increased Klf5 by 2.3-fold but not Klf6 (Figure 1E). Thus, LPS induces cardiac Klf5 expression directly.
KLF5 and c-Jun have opposite functions on PPARα gene expression and compete for binding to the Ppara promoter.

In order to identify whether c-Jun and KLF5 modulate Ppara gene expression in a synergistic or competitive fashion we generated an adenovirus that expresses a constitutively active form of c-Jun (Ad-cjunAsp) (Online Figure II). In this isoform Ser58, Thr62, Ser63, Ser73, Thr91 and Thr93 of the transactivation domain have been substituted with the phospho-mimetic aspartic acid. Therefore c-Jun is constitutively active without phosphorylation by JNK27. Infection of HL-1 cells with Ad-cjunAsp reduced Ppara mRNA levels by 40% (Figure 2A) and protein levels by 60% (Figure 2B and Online Figure III). On the other hand, treatment of HL-1 cells with adenovirus expressing KLF5 (Ad-KLF5) increased Ppara gene expression levels by 4-fold (Figure 2C) and protein levels by 2.5-fold (Figure 2D and Online Figure III). Thus, c-Jun and KLF5 have opposite regulatory roles on Ppara expression.

We performed chromatin immunoprecipitation (ChIP) to investigate whether the inhibitory effect of c-Jun and the positive effect of KLF5 on Ppara gene expression are due to their direct binding on the Ppara promoter. Treatment of HL-1 cells with Ad-cjunAsp increased enrichment of the region A of the Ppara promoter with c-Jun (Figure 2E). The same region was also occupied by KLF5 when HL-1 cells were treated with Ad-KLF5 (Figure 2F). Conversely, the region B of the Ppara promoter was occupied by c-Jun (Figure 2G) but not KLF5 (Figure 2H). As c-Jun and KLF5 have opposite effects on Ppara expression and are both activated in the hearts of LPS-treated mice, we treated HL-1 cells with LPS and assessed binding of endogenous c-Jun and KLF5 on the region A. LPS treatment promoted c-Jun binding, which in turn prevented KLF5 binding on the region A (Figure 2I).

Cardiac myocyte-specific Klf5 ablation alters cardiac function.

Our in vitro findings implicated KLF5 in activation of Ppara expression. In order to assess this in vivo we generated a mouse line with cardiac myocyte-specific deletion of Klf5 (aMHC-Klf5−/−) by crossing αMHC-Cre mice with mice that have Klf5 exons 2 and 3 flanked with loxP sites (floxed mice)24. Cardiac Klf5 mRNA levels were reduced by approximately 40% in aMHC-Klf5−/− mice (Figure 3A). The partial downregulation of Klf5 gene expression was likely due to the presence of other cell types in the heart, such as fibroblasts and endothelial cells. Therefore, we isolated primary mouse cardiac myocytes and assessed Klf5 mRNA levels, which were 85% lower compared to control cardiac myocytes isolated from floxed mice (Figure 3B). Klf5 expression was not reduced in skeletal muscle, intestine, kidney, white adipose tissue, or brain of aMHC-Klf5−/− mice (Figure 3A).

We then treated aMHC-Klf5−/− mice with LPS. Cardiac myocyte-specific ablation of Klf5 only partially improved cardiac function, which was still worse compared to control aMHC-Klf5−/− treated with saline (Online Figure IV-A). This partial improvement in cardiac function was independent from changes in cardiac Ppara gene expression (Online Figure IV-B).

Changes in cardiac gene expression in aMHC-Klf5−/− mice.

We next performed microarray analysis on hearts from aMHC-Klf5−/− and floxed control mice. Hierarchical clustering of the microarray data revealed two distinct expression signatures (Figure 3C). We identified 228 up-regulated and 79 downregulated genes in aMHC-Klf5−/− hearts, with at least 2-fold change (Online Figure V). Gene ontology analysis for classification of the differentially expressed genes based on the biological process suggested that 36 of the downregulated genes are involved in metabolic pathways (Figure 3D & Online Table I). Two of these genes, acyl-CoA thioesterase (Acot) 3 and Acot4, are related to FAO while 6 genes are associated with carbohydrate metabolism: serine (or cysteine) peptidase inhibitor, clade A, member 1A (Serpina1a), Serpina 1b, phosphorylase kinase α 1 (Phka1) and solute carrier family 3 member 2 (Online Table I). Gene ontology analysis for the upregulated transcripts showed 39 metabolism-related genes (Online Table II). Among these genes, 13 were associated with lipid metabolism, 2 with glucose metabolism and 2 genes were related to both glucose and lipid metabolism (Figure 3E &
Pathway analysis indicated a strong association of downregulated genes with the pathways of complement and coagulation, FA elongation, biosynthesis of unsaturated FAs, linoleic metabolism and RNA degradation (Figure 3F). Pathway analysis showed that upregulated genes are involved in carbohydrate digestion and absorption, as well as in linoleic acid metabolism (Figure 3G).

In addition to gene ontology analysis, we performed ingenuity pathway analysis for the whole genome microarray dataset to predict transcriptional networks and molecular relationships among genes. This analysis also revealed that many of the downregulated cardiac genes of the aMHC-Klf5<sup>−/−</sup> mice affect lipid metabolism (Figure 4A & Online Table III).

aMHC-Klf5<sup>−/−</sup> mice have reduced cardiac Ppara and FAO.

We confirmed with RT-PCR that deletion of cardiac myocyte Klf5 was associated with reduced cardiac Ppara expression levels in both male (30%) and female (45%) mice (Figure 4B). Cardiac PPARα protein levels were reduced by approximately 60% in aMHC-Klf5<sup>−/−</sup> mice (Figure 4C and Online Figure III). Similarly, the expression of a broad spectrum of FAO-related genes was reduced in the hearts of aMHC-Klf5<sup>−/−</sup> mice (Figure 4D). The expression of PPARγ coactivator (Pgc1α) was reduced by 45% in male and 25% in female mice, acyl-CoA oxidase (Acox) was reduced by 30% in male and 35% in female and carnitine palmitoyl-transferase (Cpt)1b was reduced by 45% in male and 35% in female mice. On the other hand, cardiac Pgc1b, Ppard and Pparg mRNA levels were not altered significantly (Figure 4D). The aMHC-Klf5<sup>−/−</sup> hearts had a trend for reduced FA uptake (Online Figure VI-A, B), and the expression of lipid uptake-related genes was reduced (Figure 4E). Cardiac expression of cluster of differentiation (Cd)36 was reduced by 40% in male and 35% in female aMHC-Klf5<sup>−/−</sup> mice, and lipoprotein lipase (Lpl) was reduced by 20% in male and female aMHC-Klf5<sup>−/−</sup> mice. The gene expression levels of enzymes that catalyze cardiac TG formation were also reduced. Diacylglycerol acyltransferase (Dgat)1 expression was reduced by 25% in male and 30% in female aMHC-Klf5<sup>−/−</sup> mice. Cardiac Dgat2 mRNA levels were 45% lower in both male and female aMHC-Klf5<sup>−/−</sup> mice (Figure 4F). These changes were consistent with reduced (25%) cardiac TG content in aMHC-Klf5<sup>−/−</sup> mice (Online Figure VII). Gene expression levels of lipid droplet-associated proteins, perilipin (Plin) 2 and Plin5, were not altered (Figure 4F). The downregulation in Pgc1 expression was also seen at the protein level, which was 40% lower in aMHC-Klf5<sup>−/−</sup> mice, while CPT1, DGAT1 and ATGL protein showed a trend towards lower levels in aMHC-Klf5<sup>−/−</sup> mice (Figure 4G & Online Figure III). Reduced PPARα expression levels were associated with increased (60%) phosphorylation of adenosine monophosphate kinase (AMPK) (Figure 4G & Online Figure III), reduced cardiac FAO (Figure 4H) and increased glucose oxidation levels (Figure 4I) although glucose uptake was not altered in aMHC-Klf5<sup>−/−</sup> hearts (Online Figure VI-C, D). These findings suggest that cardiac FA metabolism is suppressed in aMHC-Klf5<sup>−/−</sup> mice, which is consistent with cardiac Ppara downregulation.

Older aMHC-Klf5<sup>−/−</sup> mice have lower cardiac ATP content and increased TG accumulation.

The reduced FAO-related gene expression profile of the aMHC-Klf5<sup>−/−</sup> mice was associated with reduced ATP content (Figure 5A). Reduced cardiac ATP content in 9-12 months old aMHC-Klf5<sup>−/−</sup> mice was accompanied by increased accumulation of cardiac TG (Figure 5B). Increased cardiac TG content in older aMHC-Klf5<sup>−/−</sup> mice was associated with reversal of the expression of Dgat1, as well as of other fatty acid metabolism-related genes, such as Ppara, Cd36, Pgc1a and Aox (Online Figure VIII). Mitochondrial DNA content (Figure 5C), complex I activity (Figure 5D) and complex IV activity (Figure 5E) were not altered in the hearts of aMHC-Klf5<sup>−/−</sup> mice.
αMHC-Klf5−/− mice develop cardiac dysfunction.

Because cardiac myocyte-specific deletion of Klf5 reduces Ppara, FAO and cardiac ATP levels, we hypothesized that αMHC-Klf5−/− mice would have compromised cardiac function. Indeed, although young mice (2-3 months old) had normal cardiac function (Figure 6A-C), mice begin to develop cardiac dysfunction at the age of 6 months (Figure 6D-F). As the mice aged further (8-12 months old), the dysfunction progressed (Fig. 6G) with signs of dilated cardiomyopathy, as shown by reduced fractional shortening (Fig. 6H) and increased left ventricular internal dimension during both diastole (Fig. 6I) and systole (Fig. 6J). Left ventricular posterior wall thickness during either diastole or systole was not different in 6 (Online Figure IX) and 8-12 months old (Figure 6K, 6L) αMHC-Klf5−/− mice. Heart weight/tibia length ratio was slightly increased (Fig. 6M) in αMHC-Klf5−/− mice. The mRNA levels of heart failure marker brain natriuretic peptide (BNP) were increased in both young (Fig. 6N) and old αMHC-Klf5−/− mice (Fig. 6O), while atrial natriuretic factor (ANF) gene expression was increased in old mice (Fig. 6O). The expression of αMHC and bMHC was not altered either in young or old αMHC-Klf5−/− mice (Fig. 6N, 6O). Thus, αMHC-Klf5−/− mice have lower cardiac FAO-gene expression profile, increased lipid accumulation and dilated cardiomyopathy.

KLF5 mediates altered cardiac Ppara gene expression in diabetes.

Insulin deficient diabetes alters lipid metabolism of the heart and other organs. Ingenuity pathway analysis of the whole genome microarray data indicated that several downregulated genes in the hearts of αMHC-Klf5−/− mice are involved in the post-translational regulation of insulin signaling proteins28, 29, such as AKT, phosphoinositide 3-kinase (PI3K), p38 MAPK and extracellular signal-regulated kinase (ERK)1/2 (Figure 7A). To test whether KLF5 mediates cardiac metabolism-related gene expression changes in diabetes, we induced diabetes in wild type mice (C57 BL/6) with STZ intraperitoneal (ip) injections. Six weeks post-STZ administration, diabetic mice with increased plasma glucose levels (CTRL: 149 ± 6.9 mg/dl, STZ: 450 ± 61 mg/dl; p<0.001; n=6) had mild cardiac dysfunction as shown by reduced fractional shortening levels (Figure 7B). Hyperglycemia reduced cardiac KLF5 protein levels (Figure 7C), as well as Klf5 and Ppara gene expression levels by 35% and 55%, respectively (Figure 7D). Induction of hyperglycemia in αMHC-Klf5−/− mice (Online Figure X-A) with STZ administration did not reduce further cardiac Ppara gene expression levels (Online Figure X-B), neither did it worsen cardiac function, compared to diabetic wild type mice (Online Figure X-C).

Previous studies30, 31 have shown that cardiac Ppara expression is reduced in leptin-deficient B6.V-Lepob/J (ob/ob) mice, which is a model of Type 2 diabetes. Therefore, we tested whether this was associated with decreased Klf5 expression. We measured gene expression levels in the hearts of hyperglycemic 12-weeks old ob/ob mice. Indeed, these mice had reduced cardiac Klf5 (60%) and Ppara (70%) mRNA levels (Figure 7E).

Correction of hyperglycemia restores cardiac Klf5 and Ppara gene expression levels.

We reduced plasma glucose levels in diabetic mice using pharmacologic or ASO-mediated inhibition of the SGLT232. Both dapagliflozin (Figure 7F) and SGLT2 ASO (Figure 7G) administration corrected plasma glucose levels and normalized cardiac Klf5 and Ppara expression levels in STZ-treated wild type mice compared to mice treated with STZ alone (Figures 7H and 7I). Restoration of plasma glucose and cardiac Klf5 and Ppara gene expression levels was accompanied by prevention of cardiac dysfunction that was observed in wild type mice treated with STZ alone (Online Figure XI-A). On the other hand, despite normalization of plasma glucose levels (Online Figure XI-B), neither cardiac function (Online Figure XI-A) nor cardiac Ppara gene expression levels (Online Figure XI-C) were improved in αMHC-Klf5−/− mice treated with STZ and dapagliflozin.
Klf5 and Ppara gene expression levels are increased in later stages of diabetes.

Our data indicate that cardiac Klf5 and Ppara gene expression levels are reduced in the early stage of diabetes (6 weeks post-STZ administration). Although, our findings are consistent with previous studies showing downregulation or lack of change in cardiac Ppara gene expression during diabetes, other reports have associated diabetes with increased cardiac Ppara expression in mice and increased cardiac FAO in diabetic humans. As it has been suggested that differential cardiac Ppara gene expression in diabetes may reflect differences in disease severity or duration, we measured cardiac Klf5 and Ppara expression levels in mice 12 weeks post-STZ administration. We found that both Klf5 (Online Figure XII-A) and Ppara (Online Figure XII-B) gene expression levels were increased at this later stage of diabetes. Accordingly, we measured cardiac Klf5 and Ppara gene expression levels in the early stages of hyperglycemia in db/db mice. Hyperglycemia begins in db/db mice at the age of 5 weeks and increases further 1 week later (Online Figure XII-C). Cardiac Klf5 (Online Figure XII-D) and Ppara (Online Figure XII-E) expression levels were reduced in 5 weeks old and increased in 6 weeks old db/db mice. Thus, Klf5 gene expression parallels both downregulation of Ppara in the early stages of diabetes, as well as its upregulation in the late stages of the disease.

**DISCUSSION**

We identified KLF5 as a regulator of cardiac Ppara that controls cardiac FAO. We also showed that cardiac myocyte-specific Klf5 ablation reduces cardiac FAO and ATP content and leads to cardiac dysfunction. Moreover, we suggest that KLF5 regulates cardiac Ppara expression in Type 1 diabetes.

Balanced FA metabolism is critical for normal cardiac function. Either deficiency or excess of cardiac FAO can lead to organ dysfunction characterized either as energetic starvation that occurs in heart failure and sepsis or lipotoxicity that is observed in obesity and diabetes. Here we report that KLF5 is a transcriptional regulator of Ppara, which implicates it as a novel regulatory protein for cardiac FA metabolism.

KLFs regulate proliferation, differentiation, development, and cell death. KLF5 either increases or reduces lipid metabolism in several tissues, such as adipose tissue, skeletal muscle and lung. However, the role of KLF5 in cardiac myocyte metabolism has not been examined. A previous study showed that cardiac myocyte-specific Klf5 ablation did not prevent pressure overload cardiac hypertrophy. Cardiac myocyte Ppara downregulation, which occurs in our αMHC-Klf5-/- mice, may account for the lack of protection from hypertrophy in these mice, as shown by increased cardiac hypertrophy in Ppara-/- mice. As homozygous Klf5-/- mice are lethal, in vivo systemic metabolic studies were performed in global heterozygote Klf5+/- mice. Klf5+/- mice have defects in development of WAT, as KLF5 is crucial for adipocyte differentiation. Thus, Klf5+/- mice are resistant to high-fat diet-induced obesity, hypercholesterolemia and glucose intolerance, although they consume more food compared to wild type mice. However, the effects of haploinsufficiency on cardiac FAO were not studied.

Our initial findings linking cardiac Klf5 upregulation with inhibition of Ppara and cardiac energy production in LPS-treated mice and cardiac myocyte cell lines led us to the hypothesis that KLF5 is a repressor of Ppara expression. However, we found that KLF5 activates cardiomyocyte Ppara. The observed Klf5 upregulation in the hearts of LPS-treated mice may be attributed to other mechanisms, which pertain to inflammatory pathways that are activated in LPS-treated animals. Nevertheless, LPS increases Klf5 expression, which induces NF-κB and inflammation in intestinal epithelial cells. Accordingly, the increased levels of cardiac Klf5 expression that we measured during later stages of diabetes (Online Figure XII) may be associated with increased inflammation that develops during progression of the disease.
Binding of c-Jun in the Ppara promoter region that KLF5 could also occupy may constitute a critical event that explains the lack of upregulation of Ppara expression despite the increased levels of KLF5 in LPS-treated mice. Moreover, the increased levels of KLF5 that we observed in the hearts of LPS-treated mice and cardiac myocytes may represent a compensatory response of cardiac myocytes to the suppression of Ppara gene expression and the energetic deficiency.

Cardiac KLF5 is a positive regulator of Ppara and changes in Klf5 expression induce parallel changes in Ppara gene expression in non-LPS treated mice. Cardiac myocyte-specific Klf5 ablation downregulated the expression of Ppara and a broad range of cardiac FAO-related genes, leading to reduced cardiac FAO, lower ATP levels, increased cardiac TG accumulation, and cardiac dysfunction. This finding is consistent with previous studies showing that excessive cardiac lipid accumulation can lead to dilated cardiomyopathy in animal models and humans. Interestingly, cardiac Ppara gene expression reverses as the aMHC-Klf5−/− mice become older and TG accumulate in their hearts. Cardiac Ppara expression reduces with aging. Thus, our observation may indicate potential non-KLF5-dependent mechanisms that are triggered by cardiac TG expansion and influence PPARα activation as described before. The increased glucose oxidation levels that we observed in aMHC-Klf5−/− mice may also exacerbate further cardiac dysfunction, as shown previously. The method we used to assess cardiac fatty acid and glucose oxidation is based on incubation of whole hearts obtained from mice in a buffer that contains radiolabeled (14C) palmitate or glucose and measurement of 14CO2 that is released. The data we obtained with this method are consistent with the decreased cardiac fatty acid metabolism-related gene expression profile and a trend for reduced cardiac fatty acid uptake that we observed. Indeed, this is an in vitro approach that is performed in non-working hearts and may not sense fully the metabolic alterations that occur in a working heart. Thus, future studies with isolated working hearts perfused via the coronary arteries will help to complete the assessment of the cardiac metabolic effects of cardiac myocyte Klf5 ablation.

Changes in cardiac energetics and PPARα vary depending on the type of cardiac disease. Failing hearts show deficit in energy production and alterations in the source of energy substrates. Ppara−/− mice have decreased cardiac fatty acid metabolism and decreased longevity but normal cardiac function at baseline. However, starvation causes contractile dysfunction and lower cardiac ATP levels in Ppara−/− mice, primarily due to defective fatty acid uptake. Heart failure is accompanied by lower mitochondrial oxidative metabolism and ATP levels. The extent of the energetic deficiency during heart failure is influenced by the stage and cause of heart failure. Pressure overload-induced cardiac hypertrophy is accompanied by reduced PPARα levels, lower FAO and elevated glycolysis. Pharmacological PPARα activation with fenofibrate administration attenuates cardiac hypertrophy in mice and rats. Increased flux of FA from intracellular TG to mitochondria for FAO also improves contractility in hearts with pressure overload. On the contrary, Ppara haploinsufficiency attenuates pressure overload hypertrophy and failure. Ischemia-related heart failure either activates or inhibits PPARα, depending on the timing and experimental model. Mitochondrial oxidative metabolism is reduced during ischemia due to lower oxygen supply. However, cardiac FAO is induced during reperfusion of the ischemic heart. Pharmacological activation of PPARα 8-12 weeks post-myocardial ischemia aggravates cardiac hypertrophy. Similarly, isolated hearts from either aMHC-Ppara mice or mice that are subjected to ischemia/reperfusion and are treated with a PPARα agonist have lower cardiac power and increased infarct size. On the other hand, PPARα activation improves contractile function and reduces infarct size in isolated perfused rat hearts following ischemia/reperfusion. Beneficial effects on infarct size and cardiac performance have also been reported in rats following in vivo ischemia/reperfusion and treatment with PPARα agonist. Thus, PPARα can have either protective or aggravating effects in cardiac function.

We assessed Klf5 and Ppara expression in diabetic animal models. In agreement with other studies, we found that in animal models of both insulin-deficient and Type 2 diabetes, cardiac Ppara mRNA levels were reduced in the early stage of the disease. In both cases this was associated with reduced Klf5 gene expression. KLF5-mediated regulation of Ppara expression was dependent on glucose changes. This
was demonstrated by correction of hyperglycemia with SGLT2 inhibition that restored normal cardiac Klf5 and Ppara gene expression levels and prevented cardiac dysfunction in a Klf5-dependent manner. Cardiac cells can take up glucose via both the insulin-dependent GLUT4, the insulin-independent GLUT1, and nonspecific pinocytosis. Glucose uptake is not completely inhibited in Type 1 diabetes animal models as they can still take up glucose via GLUT1, while membranous GLUT4 protein levels are reduced by approximately 35%. Therefore, the observed reduction in Klf5 and Ppara during the early stages of insulin-dependent diabetes may reflect increased utilization of glucose for energy production in cardiac myocytes instead of the preferred FAs. Further studies remain to be performed in order to elucidate whether downregulation of cardiac Ppara gene expression in the early stage of diabetes is accompanied by concomitant reduction of cardiac fatty acid oxidation, which reverses afterwards.

Our findings linking hyperglycemia in the early stage of diabetes with reduced Ppara mRNA levels deviate from previous studies linking diabetes with increased cardiac Ppara mRNA or protein levels in STZ-treated wild type mice or diabetic mice with mutated leptin receptor (db/db). Another study also showed that diabetic patients have increased myocardial FAO. On the other hand, our data are consistent with studies showing that cardiac Ppara expression are reduced in the diabetic Akita mice (C57BL/6 background), as well as in rats and in isolated hearts obtained from STZ-treated wild type mice. The discrepancy between these studies may be attributed to different experimental conditions, such as the age of mice, genetic background or duration of hyperglycemia. Nevertheless, it has been proposed that the intensity of diabetes and availability of plasma lipids may account for differential effects of the disease on cardiac Ppara expression levels. Our STZ experiments were performed in 10 weeks old C57BL/6J mice. Animals in previous studies that showed increased cardiac Ppara expression were of mixed C57BL/6 × CBA/J or C57BLKS background. When the duration of hyperglycemia was extended to 3 months in our experiments, cardiac Klf5 and Ppara gene expression levels were increased. Accordingly, early stage of hyperglycemia in db/db mice was accompanied by reduced cardiac Klf5 and Ppara expression, which is reversed afterwards. Thus, inhibition of Klf5 and Ppara gene expression levels may represent an early cardiac response during diabetes that is followed by reversal of both Klf5 and Ppara expression at a later stage depending on the intensity of hyperglycemia and availability of fatty acids. Nevertheless, changes in cardiac Klf5 and Ppara gene expression are parallel during both early and late stage of diabetes.

In summary, our findings show that KLF5 is a positive transcriptional regulator of cardiac Ppara and its inhibition leads to cardiac dysfunction. Moreover, Klf5 expression changes parallel those of cardiac Ppara in diabetes. Thus, cardiac KLF5 emerges as a potential therapeutic target for several types of cardiac dysfunction that are associated with PPARα alterations and energetic deficiency.

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**DISCLOSURES**
There is no conflict of interest.
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FIGURE LEGENDS

Figure 1: Cardiac KLF5 is upregulated in sepsis – (A) Predicted AP-1 (yellow) and KLF (framed) binding sites on mouse Ppara promoter. (B-C) Cardiac mRNA levels of Klf isoforms (B) and protein levels of KLF5 and β-actin (C) in 10-12-weeks old C57BL/6 mice treated with 5 mg/kg LPS or saline (CTRL) (n=4-5; *p<0.05; **p<0.01; ***p<0.001 vs CTRL). (D-E) Ppara, Klf5 and Klf6 mRNA levels in HL-1 cells (D) treated with 1μg/ml LPS or saline (CTRL) for 9h (n=6; *p<0.05 vs. CTRL) or in aMHC-Ppargc mice (E) treated with 5mg/kg LPS or saline (CTRL) for 8-10h (n=5; *p<0.05; **p<0.01 vs. CTRL).

Figure 2: KLF5 and c-Jun have opposite effects on Ppara expression and compete for binding on Ppara promoter (A-D) Ppara and Klf5 mRNA (A, C) and protein (B, D) levels in HL-1 cells treated with Ad-cJunAsp (A, B) or Ad-KLF5 (C, D) (n=6; *p<0.05; **p<0.01; ***p<0.001 vs CTRL). (E-I) Enrichment of -792/-772 bp region (E, F) or -719/-698 bp region (G, H) of mouse Ppara promoter with c-Jun (E, G) or KLF5 (F, H) of chromatin samples from HL-1 cells treated with Ad-GFP (CTRL) and either Ad-cJunAsp (E, G) or Ad-KLF5 (F, H); **p<0.01 vs CTRL. (I) Enrichment of -792/-772 bp region of mouse Ppara promoter with c-Jun or KLF5 of chromatin samples from HL-1 cells treated with 1 μg/ml LPS or saline (CTRL); *p<0.05 vs CTRL. Data for all bar graphs are represented as means ± SEM (statistical analysis: t-test).

Figure 3: Cardiac myocyte-specific ablation of KLF5 induces a distinct transcriptome profile – (A, B) Klf5 mRNA in the heart, skeletal muscle, intestine, kidney, white adipose tissue, brain (A) and primary cardiac myocytes (B) of aMHC-Klf5-/- mice (n=3; *p<0.05 vs floxed). (C) Hierarchical clustering for differentially expressed mRNAs detected by whole genome microarray analysis of cardiac mRNA obtained from aMHC-Klf5-/- mouse array that are related to FA metabolism. (D-G) Ingenuity pathway analysis of genes regulated over 2-fold in the aMHC-Klf5-/- mouse array that are related to FA metabolism. (D-G) Gene ontology analysis for classification of the downregulated (D) or upregulated (E) genes based on the metabolic process that they are associated with and pathway analysis for downregulated (F) and upregulated (G) genes detected with whole genome microarray analysis of cardiac mRNA obtained from aMHC-Klf5-/- mice and control floxed mice. For all bar graphs are represented as means ± SEM (statistical analysis: t-test).

Figure 4: Cardiac myocyte-specific ablation of Klf5 inhibits the expression of genes that are associated with FA metabolism – (A) Ingenuity pathway analysis of genes regulated over 2-fold in the aMHC-Klf5-/- mouse array that are related to FA metabolism. (B) Cardiac Klf5 and Ppara mRNA levels of 10- to 12-week-old aMHC-Klf5-/- male and female mice (n=5; **p<0.01; ***p<0.001 vs same gender floxed mice). (C) Cardiac PPARα and β-actin protein levels of 10- to 12-week-old floxed and aMHC-Klf5-/- male mice. (D-F) Cardiac mRNA levels for FA oxidation- (Pparg-1a, Pparg-1β, Pparg, Ppard, Acox and Cpt1b) (D), lipid uptake- (Cd36, Lpl and Angptl4) (E) and lipid storage-related genes (Dgat1, Dgat2, Plin2, Plin5) (F) (n=5; *p<0.05, **p<0.01, ***p<0.001 vs same gender floxed mice). (G) Cardiac PGC-1, CPT-1, DGAT-1, ATGL, phosphorylated AMPK, total AMPK, and GAPDH protein levels of 10- to 12-week-old floxed and aMHC-Klf5-/- male mice. Data for all bar graphs are represented as means ± SEM (statistical analysis: t-test).

Figure 5: Cardiac myocyte-specific ablation of Klf5 reduces ATP content and promotes cardiac TG accumulation - (A-E) ATP levels in cardiac muscle (A), cardiac TG levels normalized to tissue weight (B), ratio of cardiac mitochondrial gene DNA (ATPase6) to nuclear gene DNA (β-actin) (C), complex I: citrate synthase activity ratio (D) and complex IV: citrate synthase activity ratio (E) normalized citrate synthase activity in 8-12 months old aMHC-Klf5-/- and floxed (WT) mice (n=5-7; *p<0.05). Data for all bar graphs are represented as means ± SEM (statistical analysis: t-test).

Figure 6: Cardiac myocyte-specific ablation of Klf5 impairs cardiac function - (A-F) Fractional shortening (A, D), left ventricular internal dimension during diastole (B, E), left ventricular internal dimension during systole (C, F), in 2-3 months old (A-C) and 6 months old (D-F) aMHC-Klf5-/- and floxed.
(WT) mice. (G-M) Photographs of echocardiograms (G), fractional shortening (H), left ventricular internal dimension during diastole (I), left ventricular internal dimension during systole (J) left ventricular posterior wall during diastole (K) left ventricular posterior wall during systole (L), and heart weight/tibia length ratio (M) in 8-12 months old αMHC-Klf5−/− and floxed (WT) mice (n=7-8; *p<0.05). (N, O) Cardiac mRNA levels for Bnp, Anf, αMHC and βMHC genes in 2-3 months old (N) and 11-12 months old (O) male floxed and αMHC-Klf5−/− mice (F) (n=5; *p<0.05, **p<0.01 vs floxed mice).

Figure 7: Diabetes inhibits cardiac Klf5 and Ppara gene expression – (A) Ingenuity pathway analysis of cardiac genes regulated over 2-fold in the aMHC-Klf5−/− mouse array that have direct or indirect association with insulin signaling and glucose metabolism proteins. Highlighted with bold fonts within the diagram are proteins that modulate insulin signaling. (B) Fractional shortening of C57BL/6 mice 6 weeks post-STZ or saline (CTRL) administration (n=5; *p<0.05 vs CTRL). (C) Western blot analysis for cardiac KLF5 and β-actin protein levels in C57BL/6 mice 6 weeks post-STZ administration (n=3; ***p<0.001 vs CTRL). (D) Cardiac Klf5 and Ppara mRNA levels in floxed and aMHC-Klf5−/− mice 6 weeks post-STZ administration (n=5; *p<0.05, **p<0.01 vs CTRL). (E) Cardiac Klf5 and Ppara mRNA levels in 12 weeks old ob/ob mice compared with wild type C57BL/6 mice (n=4-5, *p<0.05, ***p<0.001 vs wt). (F-I) Plasma glucose levels (F, G) and cardiac Klf5 and Ppara mRNA levels (H, I) in wild type mice treated with STZ (6 weeks prior to glucose measurement), dapagliflozin (F, H), antisense oligonucleotides against SGLT2 (SGLT2-ASO) (G, I) and combination of either STZ with dapagliflozin (F, H) or STZ with SGLT2-ASO (G, I) (n=5, **p<0.01, ***p<0.001 vs CTRL).
NOVELTY AND SIGNIFICANCE

What Is Known?

- Sepsis downregulates cardiac peroxisome proliferator-activated receptor (Ppar) α gene expression.
- Cardiac PPARα is upregulated in diabetic cardiomyopathy and is considered as the major driver of increased cardiac fatty acid oxidation.

What New Information Does This Article Contribute?

- Krüppel-like factor (KLF) 5 is a positive regulator of Ppara gene transcription.
- LPS-induced sepsis prevents binding of KLF5 in the Ppara promoter.
- Klf5 and Ppara gene expression are downregulated at the early stage of diabetes and increased as the disease progresses.

Cardiac fatty acid oxidation accounts for 70% of the ATP that is produced in the heart. PPARα is a nuclear receptor with a central role in the transcriptional regulation of various proteins that contribute in cardiac fatty acid metabolism. Sepsis, systemic inflammatory response that follows bacterial infection, suppresses cardiac fatty acid oxidation, which is accounted for by downregulation of cardiac myocyte PPARα. This study identified KLF5 as a positive regulator of Ppara gene expression. During sepsis KLF5 binding on Ppara gene promoter is prevented. Cardiac myocyte-specific Klf5 ablation reduced cardiac fatty acid oxidation and led to cardiac dysfunction in older mice. Ppara gene expression is also altered in diabetes. This study showed that a dynamic oscillation of cardiac Ppara gene expression occurs in diabetes. The initial response is constituted by downregulation of cardiac Ppara and is followed by increased Ppara expression as the disease progresses. Both early downregulation and late upregulation of Ppara are associated with respective changes of cardiac Klf5 gene expression. Further delineation of the metabolic profile of the early cardiac response in diabetes may indicate new targets for treating diabetic cardiomyopathy.
Figure 4
Cardiac Myocyte KLF5 Regulates Ppara Expression and Cardiac Function
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SUPPLEMENTAL MATERIAL

Mouse generation and experiments - All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Temple University and Columbia University. Mice were maintained under appropriate barrier conditions in a 12hr light-dark cycle and received food and water ad libitum. Mice were anesthetized by isofluorane inhalation. Mouse hearts were harvested, flash frozen and stored at -80°C until further use. All analyses involving animals were performed with at least 5 mice per experimental group. Mice with exons 2 and 3 of the mouse klf5 gene flanked with loxP sites (floxed KLF5 mice) were kindly provided by Dr. Jeffrey A. Whitsett. These mice were crossed with mice overexpressing Cre recombinase driven by the cardiomyocytes-specific aMHC promoter (aMHC-Cre), generating mice with cardiomyocytes-specific klf5 gene deletion (aMHC-Klf5^-/-). For all experiments that aMHC-Klf5^-/- mice were used, the control group was floxed Klf5 mice.

Wild type C57BL/6 mice obtained from the Jackson Laboratory were treated IP with 5 mg/kg E. coli lipopolysaccharides (LPS) to mimic sepsis. Control mice were treated with equal volumes of saline. Cardiac function was assessed by 2D echocardiography 5-6 h post-LPS administration and mice were sacrificed 2-3 h later (7-9 h post LPS injection). C57BL/6 or aMHC-Klf5^-/- mice were injected IP with 180 mg/kg STZ to mimic type-I diabetes (insulin dependent), which was confirmed by measurement of blood glucose levels with a glucometer. Mice were considered diabetic when fasting glucose levels exceeded 250 mg/dl or when feeding glucose levels exceeded 400 mg/dl. Control mice were treated with equal volumes of saline. Analyses were performed 6 or 12 weeks post-STZ administration and mice were sacrificed afterwards following anesthesia with isofluorane inhalation.

Induction of insulin deficiency - Mice were made diabetic by STZ treatment similar to that described by Kunjathoor, Wilson, and LeBoeuf and adopted by the Diabetes Complications Consortium. Mice were divided into two groups; one group was treated with STZ (Sigma
Chemical Co., St. Louis, MO), the other group was treated with vehicle. STZ was dissolved in sterile citrate buffer (pH 4.5) and used within 20 min of preparation. The solution was injected IP into mice (50 mg/kg, ~200 μl) for five consecutive days. Ten days after the last STZ injection 4-hours fasting glucose levels were measured. STZ-injected mice with glucose levels >13.9 mmol/l (>250mg/dl) were considered to be diabetic.

**Dapagliflozin treatment** - Dapagliflozin, an oral SGLT2 inhibitor (SGLTi, provided by ISIS Pharmaceuticals, CA) was dissolved in DMSO. Mice were treated with SGLT2i at a dose of 5 mg/kg/day in drinking water. Control mice were treated with vehicle.

**Antisense oligonucleotide (ASO) treatment** - In order to inhibit the sodium glucose co-transporter 2 (SGLT2), expression mice were injected with the ASO, ISIS 388625 (sequence 5΄-TGTTCCAGCCCA-3΄) at 20 mg/kg body weight 10 days after the last STZ injection. Control mice were injected with ASO, ISIS-141923 (5΄-CCTTCCCTGAAGGTTCCTCC-3΄), which does not have perfect complementarity to any known gene in public databases. All ASOs were dissolved in sterile saline for IP injection.

**Echocardiography** - Two-dimensional echocardiography was performed on anesthetized (inhaled isofluorane) 10- to 12-week-old (n = 5-10 per group) mice (VisualSonics Vevo 2100). Echocardiographic images were recorded in a digital format. Images were then analyzed off-line by a single observer blinded to the respective treatments of mice⁴.

**RNA purification and gene expression analysis** - Total RNA was purified from hearts or HL-1 cells, which were kindly provided by Dr. William Claycomb⁵, using the TRIzol reagent according to the instructions of the manufacturer (Invitrogen). DNase-treated RNA was used for cDNA synthesis using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). cDNA was analyzed with quantitative real-time PCR that was performed with SYBR Select Master Mix (Applied Biosystems). Incorporation of the SYBR green dye into the PCR products was monitored
in real time with an Mx3000 sequence detection system (Stratagene). Samples were normalized against 18S. The sequences of the primers have been described previously\(^6,7\). New primers are described in Online Table IV.

**Whole genome microarray analysis** – The microarray analysis for cardiac mRNA obtained from LPS-treated C57BL/6 mice was performed by Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL). Total RNA samples were sent to ORB for analysis using mouse exonic evidence-based oligonucleotide (MEEBO) microarrays (Lot 20833). MEEBO microarrays were printed by Microarrays Inc. (Nashville, Tennessee) and contained 38,083 70-mer oligonucleotides probes complementary to constitutive exons of most mouse genes, as well as alternatively spliced exons, and control sequences. More information on the MEEBO oligonucleotide is available at http://alizadehlab.stanford.edu/. Biotin-labeled complementary RNA (cRNA) was prepared from total RNA. Briefly, an oligonucleotide containing a 5'-T7-promoter sequence and a 3' T24VN sequence was used to prime reverse transcription of RNA catalyzed by Superscript II (Invitrogen, Carlsbad, CA). Double-stranded cDNA was prepared from the 1st strand product\(^8\), and purified on a PCR purification column (Qiagen, Valencia, CA).

The double-stranded cDNA was then used as a template for *in vitro* transcription with T7 RNA polymerase using a high yield transcription kit (Ambion) and including biotin-16-UTP (Ambion) in the reaction mixture. Biotinylated cRNA samples were fragmented, diluted in a formamide-containing hybridization buffer, and loaded on to the surface of MEEBO microarray slides enclosed in custom hybridization chambers. The slides were hybridized for 16-18 hours under constant rotation in a Model 400 hybridization oven (Scigene, Sunnyvale, CA). After hybridization, the microarray slides were washed under stringent conditions, stained with Streptavidin-Alexa-647 (Invitrogen), and scanned using an Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). Spot intensities for each probe were calculated by subtracting median local background from median local foreground for each spot. Spot intensities
were transformed by taking the base 2 logarithm of each value. The spot intensities were then
normalized by subtracting the 70th percentile of the spot intensities of probes against mouse
constitutive exons and adding back a scaling factor (grand mean of 70th percentile). After
removing data for low quality spots, control sequences, and non-mouse probes, 34,945 mouse
probe intensities remained. The mouse probes intensities were filtered to identify all probes with
intensity above a normalized threshold $\log_2 (3\times \text{standard deviation of raw local background}) +$
mean of log2-transformed negative controls], to arrive at 12,495 probes above threshold in all
samples from at least one treatment group.

For statistical analysis, samples were binned in 2 treatment groups. The log2-transformed
and normalized spot intensities for the detectable probes were examined for differences between
the treatment groups by 1-way ANOVA using National Institute of Ageing (NIA) Array Analysis
software. The statistical significance was determined using the False Discovery Rate (FDR)
method. It is the proportion of false positives among all probes with P values lower or equal to the
P value of the probes that we consider significant. It can also be viewed as an equivalent of a P-
value in experiments with multiple hypotheses testing. FDR is an intermediate method between
the P-value and Bonferroni correction (multiplying P-value by the total number of probes). The
equation is:

$$FDR_r = \min \left[ \frac{p_i N}{i} \right]_{i \geq r}$$

where $r$ is the rank of a probe ordered by increasing P values, $p_i$ is the P value for probe with rank
i, and N is the total number of probes tested. FDR value increases monotonously with increasing
P value.

Gene ontology categories showing significant over-representation of differentially
expressed genes were determined using GenMAPP software (Gladstone Institute, San
Francisco, CA) for 12,495 detectable probes with current Entrez Gene IDs. Specifically, the MAPPfinder module of GenMAPP was first used to map all detectable probes, based on their gene targets, to GO and Local MAPP categories. Then MAPPfinder compared the relative representation in each functional group of genes associated with probes meeting one of the differential expression criteria to the relative representation of genes associated with the full set of 12,495 detectable probes. Significance was determined by permutation of Z scores with correction for multiple comparisons as described in the GenMAPP software manual. Genes meeting the different criteria were further analyzed using online Gene set Analysis Toolkit (Dr. Bing Zhang’s group, Vanderbilt University) in order to identify the Wiki, KEGG, Pathway Commons and GO functional pathways showing an over-representation of differentially expressed genes. Data for the detectable probes were clustered using Cluster 3.0 software\textsuperscript{10}. The data was pre-processed by three consecutive rounds of gene median centering and then hierarchically clustered using centered correlation as the similarity metric and average linkage as clustering method. Intensity scale shown is arbitrary. The microarray profiling data for cardiac mRNA obtained from LPS-treated mice have been deposited in the Gene Expression Omnibus database under accession number GSE63920.

The microarray analysis for cardiac mRNA obtained from αMHC-Klf5\textsuperscript{-/-} mice was performed by Arraystar. RNA quantity and quality were measured by NanoDrop ND-1000. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY\texttrademark Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3’ bias utilizing a random priming method. The labeled cRNAs were purified by RNeasy Mini Kit (Qiagen). The concentration and specific activity of the
labeled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000. 1 μg of each labeled cRNA was fragmented by adding 5 μl 10 × Blocking Agent and 1 μl of 25 × Fragmentation Buffer, then heated the mixture at 60 °C for 30 min, finally 25 μl 2 × GE Hybridization buffer was added to dilute the labeled cRNA. 50 μl of hybridization solution was dispensed into the gasket slide and assembled to the microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned with using the Agilent DNA Microarray Scanner (part number G2505C). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed with using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, mRNAs that at least 1 out of 8 samples have flags in Present or Marginal (“All Targets Value”) were chosen for further data analysis. Differentially expressed mRNAs with statistical significance between two groups were identified through Volcano Plot filtering. Hierarchical Clustering was performed using the Agilent GeneSpring GX software (version 11.5.1). GO analysis and Pathway analysis were performed in the standard enrichment computation method.

The microarray profiling data for cardiac mRNA obtained from aMHC-Klf5−/− and control floxed mice have been deposited in the Gene Expression Omnibus database under accession number GSE63839.

**Protein purification and analysis** - Isolated heart tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitors (aprotinin, bestatin, E-64, leupeptin, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, β-glycerophosphate – Pierce Biotechnology). Total protein extracts (25 or 40 μg) were applied to SDS-PAGE and transferred onto PVDF membranes. Antibodies were obtained from Santa Cruz (β-actin, GAPDH), Cell Signaling (tAMPK, pAMPK, ATGL), Alpha Diagnostic (CPT1) and Abcam (PPARα, DGAT1).
**Measurement of mitochondrial DNA** - Mitochondrial DNA was quantified by calculating the ratio of mitochondrial gene copy number (ATPase6) to nuclear gene copy number (β-actin). Cardiac DNA was extracted from frozen tissue. Specifically, cardiac tissue was added in DNA buffer (0.5% SDS, 0.1M NaCl, 0.05M Tris, pH8.0, 3mM EDTA) and incubated at 60°C for 2 hrs. 75μl 8M potassium acetate and 500μl chloroform were added and samples were centrifuged at 9,500 rpm for 5 min. DNA was precipitated from the aqueous phase by addition of 100% ethanol and centrifugation and washed twice with 75% ethanol. The DNA pellet was diluted in ddH2O. 20ng of DNA were used for PCR analysis.

**Cardiac glucose and FFA uptake** - FFA and glucose uptake were assessed in mice that were injected with PBS or P407 and then fasted for 16 h. [9,10-14C(N)]-oleate (PerkinElmer Life Sciences) was complexed to 6% FA-free BSA (Sigma). Mice were injected intravenously with 1 μCi [9,10-14C(N)]-oleate-BSA and blood was collected at 0.5, 1, 3, and 5 min after injection. Five minutes after injection, the body cavity was perfused with 10 ml of PBS by cardiac puncture and tissues were excised. Tissue was homogenized in PBS and radioactive counts were measured. Basal glucose uptake was measured in hearts following an intravenous administration of 2.5 μCi of 2-deoxy-D-[1-3H]glucose (PerkinElmer Life Sciences). Blood was collected 2, 30, and 60 min following injection. At 60 min, hearts were perfused with PBS, tissues were excised, and radioactive counts were measured. For all turnover studies, radioactivity per gram of tissue was normalized to the respective 30 s or 2 min plasma counts (injected dose).

**FA and glucose oxidation** – FA and glucose oxidation were measured in whole hearts isolated from 9-12 week old mice. Hearts were incubated at 37°C for 2h in modified Krebs-Ringer buffer (MKR: 115 mM NaCl, 2.6 mM KCl, 1.2 mM KH2PO4, 10 mM NaHCO3, 10 mM HEPES, pH 7.4) that contained 2% BSA, 0.6 mM palmitate, 6 mM glucose, 5 μCi/ml D-[14C(U)]-glucose or 1 μCi/ml Palmitic Acid, [14C(U)] and was gassed with 95% O2 and 5% CO2. Tissue-containing reaction buffer was incubated at 37°C for 2h. The reaction was terminated and CO2 was released.
by administration of perchloric acid. Released CO\textsubscript{2} was trapped in a KOH-soaked Whatman paper that was placed in a central well of the flask. Palmitate or glucose oxidation was determined by measuring the amount of \textsuperscript{14}CO\textsubscript{2}.

**Cardiac ATP measurement** – Cardiac pieces of 10 mg were used to determine ATP levels. Heart pieces were dissolved in ice-cold 0.1% Trichloro-acetic acid and centrifuged. Supernatant was resuspended in 50mM Tris-acetate containing 2mM EDTA, pH 7.8 and a portion was used to measure ATP. The measurement was performed with the ATP determination kit (Invitrogen) according to the instructions of the manufacturer.

**Isolation of primary cardiomyocytes** – Adult mouse cardiomyocytes were isolated from ventricles of WT and \textit{αMHC-Klf5}\textsuperscript{-/-} mice as described previously\textsuperscript{11} with minor modifications. Hearts from heparinized mice (90 USP; ip) were cannulated through the aorta. Hearts were perfused with perfusion buffer (120.4 mM NaCl, 14.7 mM KCl, 0.6 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.6 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}, 10 mM Hepes, 4.6 mM NaHCO\textsubscript{3}, 30 mM taurine, 10 mM BDM, 5.5 mM glucose; pH 7.0) for 3 min followed by perfusion buffer containing 19250 units Collagenese type II (Worthington), 5-6 mg trypsin and 0.02 mM CaCl\textsubscript{2} for 7 min. Ventricles were gently teared in small pieces, perfusion buffer containing 5 mg/ml BSA and 0.125 mM CaCl\textsubscript{2} was added and filtered with 100 \textmu m nylon. The filtrate was pelleted by gravity for 5 min, centrifuged for 30 sec at 0.7 rpm and the pellet resuspended in perfusion buffer containing 5 mg/ml BSA and 0.225 mM CaCl\textsubscript{2}. The cells were pelleted by gravity for 10 min, centrifuged for 30 sec at 0.7 rpm and the pellet resuspended in perfusion buffer containing 5 mg/ml BSA and 0.525 mM CaCl\textsubscript{2}. The cells were pelleted by gravity for 10 min, centrifuged for 30 sec at 0.7 rpm and the pellet resuspended in perfusion buffer containing 5 mg/ml BSA and 1.025 mM CaCl\textsubscript{2} and plated on laminin-coated (10 \mu g/ml) 6 wells plates and used the next day.
**Complex I and complex IV activity measurement** – Complex I & IV enzyme activities were measured using the Complex I or Complex IV IV rodent enzyme activity microplate assay kit (abcam) according to the instructions of the manufacturer.

**Construction of Recombinant Adenoviruses** – The recombinant adenovirus that expresses KLF5 was kindly provided by Dr. Ceshi Chen\(^\text{12}\). The plasmid that contained the cDNA of constitutively active c-Jun (c-Jun\(^\text{Asp}\)) was kindly provided by Dr. Dick Bohmann\(^\text{13}\). The c-Jun\(^\text{Asp}\) cDNA was extracted by digestion with XhoI and NotI restriction enzymes and cloned into the same restriction sites of the pAdTrackCMV vector. The recombinant adenoviruses were constructed as described\(^\text{14}\) using the Ad-Easy-1 system where the adenovirus construct is generated in bacteria BJ-5183 cells. Correct clones were propagated in RecA DH5\(\alpha\) cells. The recombinant adenoviral vectors were linearized with PacI and used to infect human embryonic kidney 293 (HEK-293) cell cultures. Following large scale infection of HEK-293 cell cultures, the recombinant adenoviruses were purified by two consecutive cesium chloride ultracentrifugation steps, dialyzed, and titrated. Usually, titers of 0.5 – 2 × 10\(^{10}\) plaque-forming units (pfu)/ml were obtained.

**Infection of cells with recombinant adenoviruses** – HL-1 cells were grown as described previously\(^\text{5}\). Cells were infected with Ad-cJun\(^\text{Asp}\), Ad-KLF5 or control adenovirus-expressing GFP (Ad-GFP) at a 10 multiplicity of infection (MOI) in medium supplemented with 2% Heat-Inactivated Horse Serum and 1% Penicillin/Streptomycin. Medium was removed 6 hours post-infection and fresh 10% FBS-containing medium was added. Cells were harvested 48h post-infection.

**Chromatin immunoprecipitation (ChIP)** - ChIP experiments were performed as described previously\(^\text{15}\). In brief, HL-1 cells were fixed with 1% formaldehyde for 10 min at room temperature and lysed with the addition of cell lysis buffer (50mM Hepes-KOH pH 7.5, 140 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1mM Na\(_3\)VO\(_4\),1mM NaF) and incubation at 4 °C for 10 min. The nuclei were washed once with washing buffer (10 mM Tris-HCl
pH 8, 200mM NaCl, 1mM EDTA, 0.5mM EGTA, 1mM Na₃VO₄, 1mM NaF). The nuclei were lysed in nucleus lysis buffer (10mM Tris-HCl pH8, 100mM NaCl, 1mM EDTA pH8, 0.5mM EGTA pH8, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine, 1mM Na₃VO₄, 1mM NaF) followed by sonication using a Bioruptor (Diagenode), and chromatin was pre-cleared by the addition of nine volumes of nucleus lysis buffer and magnetic Dynabeads. One per cent of the chromatin was kept as input. We coupled 10 μg antibody with 100 μl of beads for 12h in reaction buffer, and the complex was added to pre-cleared chromatin followed by overnight incubation at 4 °C with rotation. We washed the complexes bound to the beads using buffers with increasing salt concentration: six times with RIPA wash buffer (50 mM Hepes-KOH pH7.6, 300mM LiCl, 1mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate) and twice with TE wash buffer (10mM Tris-HCl pH8, 1mM EDTA, 50mM NaCl) followed by elution (0.1M NaHCO3, 1% SDS) and treatment with RNase and proteinase K. The cross-links were then reversed, and the DNA was precipitated using ethanol and glycogen for further analysis with RT-PCR.
Online Figure I - Densitometric quantitation of KLF5 and KLF6 normalized with β-actin in protein samples obtained from hearts of 10- to 12-week-old C57BL/6 mice that were treated with 5 mg/kg LPS; *p<0.05, n=3. Data are represented as means ± SEM.
Online Figure II - Western blot for c-Jun of protein lysate from HL-1 cells treated with adenovirus expressing a constitutively active c-Jun (cJun^{Asp}).
Online Figure III: Densitometric quantitation of PPARα and KLF5 normalized with β-actin in protein samples obtained from HL-1 cells infected with recombinant adenovirus expressing either control GFP (grey bars), constitutively active c-Jun (cJunAsp) (white bar) or KLF5 (white bars); *p<0.05, n=3-4. Data are represented as means ± SEM.

Densitometric quantitation of PPARα, PGC-1, CPT-1, DGAT-1, ATGL, and ratio of phosphorylated AMPK/total AMPK normalized with GAPDH in protein samples obtained from hearts of 10- to 12-weeks-old control floxed (black bars) or αMHC-Klf5−/− (white bars) mice; *p<0.05, n=4-5. Data are represented as means ± SEM.
Online Figure IV: Fractional shortening (A) and cardiac Ppara mRNA levels (B) of floxed and αMHC-Klf5/- mice treated with saline or 7.5 mg/kg LPS; (*p<0.05, **p<0.01, ***p<0.001 vs indicated group; n=4-5).
Online Figure V – Upregulated (A-C) and downregulated (D) genes detected by whole genome microarray analysis of cardiac mRNA obtained from αMHC-Klf5−/− mice and control floxed mice with at least 2-fold change.
Online Figure VI - A: Plasma disappearance of the $^{14}$C-oleate was measured at 30 s, 2 min, and 5 min after injection. B: Cardiac FFA uptake was assessed using $^{14}$C-oleate. C: Plasma disappearance of the $^{3}$H-2-deoxyglucose was measured at 2 min and 30 min after injection. D: Cardiac glucose uptake was assessed using $^{3}$H-2-deoxyglucose. Data were compared by Student's t-test. *P < 0.05.
Online Figure VII – Cardiac triglycerides in control floxed and αMHC-Klf5-/- 8-12 weeks old male mice (n=3).
Online Figure VIII – Cardiac mRNA levels for Ppara, Pdk4, Dgat1, Cd36, Pgc1a and Aox of 2-3, 9 and 12 months old floxed and αMHC-Klf5−/− male mice (n=4-6; *p<0.05, **p<0.01, vs floxed mice).
**Online Figure IX** - Left ventricular posterior wall during diastole (LVPWd) and left ventricular posterior wall during systole (LVPWs) in 6 months old $aMHC-Klf5^{-/-}$ and floxed mice (n=4-6).
Online Figure X – (A-C) Plasma glucose levels (A) cardiac Ppara mRNA levels (B) and fractional shortening (C) in floxed and aMHC-Klf5−/− mice 6 weeks post-STZ administration (n=4-5; *p<0.05, **p<0.01).
Online Figure XI – (A-C) Fractional shortening (A), plasma glucose levels (B) and cardiac *Ppara* mRNA levels in floxed and *aMHC-Klf5*−/− mice 6 weeks post-treatment with streptozotocin (STZ) or combination of STZ and dapagliflozin (n=4-5; *p<0.05, **p<0.01).
Online Figure XII - (A, E) Klf5 (A) and Ppara (B) mRNA levels in hearts of WT mice 12 weeks-post STZ administration. (C-E) Plasma glucose (C), cardiac Klf5 (D) and Ppara (E) mRNA levels in 5 and 6 weeks old db/db mice compared with wild type C57BLKS mice (n=4-5, *p<0.05, **p<0.01 vs wt). Data for all bar graphs are represented as means ± SEM (statistical analysis: t-test).
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<th>Gene Symbol</th>
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<th>P-value</th>
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<td>Ahcy1l2</td>
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**Online Table I** – Metabolism-related genes with reduced expression as shown with whole genome microarray analysis of cardiac mRNA obtained from the hearts of *aMHC-Klf5*<sup>-/-</sup> mice and control floxed mice.
### Online Table II - Metabolism-related genes with increased expression as shown with whole genome microarray analysis of cardiac mRNA obtained from the hearts of *aMHC-Klf5*−/− mice and control floxed mice. Black highlighted rows indicate lipid metabolism-associated genes, light grey
indicates glucose metabolism-associated genes and dark grey indicates genes that are associated with both lipid and glucose metabolism.
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<tr>
<th>Molecules</th>
<th>Categories</th>
<th>Diseases or Functions Annotation</th>
<th>p-Value</th>
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<td>ACOT1,ACOT4,CYP4A11</td>
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<td>metabolism of long chain fatty acid</td>
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<td>B4GALNT1</td>
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<td>accumulation of testosterone, drainage of testosterone, synthesis of lactosylceramide II3-sulfate, , synthesis of gangliotriaosylceramide II3-sulfate, quantity of asialo GM1 ganglioside, quantity of ganglioside GT1, GD1a, GM3, GM1, GM2</td>
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<td>CYP4A11</td>
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### Online Table III

Differentially expressed genes that are associated with lipid or carbohydrate metabolism in the hearts of \( aMHC-Klf5^{−/−} \) mice compared to floxed mice processed with Ingenuity Pathway Analysis of the dataset that was obtained with whole genome microarray analysis.

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<th>Gene Expression</th>
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<td>Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism</td>
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### Analysis method

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<td>mKLF 16 5'-GGCTGCGGCCAAAGCCTATTA-3'</td>
<td>5'-ATCAGAACCGGGGAAGACTTT-3'</td>
</tr>
<tr>
<td>ChIP</td>
<td>mPPARα prom -792/-772 5'-CCGTGAAGATCAGGAAAG-3'</td>
<td>5'-CATACGCTTCAGGGTGAG-3'</td>
</tr>
<tr>
<td></td>
<td>mPPARα prom -719/-698 5'-CTCAACCCCTGAAGGCTATG-3'</td>
<td>5'-CAACAGGTGCGCAAGGTGC-3'</td>
</tr>
</tbody>
</table>

**Online Table IV** - New primers used for RT-PCR and ChIP analyses of the present study. Some of the primers used in this study and are not included in this table were described in our previous studies\(^6,7\).
REFERENCES


