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ORIGINAL RESEARCH ARTICLE

MicroRNA-195 Regulates Metabolism in Failing Myocardium Via Alterations in Sirtuin 3 Expression and Mitochondrial Protein Acetylation

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BACKGROUND: Heart failure leads to mitochondrial dysfunction and metabolic abnormalities of the failing myocardium coupled with an energy-depleted state and cardiac remodeling. The mitochondrial deacetylase sirtuin 3 (SIRT3) plays a pivotal role in the maintenance of mitochondrial function through regulating the mitochondrial acetylome. It is interesting to note that unique cardiac and systemic microRNAs have been shown to play an important role in cardiac remodeling by modulating key signaling elements in the myocardium.

METHODS: Cellular signaling was analyzed in human cardiomyocyte-like AC16 cells, and acetylation levels in rodent models of SIRT3$^{-/-}$ and transgenic microRNA-195 (miR-195) overexpression were compared with wild type. Luciferase assays, Western blotting, immunoprecipitation assays, and echocardiographic analysis were performed. Enzymatic activities of pyruvate dehydrogenase (PDH) and ATP synthase were measured.

RESULTS: In failing human myocardium, we observed induction of miR-195 along with decreased expression of the mitochondrial deacetylase SIRT3 that was associated with increased global protein acetylation. We further investigated the role of miR-195 in SIRT3-mediated metabolic processes and its impact on regulating enzymes involved in deacetylation. Proteomic analysis of the total acetylome showed increased overall acetylation, and specific lysine acetylation of 2 central mitochondrial metabolic enzymes, PDH and ATP synthase, as well. miR-195 downregulates SIRT3 expression through direct 3′-untranslated region targeting. Treatments with either sirtuin inhibitor nicotinamide, small interfering RNA–mediated SIRT3 knockdown or miR-195 overexpression enhanced acetylation of PDH complex and ATP synthase. This effect diminished PDH and ATP synthase activity and impaired mitochondrial respiration.SIRT3$^{-/-}$ and miR-195 transgenic mice consistently showed enhanced global protein acetylation, including PDH complex and ATP synthase, associated with decreased enzymatic activity.

CONCLUSIONS: Altogether, these data suggest that increased levels of miR-195 in failing myocardium regulate a novel pathway that involves direct SIRT3 suppression and enzymatic inhibition via increased acetylation of PDH and ATP synthase that are essential for cardiac energy metabolism.

Key Words: heart failure ■ metabolism ■ microRNAs ■ oxidative phosphorylation ■ sirtuins

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Clinical Perspective

What Is New?

- Acetylome analysis of failing human myocardium revealed hyperacetylation of mitochondrial enzymes including pyruvate dehydrogenase and ATP synthase.
- This was accompanied by induction of microRNA-195 (miR-195), decreased mitochondrial deacetylase sirtuin 3 (SIRT3), and global protein hyperacetylation.
- miR-195 suppresses SIRT3 expression through direct 3′-untranslated region targeting.
- Pharmacological inhibition of SIRT3, SIRT3 knockdown, and miR-195 overexpression all enhanced pyruvate dehydrogenase and ATP synthase acetylation, diminished enzymatic activity, and impaired mitochondrial respiration.
- SIRT3$^{-/-}$ and miR-195 transgenic mice showed hyperacetylation and decreased pyruvate dehydrogenase and ATP synthase enzymatic activity.
- Thus, a novel pathway of cardiac energy metabolism controls SIRT3 suppression and hyperacetylation of pyruvate dehydrogenase and ATP synthase through miR-195 in failing myocardium.

What Are the Clinical Implications?

- Our data reveal a novel molecular pathway controlling cardiac metabolism in failing myocardium.
- We demonstrate an important mechanistic role of a specific miR-195 controlling cardiac energy metabolism through the induction of hyperacetylation of key mitochondrial enzymes in human heart failure.
- This suggests miR-195 as potential therapeutic target in heart failure.
- Furthermore, we show that protein hyperacetylation resulting from dysregulated sirtuin deacetylase levels is a distinct molecular regulator of mitochondrial function.
- Altogether, this study identifies several new targets contributing to impaired energy metabolism in the failing myocardium.

Heart failure (HF) is a clinical syndrome with rising incidence and prevalence and high morbidity and mortality rates. Because of cardiac dysfunction, multiple organ systems are affected resulting in malfunction with devastating consequences.[1] Besides functional and structural changes in the failing myocardium, cardiac metabolism is impaired, resulting in energy depletion.[2] Through physiological myocardial energy metabolism, the heart acquires 6 kg of ATP per day to sustain its regular functions. A deprivation of cardiac ATP stores is tightly coupled to progressive mechanical failure.[2] Mitochondrial metabolic dysfunction plays a central role, because it constitutes the cellular organelle that coordinates multiple metabolic systems and enzymes involved in substrate utilization and oxidative phosphorylation. The primary sources for ATP synthesis in the normal myocardium are fatty acids (≈70%) with glucose, lactate, and ketone bodies accounting for the rest.[3] In the failing myocardium, there is a shift toward enhanced glycolytic flux[4] and reduced fatty acid oxidation,[5–7] oxidative phosphorylation, and ATP synthesis[8–10].

Protein acetylation is the transfer of an acetyl group from acetyl coenzyme A (CoA) to the ε-amino group on lysine residues that neutralizes the positive charge of lysine. This molecular mechanism was first discovered as a posttranslational modification affecting chromatin remodeling and transcription in histones.[11,12] Recent studies revealed that acetylation is also a widespread and evolutionarily conserved posttranslational modification of extranuclear proteins[13]. This modification regulates several cellular functions such as energy production, oxidative stress, angiogenesis, autophagy, and cell death and survival.[14] High-throughput proteomic assessments showed that protein acetylation has profound regulatory consequences in enzymes involved in major metabolic pathways.[13,15–17]

In mammalian cells, 7 sirtuin (SIRT1–7) homologs of the yeast *Sirt2* gene regulate protein translational modifications including acetylation, ADP-ribosylation, malonylation, and succinylation.[18] The dependence of sirtuins on NAD^+ makes their enzymatic activity particularly sensitive to dynamic energy fluctuations in metabolism.[19,20] The sirtuin inhibitor nicotinamide (NAM) has been involved in the regulation of energetic metabolism by directly regulating sirtuin activity.[21–25] Of the SIRT family, SIRT3, SIRT4 and SIRT5 are present in mitochondria.[26–29] SIRT3 is the major mitochondrial deacetylase, whereas SIRT4 functions as an ADP-ribosyltransferase,[30,31] and SIRT5 mediates malonylation and succinylation.[32,33] Full-length SIRT3 is a 44-kDa protein with a mitochondrial localization sequence that is cleaved to generate a 28-kDa active SIRT3 deacetylase when imported into mitochondria.[34–36] SIRT3 has been shown to deacetylate and activate the enzymes of key mitochondrial metabolic pathways, such as fatty acid oxidation (long-chain acyl coenzyme A dehydrogenase[37]), tricarboxylic acid cycle (succinate dehydrogenase [38]), isocitrate dehydrogenase 2[31]), and acetyl-CoAsynthetase 2[39]), electron transport train enzymes (NDUFA9 and NDUFS1[40]), and the antioxidant manganese superoxide dismutase.[41,42] The role of SIRT3 in protein deacetylation and involvement in a wide variety of physiological functions and diseases has indicated this deacetylase as a potential therapeutic application.

MicroRNA-mediated control of metabolic processes has recently gained increasing interest. In prior studies, microRNA-195 (miR-195) has been shown to be induced in response to transverse aortic constriction[43,44]

and was shown to be a miR induced in plasma, serum, and myocardium of patients with advanced HF.[45] miR-195 is a member of the micro-15/16/195/424/497 family, stress inducible and activated in multiple diseases, such as cancers, HF, and schizophrenia.[46] Overexpression of miR-195 is sufficient to induce a dose-dependent hypertrophy in cultured cardiomyocytes, and cardio-specific miR-195 overexpression resulted in pathological cardiac growth with disorganization of cardiomyocytes and the development of HF.[43] It is interesting to note that miR-195 regulates SIRT1 by targeting the 3′-untranslated region (3′-UTR) of SIRT1 mRNA in cardiomyocytes[47,48] through a binding motif that is not unique to SIRT1.

In this article, we aimed to analyze the function of miR-195 in regulating SIRT3 expression and SIRT3-mediated deacetylation of specific targets and study the impact of acetylation on the cardiac acetylome, myocardial metabolism, and function.

METHODS

The data, analytic methods, and study materials will be or have been made available to other researchers for purposes of reproducing the results or replicating the procedure. The authors declare that all supporting data are available within the article and its online supplementary files.

Patient Cohort

Patients with advanced HF were recruited at Columbia University Medical Center. Myocardial specimens were collected from all patients (n=12) at the time of left ventricular assist device implantation for end-stage HF. Control myocardial samples (n=5) were obtained from deidentified specimens collected from nonfailing hearts determined to be unusable for cardiac transplantation because of acute recipient issues or donor coronary artery disease, but without evidence of previous cardiac disease (Table I in the online-only Data Supplement).

The present study was approved by the Institutional Review Board of Columbia University. All patients provided written informed consent before inclusion in the study.

Animal Studies

Transverse aortic constriction (TAC) and myocardial infarction (MI) models were used. Transverse aortic banding or ligation of the left descending coronary artery or sham surgery was performed in C57B/L6 mice (Jackson Laboratory; age, 10–12 weeks). Animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally. Pressure-controlled ventilation was initiated after intubation at 15 cm H_2O. After chest opening, a 9-0 prolene suture was placed around the aortic arch or around the left anterior descending coronary artery 2 mm below the left atrium and ligated. Sham surgery was performed without ligation. Chest closure was performed by suturing together adjacent ribs and the skin. Mice were euthanized 6 weeks after surgery.

In SIRT3 studies, control mice 129S1/SvlmJ and SIRT3 knockout mice 129-Sirt3<tm1.1Fwa>/J were euthanized at 10 to 12 weeks of age. In miR-195 studies, a cardiac-specific expression plasmid containing the α-myosin heavy chain, human growth hormone poly(A)+ signal and a mouse genomic fragment flanking the miR of interest was used for the generation of transgenic mice (kind donation from the Olson laboratory, UT Southwestern Dallas). Mice were monitored by echocardiography for the development of cardiac failure at 8 to 10 weeks of age, and euthanized at 10 to 12 weeks of age.

The protocol (AAAR3420) was approved by the Columbia University Institutional Animal Care and Use Committee.

Cell Culture

AC16 human cardiomyocyte-like cells[49] were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum, and grown in a CO_2 incubator maintained at atmospheric oxygen levels and 5% CO_2.

AC16 cells were exposed to 100 nmol/L angiotensin II (Ang II) (Sigma-Aldrich) or vehicle (phosphate-buffered saline [PBS]). Whole-cell lysates were prepared after 48 hours of treatment. NAM (Sigma-Aldrich) was dissolved in PBS. Cells were treated with different concentration of NAM (10, 20, and 40 mmol/L) or vehicle (PBS) as described[50] for 20 hours.

Small Interfering RNA and Vector Transfection

Cells were transfected with small interfering RNA (siRNA) targeting SIRT3 or nontargeting control siRNA (Dharmacon); Firefly/Renilla Duo-Luciferase reporter vector with SIRT3 3′-UTR sequences or vector alone; precursor miR-195 clones in nonviral vectors or precursor miR scrambled control clones (GeneCopoeia).

Knockdown of miR-195

Cultures at 30% confluence were transfected with anti-miR microRNA inhibitor of miR-195 or nontargeting control (Ambion). The anti-miR microRNA inhibitor negative control contains a random sequence validated to produce no identifiable effects on known functions of microRNAs.

Mitochondria Isolation

Cells were washed and harvested in PBS. Cell pellet collected by centrifugation (2000 rpm, 5 minutes, 4°C) were resuspended in radioimmunoprecipitation assay buffer (Thermo Scientific), supplemented with protease inhibitor (cOmplete tablet, Roche), and incubated on ice for 30 minutes. After centrifugation (13 200 rpm, 30 minutes, and 4°C) the supernatant was saved as whole-cell lysate. Mitochondria isolation kit (Thermo Scientific) was used for mitochondria fraction preparation.

Immunoprecipitation and Immunoblot Analysis

Total protein (100 μg) from whole-cell lysate or mitochondria fraction was immunoprecipitated. The extract was incubated

for 16 hours at 4°C with antiacetylated lysine (Cell Signaling) followed by addition of protein G beads and incubated further for 6 hours at 4°C. The beads were centrifuged at 2000 rpm for 2 minutes and washed 3 times in PBS buffer. The beads were recovered by centrifugation and aliquots of pellets were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. For Western blotting, antiacetylated lysine (Cell Signaling), anti-SIRT2, anti-SIRT3, anti-SIRT5, anti-SIRT6 (Cell Signaling), anti-Cytochrome c oxidase IV (Cell Signaling), Horseradish peroxidase–conjugated anti-GAPDH (Cell Signaling), anti-APT5A (Abcam), and anti-PDH cocktail antibodies (Abcam) were used for detection.

Enzymatic Activity Assay

The pyruvate dehydrogenase enzyme activity microplate assay kit (Abcam) was used. In brief, samples were prepared through detergent extraction, loaded on a capture antibody–precoated microplate, and incubated 3 hours at room temperature. Then wells were washed, and assay solution was added. Absorbance at 450 nm was measured with 20-second intervals.

ATP synthase enzyme activity microplate assay kit (Abcam) was used. In brief, samples were prepared through detergent extraction, loaded on a capture antibody–precoated microplate, and incubated 3 hours at room temperature. Then wells were washed, and lipid mix was added. Following 45 minutes of incubation at room temperature, reagent mix was mixed into each well. Absorbance at 340 nm was measured at 1-minute intervals.

Measurement of Cellular Respiration

The oxygen consumption rate was determined using a Seahorse Bioscience XF24 Extracellular Flux analyzer. AC16 cells were plated on XF24 microplates at 5.0×104 cells/well in low-glucose (1 g/L) Dulbecco modified Eagle medium supplemented with 1% fetal bovine serum, penicillin (10 U/mL), and streptomycin (10 U/mL) 24 hours before measurement. Intact cellular respiration was assayed under basal conditions (10 mmol/L D-glucose, 10 mmol/L pyruvate, 0% serum) and after the administration of various drugs as follows: mitochondrial inhibitor oligomycin (oligo) (1 μmol/L), mitochondrial uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (1 μmol/L), respiratory chain inhibitor antimycin A (1 μmol/L), and rotenone (1 μmol/L).

Respiratory parameters were quantified by subtracting respiration rates at times before and after addition of electron transport chain inhibitors according to Seahorse Biosciences; basal respiration: baseline respiration minus antimycin A–dependent respiration; ATP turnover: baseline respiration minus oligo-dependent respiration; H+ leak: oligo-dependent respiration minus antimycin A–dependent respiration; respiratory capacity: carbonylcyanide *p*-trifluoromethoxyphenylhydrazone–dependent respiration minus antimycin A–dependent respiration.

Quantitative Real-Time Polymerase Chain Reaction Assays

Equivalent amounts (2 μg) of purified RNA were used as a template to synthesize cDNA using oligo-d(T) primers and SuperScript III/RNaseOUT Enzyme Mix (Invitrogen). Relative levels were calculated using $\Delta C\tau$ method. Primer sequences are provided in Table II in the online-only Data Supplement.

For miR quantification, total RNA was purified using RNeasy Mini Kit (QIAGEN). miR abundance was assessed by quantitative real-time polymerase chain reaction using All-in-One miR qRT-PCR Reagent Kits and Validated Primers (GeneCopoeia). miR-191 was used as normalization.

Luciferase Assay

Cells were cotransfected with the luciferase constructs and either a scrambled control miR or miR-195 overexpression plasmids. Cells were harvested after 48 hours, and a dual luciferase assay was performed using a Luc-pair miR Luciferase kit (GeneCopoeia). The expression of renilla luciferase served as control.

Statistical Analysis

Results were expressed as mean±SEM. Probability values of $P<0.05$ were considered significant. Comparison between 2 groups was calculated and statistically compared using the 2-tailed Student test. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.).

RESULTS

Increased miR-195 Suppresses SIRT3 Expression and Increases Acetylation

Through an unbiased screening of myocardial and circulating miRs in patients with advanced HF and controls, we identified a cluster of miRs differentially regulated in failing myocardium and another set in the circulation through deep sequencing of noncoding RNAs.[45] miR-195 was found to be induced in the failing myocardium of patients with HF (3.04-fold increase) and in animal models of pressure-overload cardiomyopathy following transaortic banding and ischemic myocardium (2.01-fold after TAC and 2.09-fold after MI; Figure 1A). It showed high abundance suggesting that this miR may represent a molecular signature of failing myocardium.

Computational prediction of targets of miR-195 identified a putative-binding site of miR-195 in the SIRT3 mRNA 3′-UTR (http://www.microRNA.org, as shown in Figure 1B). We, therefore, performed luciferase reporter activity assays to validate this prediction. In cardiomyocyte-like AC16 cells, miR-195 or scrambled miR overexpression vectors were cotransfected with reporter constructs containing a luciferase gene under the control of either the SIRT3 3′-UTR or control vector 3′-UTR. Luciferase activity assays showed a significant repression (–31%) in firefly/renilla ratio of SIRT3 3′-UTR vector normalized with control vector in cells overexpressing miR-195 relative to the control miR (Figure 1C). This result indicates that miR-195 directly targets at SIRT3 mRNA 3′-UTR and negatively regulates SIRT3 ex-

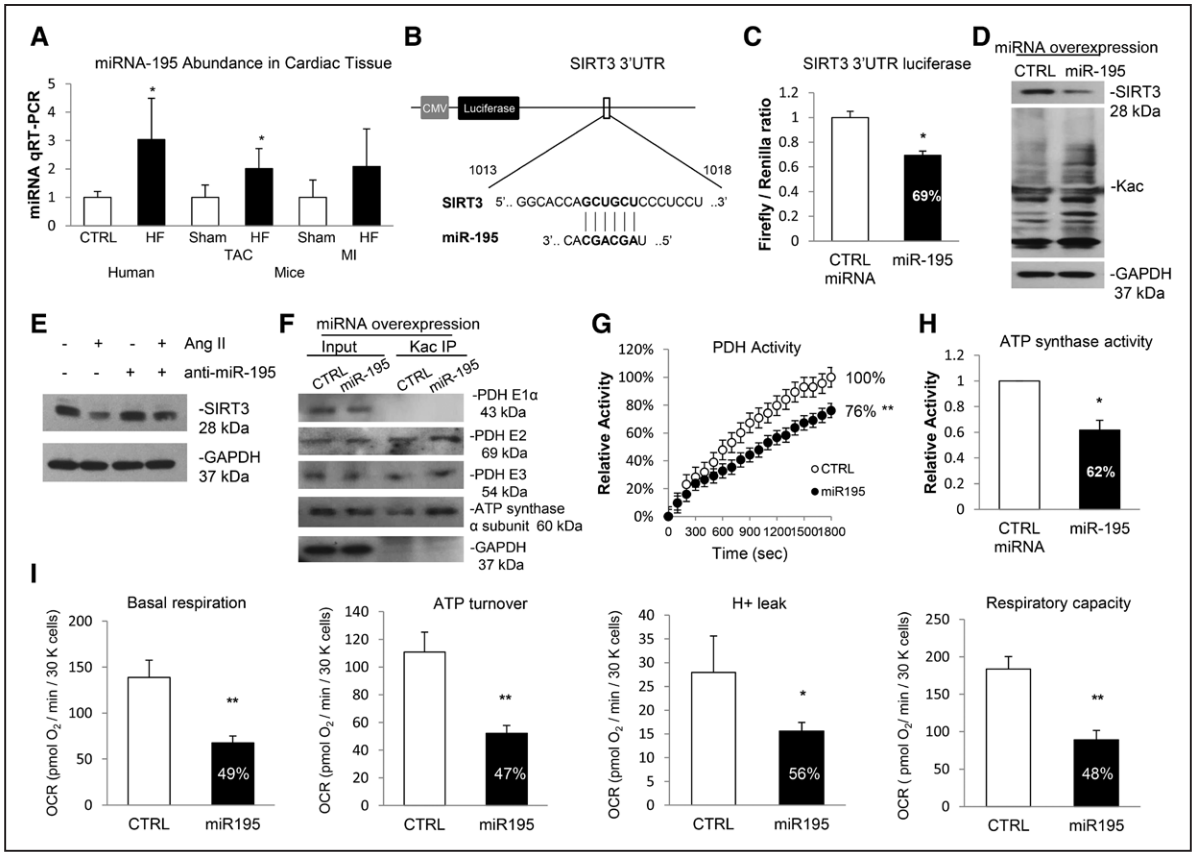

Figure 1. Increased miR-195 suppresses SIRT3 expression and increases acetylation.
A, miR-195 abundance in human cardiac tissue was assessed by qRT-PCR, which detected a 3.04-fold increase ($P<0.05$) in patients with HF. Control n=5, HF n=11. Cardiac miR-195 abundance in mouse model was assessed by qRT-PCR, which detected a 2.01-fold ($P<0.05$) and 2.09-fold elevation of the mature miR-195 in TAC HF mice and MI HF mice, in comparison with TAC Sham and MI Sham mice, respectively. miR-191 was used as normalization. **B,** Prediction of SIRT3 as a target of miR-195. The prediction was conducted by using data from microRNA target prediction systems (http://www.microRNA.org). **C,** Luciferase assay confirmed miR-195 association at SIRT3 mRNA 3′-UTR. Constructs carrying the SIRT3 3′- UTR or not (vector) were cotransfected with scramble miR control or miR-195 precursor in AC16 cells. The ratios of the firefly/renilla values for the SIRT3 construct relative to the vector construct were shown. The firefly/renilla values were calculated from 3 independent samples. Errors represent the SD derived from 3 independent experiments and $P<0.05$. **D,** miR-195 overexpression induced global protein acetylation. Cells were transfected with overexpression vector containing miR-195 precursor or scramble miR control, and the whole-cell lysates were prepared. Global acetylation level and SIRT3 expression were analyzed by Western blot, and GAPDH was used as loading control. **E,** Silencing of miR-195 rescues SIRT3 expression in response to Ang II stimulation. AC16 cells were treated with Ang II or anti-miR-195 or microRNA inhibitor negative control. Whole-cell lysates were prepared and the SIRT3 expression was analyzed by Western blot, and GAPDH was used as loading control. **F,** PDH complex and ATP synthase α-subunit were more acetylated in cells overexpressing miR-195. A representative Kac immunoprecipitation (IP) reaction from 3 independent assays was shown. Cell lysates prepared from miR-195/scramble control miR transfected cell were subjected to IP assay using antiacetylated lysine (anti-Kac). Equivalent amounts of the pellets (IP) were analyzed by Western blotting. Ten percent of the cell lysate used in the IP reaction was shown as input. **G,** miR-195 overexpression resulted in 24% decrease in PDH activity. Errors represent the SD derived from 3 independent experiments and $P<0.01$. **H,** miR-195 overexpression resulted in 38% decrease in ATP synthase activity. Errors represent the SD derived from 3 independent experiments and $P<0.05$. **I,** Basal respiration, ATP turnover, H+ leak, and respiratory capacity were all significantly decreased in AC16 cells overexpression miR-195. The OCR was measured as described before. Measurements were made in triplicate (mean and SD), and results were indicative of 3 independent experiments ($P<0.05$ or $P<0.01$). *$P<0.05$. **$P<0.01$. Ang II indicates angiotensin II; CMV, cytomegalovirus; CTRL, control; HF, heart failure; Kac, acetylated lysine; OCR, oxygen consumption rate; PDH, pyruvate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction; SIRT3, sirtuin 3; TAC, transverse aortic constriction; and 3′-UTR, 3′-untranslated region.

pression. In agreement with these data, overexpression of miR-195 resulted in a pronounced decrease in SIRT3 protein levels (–54%) accompanied by increased total protein acetylation (+53%) (Figure 1D, Figure I in the online-only Data Supplement). miR-195 expression was induced in cells transfected with miR-195 overexpres-

sion vectors as determined by quantitative real-time polymerase chain reaction (14.2-fold, Figure II in the online-only Data Supplement).

It has been reported that the expression of SIRT3 was reduced in Ang II–treated cardiomyocytes and in hearts of Ang II–induced cardiac hypertrophic mice.[51,52] It is interesting to note that other groups have shown that Ang II significantly promoted the expression of miR-195 level in mice cardiac tissues.[53,54] To investigate whether Ang II–induced miR-195 stimulation has critical impact on the downregulation of SIRT3, we assessed SIRT3 expression in cultured cells transfected with miRNA inhibitor of miR-195 in response to Ang II stimulation. Consistent with previous reports, we have confirmed the elevated miR-195 abundance in AC16 cells treated with Ang II (Figure III in the online-only Data Supplement). We also showed that the SIRT3 expression level decreased with Ang II induction (–32%). It is interesting to note that silencing of miR-195 alleviated the decrease of SIRT3 expression induced by Ang II stimulation (–12%), thereby indicating a causative role of miR-195 in modulating SIRT3 expression (Figure 1E and Figure IV in the online-only Data Supplement).

Specific hyperacetylation of pyruvate dehydrogenase (PDH) complex and ATP synthase, key enzymes of myocardial energy metabolism, were confirmed by immunoprecipitation using an antibody against acetylated lysine (Kac) followed by Western blotting, showing an increase in acetylation levels of dihydrolipoyl transacetylase (PDH E2, +75%), dihydrolipoyl dehydrogenase (PDH E3, +38%), and ATP synthase α-subunit (+77%) in response to miR-195 overexpression (Figure 1F, Figure V in the online-only Data Supplement). The enhanced acetylation resulted in a 24% decrease in PDH activity (Figure 1G) and a 38% decrease in ATP synthase activity (Figure 1H). By using a Seahorse XF24 analyzer, we quantified the respiration parameters which indicated that basal respiration (–51%), ATP production (–53%), H+ leak (–44%), and respiratory capacity (–52%) were all decreased in miR-195 overexpressed cells (Figure 1I).

Hyperacetylation and Reduced Enzymatic Activities in miR-195–Overexpressing Mice

To further investigate the role of miR-195 in vivo, we analyzed the regulation of acetylation in a cardiac-specific miR-195 overexpression mouse model. Echocardiography on animals at 8 to 10 weeks of age showed that miR-195 transgenic mice displayed decreased fractional shortening (–24%), indicating impaired cardiac function and the potential of HF during development (Figure 2A, Table III in the online-only Data Supplement). We also observed a trend toward an increase in lung weight in miR-195 overexpression mice in comparison with wild type, suggesting that the miR-195 transgenic mice have cardiomyopathy and HF (Figure VI in the online-only Data Supplement). Myocardium of miR-195 transgenic mice showed reduced SIRT3 expression (–34%) and a global increase in protein acetylation (+57%) (Figure 2B, Figure VII in the online-only Data Supplement) along with a 7.8-fold elevation in miR-195 levels (Figure VIII in the online-only Data Supplement). Immunoprecipitation assays showed increased protein acetylation of PDH E1α (+289%), PDH E2 (+151%), PDH E3 (+51%), and ATP synthase α-subunit (+53%) (Figure 2C and Figures IX and X in the online-only Data Supplement) in myocardium of miR-195 overexpression mice. PDH activity in miR-195 transgenic mice showed a 21% decrease (Figure 2D and Figure XI in the online-only Data Supplement), whereas ATP synthase activity showed a 40% decrease (Figure 2E and Figure XII in the online-only Data Supplement). Taken together, these data demonstrate a crucial role for miR-195 in cardiomyocytes and identify SIRT3 as a direct target of miR-195 leading to hyperacetylation of key metabolic proteins.

Increased Protein Acetylation and Decreased SIRT3 Expression in the Failing Myocardium

We next hypothesized that the miR-195–regulated expression of the mitochondrial deacetylase SIRT3 alters acetylation levels of key mitochondrial enzymes contributing to abnormal cardiac metabolism and function. To test our hypothesis, we analyzed SIRT3 expression and global protein acetylation in both animal models of cardiomyopathies and human failing myocardium. In myocardium of mice following TAC and MI, we detected elevated total protein acetylation (+41% after TAC and +51% after MI) that was accompanied by reduced SIRT3 levels (–19% after TAC and –39% after MI, Figure 3A and Figure XIII in the online-only Data Supplement). It is interesting to note that the myocardium of HF mice induced by TAC or MI both showed decreased SIRT3 expression levels and broad protein acetylation enhancement, indicating that the decreased SIRT3 expression and increased protein acetylation are general responses to the development of HF. In a similar manner, failing human myocardium showed broad protein acetylation levels in comparison with control myocardium (+272%, Figure 3B). Testing expression levels of various members of the sirtuin deacetylase family revealed decreased expression of SIRT3 in failing human myocardium (–46%, Figure 3B and Figures XIV and XV in the online-only Data Supplement).

We next performed a global analysis of lysine acetylation profile of mitochondrial proteins in human failing (n=4) and nonfailing myocardium (n=4). Proteomic analysis by mass spectrometry showed ly-

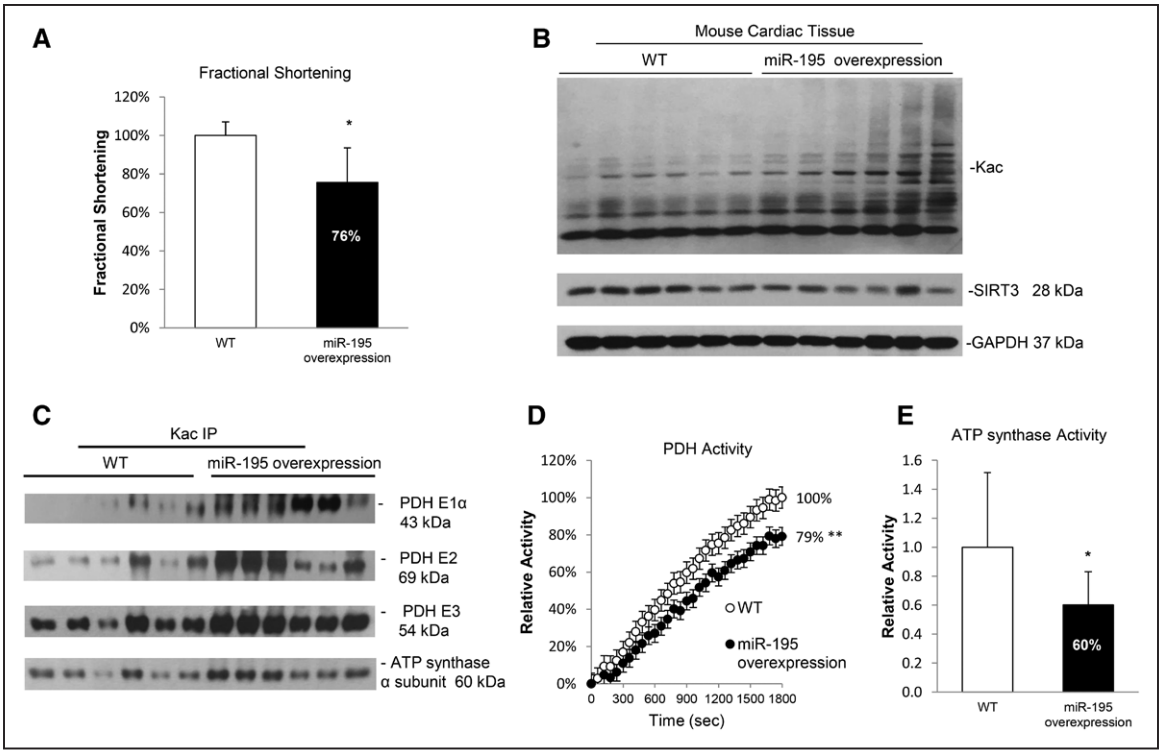

Figure 2. Hyperacetylation and reduced enzymatic activities in miR-195–overexpressing mice.
A, Echocardiographic analysis showed decreased fractional shortening ($P<0.05$) in miR-195 transgenic mice in comparison with WT littermates. Mice were monitored by echocardiography for development of heart failure at 8 to 10 weeks of age. **B,** Increased protein acetylation and decreased SIRT3 expression in miR-195 transgenic mice. Whole-cell lysates were prepared from heart tissue of WT or miR-195 overexpression mice. Global acetylation level and SIRT3 expression were analyzed by Western blotting. GAPDH was used as loading control. **C,** Induced acetylation in PDH complex and ATP synthase α-subunit in miR-195 transgenic mice. Whole-cell lysates were prepared from heart tissue of WT or miR-195 overexpression mice and subjected to IP assay using anti-Kac. Equivalent amounts of the pellets (IP) were resolved by SDS-PAGE and proteins were detected by immunoblotting. **D,** miR-195 overexpression mice showed a 21% decrease in PDH activity. Errors represent the SD derived from 3 independent experiments and $P<0.01$. **E,** miR-195 overexpression mice showed a 40% decrease in ATP synthase activity. Errors represent the SD derived from 3 independent experiments. $P<0.05$. *$P<0.05$. **$P<0.01$. IP indicates immunoprecipitation; Kac, acetylated lysine; PDH, pyruvate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SIRT3, sirtuin 3; and WT, wild type.

sine acetylation of several key enzymes involved in fatty acid and glucose metabolism (Table). Pathway network analysis for proteins with at least a 1.5-fold increase in acetylation in the failing myocardium showed that oxidative phosphorylation proteins were the most significantly acetylated (Figure 3C). Taken together, these data suggest that mitochondrial hyperacetylation in human HF is associated with changes in levels of the deacetylase SIRT3.

Inhibition of Sirtuin Deacetylase Function Increases Acetylation of Multiple Mitochondrial Proteins and Reduces Specific Enzymatic Activity of PDH and ATP Synthase

To further investigate the targets of sirtuin deacetylase, we used the potent sirtuin deacetylase inhibitor NAM in the following study. NAM may be considered a general inhibitor of the sirtuin family, and its inhibitory effects have also been demonstrated for SIRT1, SIRT2, and SIRT3.[55,56] NAM treatment (40 mmol/L) induced protein acetylation[51] in a dose-dependent manner in both total cell lysates (+67%) and the mitochondrial fraction (+174%) (Figure 4A and 4B, Figures XVI and XVII in the online only Data Supplement).

In the present study, we focused specifically on the 2 rate-limiting metabolic enzymes in acetyl coenzyme A generation and ATP production identified in the prior screening: pyruvate dehydrogenase complex and ATP synthase. Furthermore, these enzymes were shown to be hyperacetylated in miR-195 transgenic mice (Figure 2C). Immunoprecipitation assays showed that NAM treatment inhibited deacetylase activity, which increased protein acetylation of PDH E1α (+76%), PDH E2 (+117%), PDH E3 (+145%), and ATP synthase α-

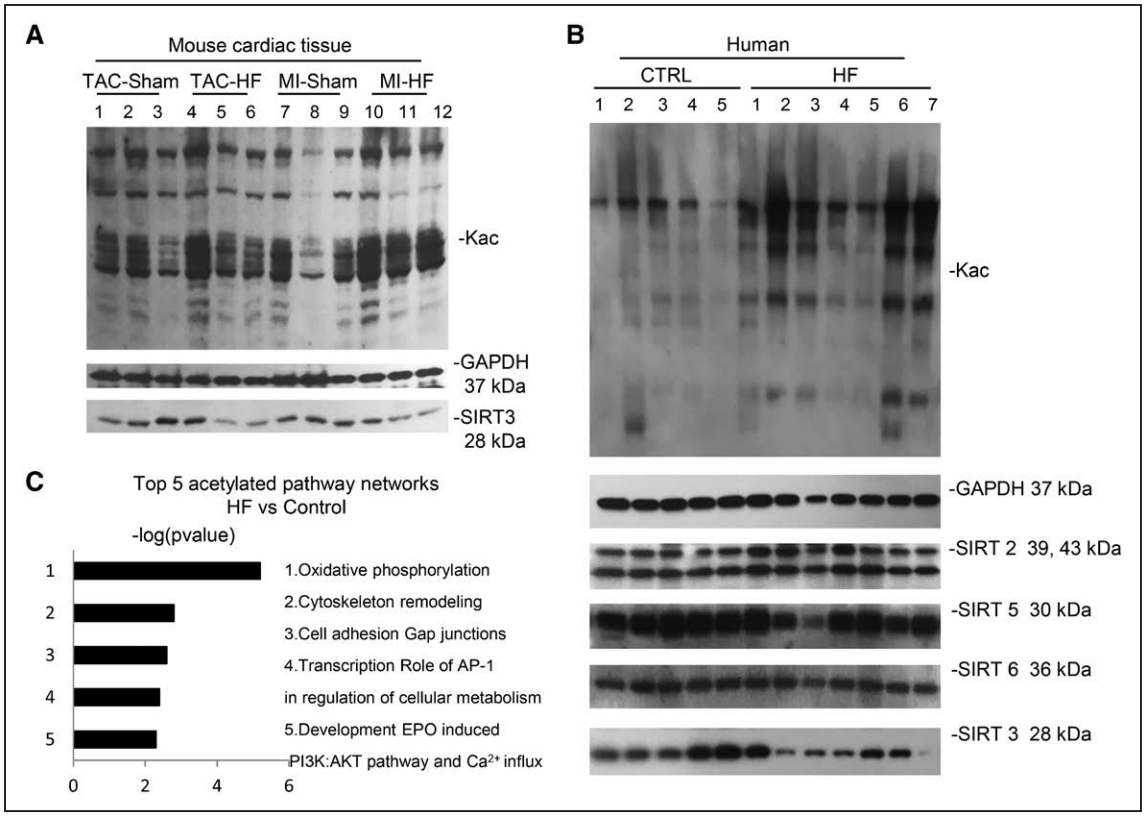

Figure 3. Increased protein acetylation and decreased SIRT3 expression in failing myocardium.
A, Increased protein acetylation and decreased SIRT3 expression in heart failure animal model. Whole-cell lysates were prepared from heart tissue of sham or TAC or MI mice. Global acetylation level and SIRT3 expression were analyzed by Western blotting. GAPDH was used as loading control. **B,** Increased protein acetylation and decreased SIRT3 expression in human samples. Whole-cell lysates were prepared from heart tissue of healthy patients or patients with HF. Global acetylation level and SIRTs expression were analyzed by Western blotting. GAPDH was used as loading control. **C,** Pathway analysis of top 5 acetylation-related pathway networks in failing myocardium in comparison with normal. Normal and failing heart tissues were homogenized and the acetylated proteins were enriched by anti-Kac antibody. Statistical analysis of the total spectra counts showed networks with at least 1.5-fold increased acetylation (P<0.01). CTRL indicates control; EPO, erythropoietin; HF, heart failure; Kac, acetylated lysine; MI, myocardial infarction; P13K, phosphatidylinositol 3-kinase; SIRT3, sirtuin 3; and TAC, transverse aortic constriction.

subunit (+50%) (Figure 4C and Figure XVIII in the online-only Data Supplement).

Consistent with these findings, acetylation of several subunits of PDH complex was associated with decreased PDH activity (–25%, Figure 4D). Given that ATP synthase is essential for mitochondrial energy generation, we tested ATP synthase activity, which was decreased in response to NAM (–45%, Figure 4E). We further measured lower oxygen consumption rate and impaired mitochondrial respiration (Figure 4F), with significant reduction in basal respiration (–45%), ATP production (–45%), H+ leak (–49%), and respiratory capacity (–47%) following NAM treatment (Figure 4G).

Because >1 SIRT protein exists in the heart, we next analyzed the specific impact of SIRT3 inhibition. Transfection with siRNA reduced SIRT3 protein expression (–93%, Figure 5A) without affecting SIRT2, SIRT5, and SIRT6 expression (Figure XIX in the online-only Data Supplement), confirming a successful specific knockdown of mitochondrial deacetylase SIRT3. siRNA-mediated SIRT3 knockdown induced total protein acetylation (+127%, Figure 5A and Figure XX in the online-only Data Supplement). Immunopecipitation assay showed that siRNA-mediated deletion of SIRT3 enhanced acetylation of PDH E1α (+29%), E2 (+124%), E3 (+46%), and ATP synthase α-subunit (+216%) (Figure 5B and Figure XXI in the online-only Data Supplement), which was further associated with a 28% decrease in PDH activity (Figure 5C) and a 40% decrease in ATP synthase activity (Figure 5D). Correlated with the enhanced acetylation state of ATP synthase, the SIRT3 knockdown resulted in lower mitochondrial respiration with reduction in basal respiration (–52%), ATP production (–52%), H+ leak (–67%), and respiratory capacity (–64%) (Figure 5E).

These data suggest that inhibition of sirtuin induces hyperacetylation of PDH complex and ATP synthase α-

Table. Proteomic Analysis of Acetylated Mitochondrial Enzymes Involved in Fatty Acid and Glucose Metabolism

Mitochondrial Enzymes With Increased Acetylation	
Enzyme Complex	**Enzyme Components and Subunits**
ECHS1	Enoyl-coenzyme A hydratase, mitochondrial ECHS1
PDH	Pyruvate dehydrogenase E1 component subunit alpha
	Pyruvate dehydrogenase E1 component subunit beta
	Dihydrolipoyl dehydrogenase, pyruvate dehydrogenase E3
ATP synthase	ATP synthase subunit alpha, mitochondrial ATP5A1
	ATP synthase subunit beta, mitochondrial ATP5B
	ATP synthase subunit d, mitochondrial ATP5H
	ATP synthase subunit e, mitochondrial ATP5I
	ATP synthase subunit g, mitochondrial ATP5L
	ATP synthase subunit O, mitochondrial ATP5O
	ATP synthase lipid-binding protein, mitochondrial ATP5G1
CPT1	Carnitine *O*-palmitoyltransferase 1
MDH	Malate dehydrogenase, mitochondrial MDH2

subunit leading to altered enzymatic activity and consequent decrease in mitochondrial function.

SIRT3 Gene Deletion Mice Showed Increased Protein Acetylation and Decreased Enzymatic Activity

To further investigate the role of SIRT3 deacetylase in vivo, heart tissue from both WT and SIRT3 gene deletion mice were tested for protein acetylation levels. We first confirmed that the SIRT3 expression was completely abolished without affecting SIRT2, SIRT5, and SIRT6 expression (Figure 6A and Figure XXII in the online-only Data Supplement). After detecting the global increase in total protein acetylation in SIRT3$^{-/-}$ mice (+174%, Figure 6A and Figure XXIII in the online-only Data Supplement), the acetylation level of SIRT3 targets, PDH complex, and ATP synthase were measured. Consistent with the results obtained by using human cardiomyocyte-like AC16 cell line, PDH E1α (+59%), E2 (+65%), E3 (+279%), and ATP synthase α-subunit (+88%) were more acetylated in SIRT3$^{-/-}$ mice (Figure 6B and Figure XXIV in the online-only Data Supplement), whereas their basal expression levels remained unchanged (Figure 6A and Figure XXIII in the online-only Data Supplement). Finally, we found that acetylation of PDH complex and ATP synthase induced by SIRT3 deletion was associated with a decrease in PDH (–40%, Figure 6C) and ATP synthase activity (–22%, Figure 6D). Together, these data suggest that SIRT3 may play a protective role in the myocardium by preventing key mitochondrial enzymes from acetylation and alteration in their activity in maintaining mitochondrial function.

Increased Acetylation and Impaired Metabolism in Failing Myocardium

We expanded this study and investigated whether PDH complex and ATP synthase have altered acetylation status and function in human failing myocardium (n=4). The expression of PDH E1α (–53%), E2 (–14%), and E3 subunit (–41%) showed a mild decrease in the heart tissue of patients with HF in comparison with normal hearts (Figure 7A and Figure XXV in the online-only Data Supplement). Moreover, an increase in the acetylation level of PDH E2 (+79%) was detected (Figure 7B, top, and Figure XXVI in the online-only Data Supplement). Both the lower expression level and the increased acetylation contribute to a reduced PDH activity in patients with HF (–56% in comparison with nondiseased myocardium; Figure 7C). In line with these changes in PDH complex, ATP synthase α-subunit also showed reduced basal expression (–39%, Figure 7A and Figure XXV in the online-only Data Supplement) and increased acetylation levels (+107%, Figure 7B, bottom, and Figure XXVI in the online-only Data Supplement), and a 25% decrease in ATP synthase activity, as well (Figure 7D), in patients with HF in comparison with normal hearts. Together, these results indicate that the alteration in PDH complex and ATP synthase function correlates with changes in both basal expression level and acetylation status contributing to mitochondrial dysfunction and cardiomyopathy.

DISCUSSION

Mitochondrial dysfunction including metabolic abnormalities and energy depletion develop in the failing myocardium and contribute to cardiac remodeling. The major finding of our study is that miR-195–controlled suppression of SIRT3 in failing myocardium results in hyperacetylation of several key mitochondrial proteins. We demonstrated that miR-195 regulates myocardial SIRT3 expression through direct targeting at the SIRT3 mRNA 3′-UTR. This effect leads to hyperacetylation of PDH and ATP synthase in failing myocardium and contributes to altered mitochondrial energy metabolism that is associated with cardiac dysfunction in humans.

We investigated the underlying mechanisms using several translational methods. First, via unbiased miR screening, we detected increased levels of miR-195 in the heart and plasma of patients with HF. Bioinformatic analysis followed by in vitro experiments in human cardiomyocyte-like AC16 cells showed that miR-195 is a direct inhibitor of SIRT3 (Figure 1). Moreover, by treating cells with the SIRT3 inhibitor NAM, SIRT3 siRNA and miR-195, we demonstrated that SIRT3 mediates deacetylation of PDH, and ATP synthase compromises their activity and impairs mitochondrial function (Figures 1, 4, and 5).SIRT3 expression was consistently reduced in human failing myocardium, in which we also observed in-

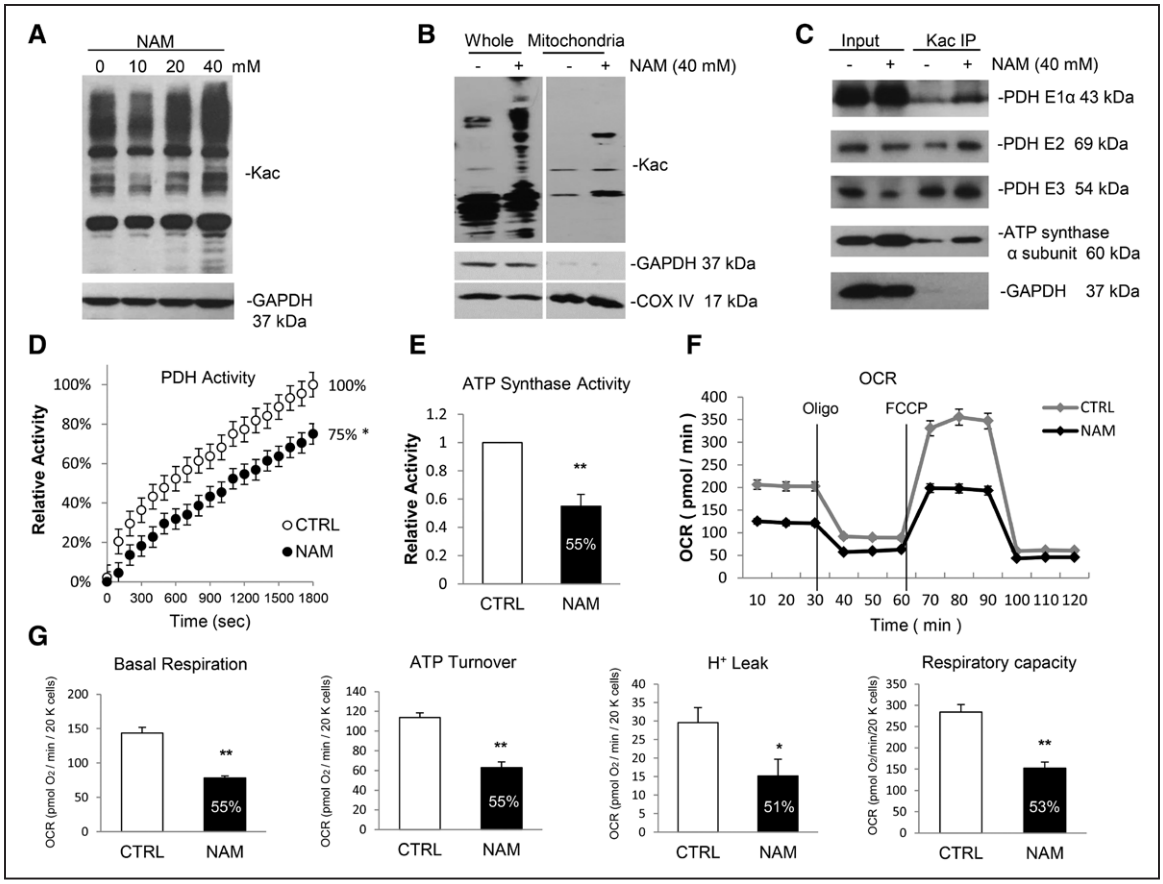

Figure 4. Sirtuin inhibitor nicotinamide (NAM) induces acetylation of multiple mitochondrial proteins and reduces enzymatic activity.

A, NAM induced acetylation in the AC16 cell line. Cells were treated with increasing concentration of NAM or vehicle (PBS). Global acetylation level was analyzed by Western blot. **B,** NAM induces acetylation of mitochondrial proteins. Whole-cell lysates and mitochondrial fraction were isolated for Western blotting analysis. **C,** Induced acetylation in PDH complex and ATP synthase α-subunit in response to NAM treatment. AC16 cells were treated with NAM or vehicle; cell lysates were prepared and subjected to IP assay using anti-Kac. Equivalent amounts of the pellets (IP) were resolved by SDS-PAGE and proteins were detected by immunoblotting. Ten percent of the cell lysate used in the IP reaction was shown as input. **D,** Twenty-five percent decrease in PDH activity in response to NAM treatment. PDH activity was measured following 20 hours of NAM treatment or vehicle. Errors represent the SD derived from 3 independent experiments and $P<0.05$. **E,** 45% decrease in ATP synthase activity in response to NAM treatment. ATP synthase activity was measured following 20 hours of NAM treatment. Errors represent the SD derived from 3 independent experiments and $P<0.01$. **F,** Oxygen consumption rate (OCR) was decreased in NAM-treated cells. The AC16 cells were seeded 24 hours before being analyzed by a Seahorse XF24 Analyzer. The OCR was measured continuously throughout the experimental period at baseline and in the presence of the indicated drugs. **G,** Basal respiration, ATP turnover, H+ leak, and respiratory capacity were all significantly decreased in NAM-treated AC16 cells in comparison with control. Measurements were made in triplicate (mean and SD), and results are indicative of 3 independent experiments ($P<0.05$ or $P<0.01$). *$P<0.05$. **$P<0.01$. COX IV indicates cytochrome c oxidase IV; CTRL, control; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (1 μmol/L); IP, immunoprecipitation; Kac, acetylated lysine; Oligo, oligomycin (1 μmol/L); PBS, phosphate-buffered saline; PDH, pyruvate dehydrogenase; ROT+AA, mixture of rotenone (1 μmol/L) and antimycin A (1 μmol); and SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

creased acetylation and decreased activities of PDH and ATP synthase (Figures 3 and 7). We also showed that miR-195 transgenic mice exhibit reduced SIRT3 expression levels associated with a global increase in total protein acetylation in comparison with WT mice (Figure 2). This finding links the previously described cardiomyopathic phenotype of these mice[43] to suppression of SIRT3, hyperacetylation of key metabolic enzymes, and energy depletion. Finally, SIRT3 knockout mice showed increased acetylation levels of PDH and ATP synthase that contributed to a decrease in enzymatic activity (Figure 6). Taken together, our data suggest a novel pathway of combined epigenetic, transcriptional, and posttranslational regulation of proteins that are involved in cardiac energy metabolism during HF development and progression. This pathway involves miR-195–mediated SIRT3 suppression and increased protein acetylation of PDH and ATP synthase. These changes inhibit PDH and ATP synthase ac-

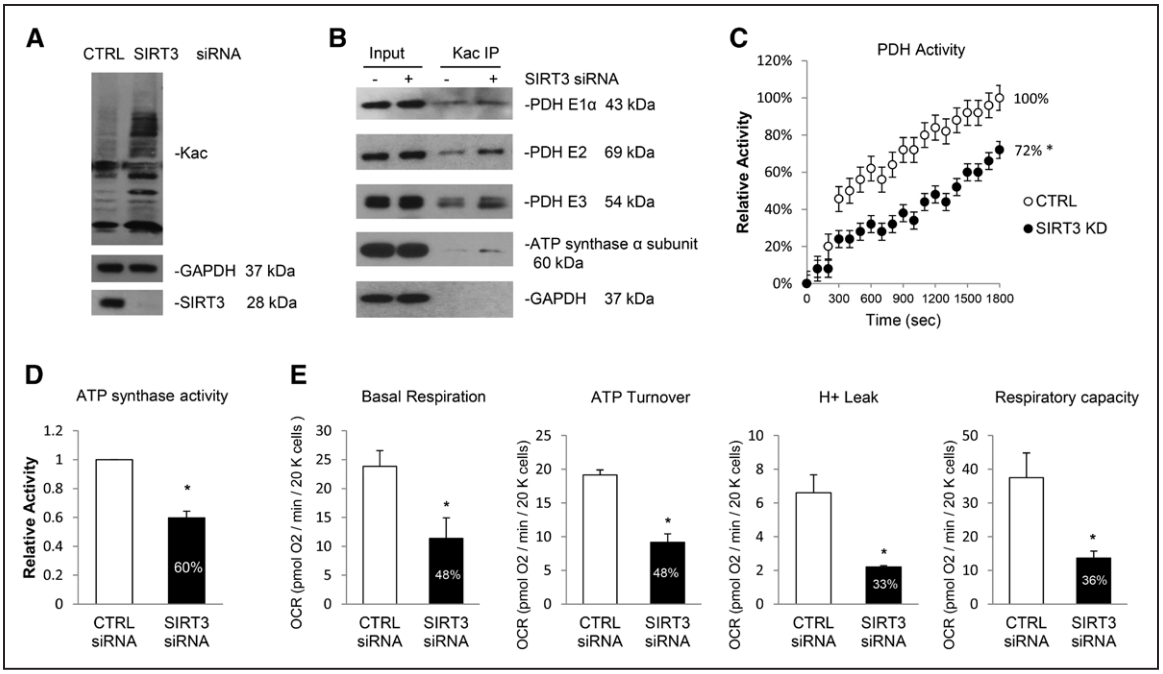

Figure 5. SIRT3 deacetylates multiple mitochondrial proteins and regulates mitochondrial metabolism. **A,** SIRT3 knockdown induced global protein acetylation. Cells were transfected with siRNAs targeting SIRT3 or nonspecific sequence, and the whole-cell lysates were prepared. Global acetylation level and SIRT3 expression were analyzed by Western blot, and GAPDH was used as loading control. **B,** PDH complex and ATP synthase α-subunit were more acetylation in SIRT3-depleted cells. A representative IP reaction from 3 independent assays was shown. Cell lysates prepared from SIRT3/CTRL siRNA-treated cells were subjected to IP assay using anti-Kac. Equivalent amounts of the pellets (IP) were analyzed by Western blotting as described above. Ten percent of the cell lysate used in the IP reaction was shown as input. **C,** SIRT3 knockdown resulted in 28% decrease in PDH activity. Errors represent the SD derived from 3 independent experiments and $P<0.05$. **D,** SIRT3 knockdown resulted in 40% decrease in ATP synthase activity. Errors represent the SD derived from 3 independent experiments and $P<0.05$. **E,** Basal respiration, ATP turnover, H+ leak, and respiratory capacity were all significantly decreased in AC16 cells depleted with SIRT3. The OCR was measured as described previously. Measurements were made in triplicate (mean and SD), and results were indicative of 3 independent experiments ($P<0.05$). *$P<0.05$. CTRL indicates control; IP, immunoprecipitation; Kac, acetylated lysine; KD, knockdown; OCR, oxygen consumption rate; PDH, pyruvate dehydrogenase; siRNA, small interfering RNA; and SIRT3, sirtuin 3.

tivity leading to impaired energy metabolism and ATP deprivation.

Mitochondria are the main cellular organelles responsible for energy balance and metabolism homeostasis, and are implicated in several human diseases. The mitochondrial deacetylase SIRT3 plays a pivotal role in the maintenance of mitochondrial function. Several recent studies revealed SIRT3 as a major regulator of the mitochondrial acetylome controlling dynamics of metabolic reprogramming and antioxidant defense mechanisms by targeting a series of key modulators and their relevant pathways. For example, acetyl-CoA synthase 2, 3-hydroxy-3-methylglutaryl-CoA synthase 2, long-chain acyl-CoA dehydrogenase, isocitrate dehydrogenase 2, glutamate dehydrogenase, NDUFA9 subunit of complex I of the electron transport train, succinate dehydrogenase (succinate dehydrogenase, complex II), OSCP (oligomycin sensitivity-conferring protein), ornithine transcarbamoylase, manganese superoxide dismutase, and cyclophilin D have all been identified as targets of SIRT3, which modulates their enzymatic activity by deacetylation.[31,37–42,57–60] The absence of SIRT3 contributes to a striking hyperacetylation of mitochondrial proteins with metabolic perturbations, and ultimately increases the susceptibility and progression of metabolic syndrome.[57,58]

Most studies have shown that SIRT3-mediated deacetylation contributes to the activation of metabolic enzymes and pathways. Our data demonstrate that loss of SIRT3 leads to the hyperacetylation of PDH and ATP synthase that is associated with decreased enzymatic activity and impaired mitochondrial respiration. However, conflicting results suggesting a positive correlation between acetylation and enzymatic activity have also been observed. SIRT3 has an important role in mitochondria function as it deacetylates and inhibits cyclophilin D activity.[59,60] Moreover, Zhao et al[61] showed that acetylation of enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, a fatty acid β-oxidation–associated enzyme, contributes to its enzymatic activity enhancement in cardiac myocytes. Long-chain acyl coenzyme A dehydrogenase acetylation has also been demonstrated to increase its activity and accelerate the fatty acid β-oxidation in the heart,[62] where-

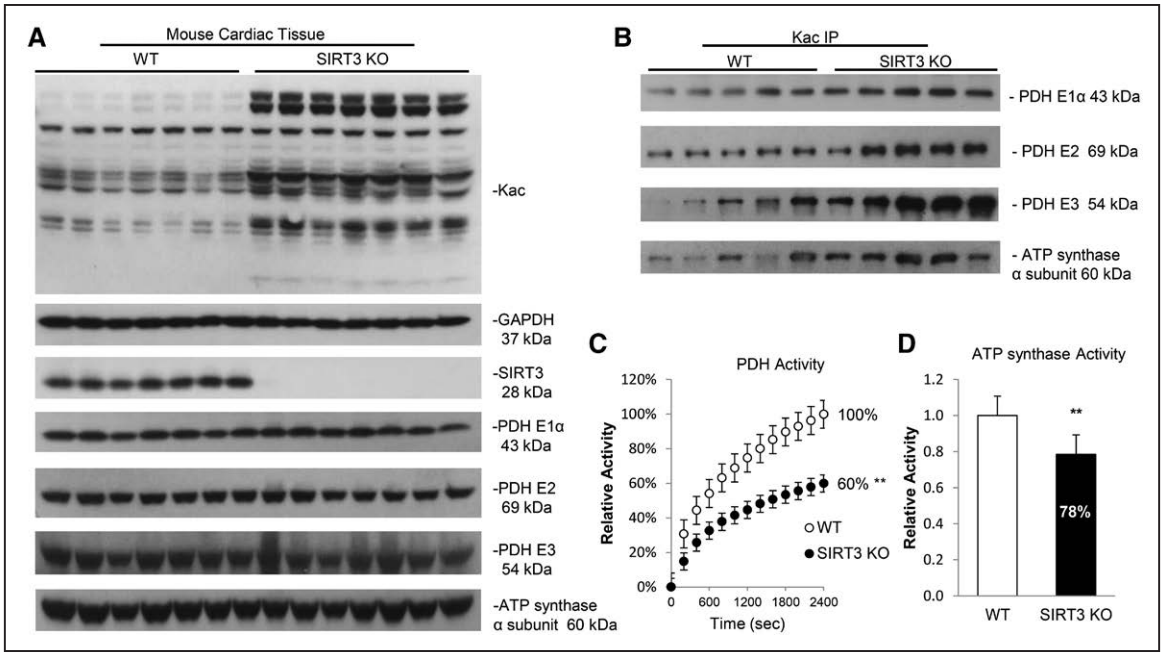

Figure 6. SIRT3 knockout (KO) mice showed increased protein acetylation and decreased enzymatic activity. **A,** Total protein acetylation level strikingly increased in SIRT3 KO mice. Whole-cell lysates were prepared from heart tissue of WT or SIRT3 KO mice. Global acetylation level, expression of SIRT3, PDH complex subunits, and ATP synthase α-subunits were analyzed by Western blotting. GAPDH was used as loading control. **B,** PDH complex and ATP synthase α-subunit were more acetylated in SIRT3 KO mice. Cell lysates prepared from WT or SIRT3 KO mice were subjected to IP assay by using anti-Kac. Equivalent amounts of the pellets (IP) were analyzed by Western blotting as describe above. Ten percent of the cell lysate used in the IP reaction was shown as input. **C,** SIRT3 KO mice showed 40% decrease in PDH activity. Errors represent the SD derived from 3 independent experiments and $P<0.01$. **D,** SIRT3 KO mice showed 22% decrease in ATP synthase activity. Errors represent the SD derived from 3 independent experiments and $P<0.01$. $^{**}P<0.01$. IP indicates immunoprecipitation; Kac, acetylated lysine; PDH, pyruvate dehydrogenase; SIRT3, sirtuin 3; and WT, wild type.

as another group reported that the hyperacetylation of long-chain acyl coenzyme A dehydrogenase reduces its enzymatic activity.[37] It remains to be explored whether the opposite effect of SIRT3-mediated metabolic alterations is associated with heart disease type specificity, cellular conditions, or microenvironment. Further studies are necessary to examine the dual regulatory roles of SIRT3 on its targets in different models of heart disease, and discuss its potential translation into therapy of HF.

The failing myocardium is characterized by changes in energy metabolism, a shift from fatty acid to glucose utilization for ATP production and overall energy depletion.[4,63] As part of these changes, we have also observed altered expression of human genes participating in mitochondrial metabolic flux in response to miR-195 overexpression (Figure XXVII in the online-only Data Supplement), which is in line with previous reports on myocardial lipotoxicity in advanced HF and the corrective impact of mechanical unloading.[63] During HF progression, myocardial metabolism is characterized by a reduction of overall oxidative capacity, reduced fatty acid and glucose oxidation rates, and an increased rate of glycolysis with resulting impairments in ATP production.[2,64,65] Alternative substrates such as ketone bodies and lactate have been postulated as additional sources of ATP production in the failing myocardium.[64,66,67] The majority of cardiac ATP production originates from mitochondrial metabolic flux through various pathways, in which SIRT3 plays a central regulatory role. It is interesting to note that the dependence of sirtuin activity on NAD^+ suggests that mitochondrial SIRT3, the metabolic sensor of changes in the energy status, might coordinate global shifts in metabolic pathways and mediate the beneficial effects in energy homeostasis accordingly, especially during energetic deficiency stages, such as caloric restriction.[68] Reduced SIRT3 expression has also been observed in obesity and diabetes mellitus,[69,70] in postinfarction HF,[71] TAC-induced HF,[72] and Dahl salt-sensitive and spontaneously hypertensive rats.[73] Loss of SIRT3 in cardiomyocytes contributes to the development of cardiac hypertrophy and HF accompanied by hyperacetylation of numerous key metabolic enzymes and proteins participating in oxidative stress.[74–76] Conversely, SIRT3-overexpressing mice are protected from hypertrophy.[42] During caloric restriction, SIRT3 mediates reprogramming in cardiac energy metabolism to allow respiration, by promoting glucose utilization, fat acid oxidation, and amino acid metabolism that fuels the tricarboxylic acid cycle and facilitates electron transport train–mediated ATP production to overcome the low

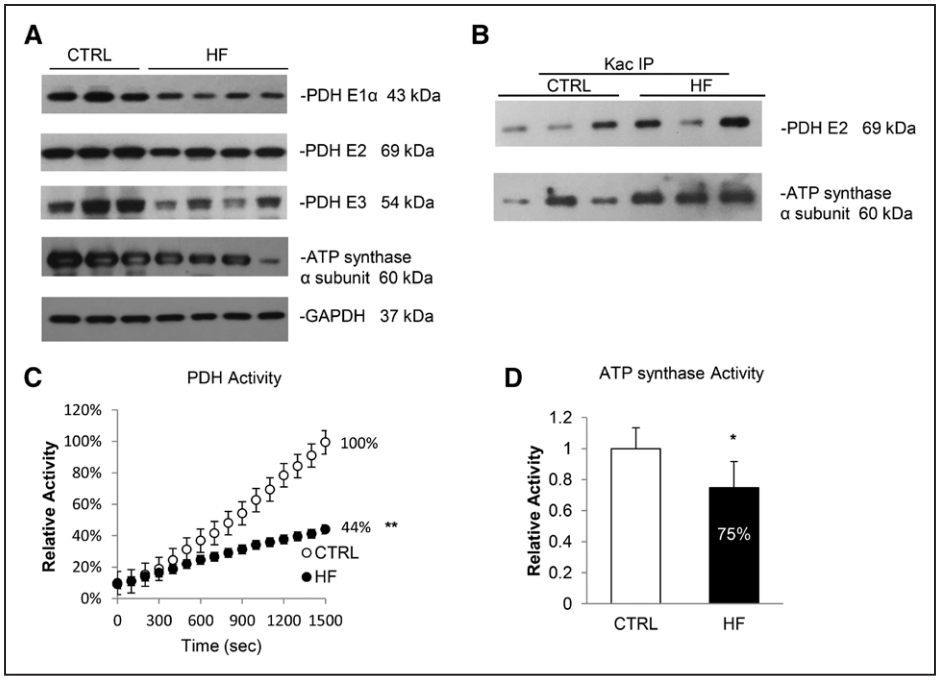

Figure 7. Increased acetylation and impaired metabolism in failing myocardium.
A, PDH complex subunits and ATP synthase α-subunits were less expressed in failing myocardium. Whole-cell lysate prepared from heart tissues of healthy patients or patients with HF were analyzed by Western blotting. GAPDH was used as loading control. **B,** PDH E2 and ATP synthase α-subunit were more acetylated in failing myocardium. Cell lysates prepared from cardiac tissues were subjected to IP assay using anti-Kac. Equivalent amounts of the pellets (IP) were analyzed by Western blotting as describe above. Ten percent of the cell lysate used in the IP reaction was shown as input. **C,** PDH activity was 56% reduced in patients with HF. Errors represent the SD derived from 3 independent experiments and $P<0.01$. **D,** ATP synthase activity was 25% reduced in patients with HF. Errors represent the SD derived from 3 independent experiments and $P<0.05$. *$P<0.05$. **$P<0.01$. CTRL indicates control; HF, heart failure; IP, immunoprecipitation; Kac, acetylated lysine; and PDH, pyruvate dehydrogenase.

energy input.[58,77] Because the derangement of these metabolic pathways and energy perturbations are associated with the progression of HF, pharmacological activators of SIRT3 might potentially ameliorate disease-specific patterns.

We have demonstrated that SIRT3 mediated deacetylation of PDH complex and ATP synthase α-subunit. This posttranslational modification alters their enzymatic activity individually and the mitochondrial respiration as the functional consequence. However, the critical lysine residues targeted for acetylation have not been determined yet. Recent studies have suggested that Lys321 of PDH E1α-subunit,[78] Lys259 and Lys480 of ATP synthase β-subunit,[79] and Lys139 of OSCP of ATP synthase[80] are potential regulatory targets. Future studies will aim at elucidating target Lys residues on PDH complex E2/E3 and ATP synthase α, the acetylation status of which is important for their enzymatic activity.

The stress-responsive miR-195 has been suggested to associate with the cardiac remodeling and the development of heart diseases. It has been shown that miR-195 expression is induced in cardiac tissue from mice in response to TAC,[43] and miR-195 is upregulated both during the early hypertrophic growth phase and the late stage of HF.[44]We also consistently observed that TAC and MI downregulate SIRT3 expression and result in global protein hyperacetylation (Figure 3A and 3B) which might be attributable to enhanced miR-195 abundance. These studies suggested unique cardiac and systemic miRs are important in cardiac remodeling and miR-195 is an excellent biomarker and genetic signature of cardiac stress profiles. Furthermore, overexpression of miR-195 is sufficient to induce cardiac hypertrophy and HF in cultured cardiomyocytes and transgenic mice,[43] indicating the specific functional effect of this miR in hypertrophic growth. Although miR-195–mediated regulation of SIRT1 has been demonstrated,[47,48] SIRT1 and SIRT3 might coordinate well-orchestrated networks to regulate cardiac stress response.

In conclusion, we here show a novel regulatory pathway in the failing myocardium that involves miR-195–controlled suppression of SIRT3 leading to hyperacetylation and mitochondrial dysfunction. These findings might provide novel targets for selective therapeutic interventions in the syndrome of HF.

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Disclosures

None.

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Circulation

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SUPPLEMENTAL MATERIAL

miR-195 Regulates Metabolism in Failing Myocardium via Alterations in SIRT3 Expression and Mitochondrial Protein Acetylation

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Supplemental Table 1.
Patient Demographic

	HF patients
n	18
Age	60.3 ± 12.4
Gender	16 M / 2 F
BMI	27.4 ± 4.9
SBP	96.9 ± 13.2
DBP	62.9 ± 12.0
EF (%)	23.0 ± 14.4
ICM	56%
DCM	44%
HTN	50%
DM	28%
HLP	61%

BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; EF: Ejection Fraction; ICM: Ischemic Cardiomyopathy; DCM: Dilated Cardiomyopathy; HTN: Hypertension; DM: Diabetes Mellitus; HLP: Hyperlipidemia.

Supplemental Table 2.
Primer sequences

Gene	Sequence
ATGL	Forward: 5’-GAAGATCACGTCCTGGAGCA-3’ Reverse: 5’-ACAGGCAGCATGTTGGAGAG-3’
HSL	Forward: 5’-CGCCACATGAGAAAACCAGT-3’ Reverse: 5’-CTATGTTGTCCTCCGCCAGA-3’
CD36	Forward: 5’-CTACCACAGTTGGTCTG-3’ Reverse: 5’-GCTGCATCTGTACCATTAATC-3’’
GLUT4	Forward: 5’-CCATTCCTTGGTTCATCGTG-3’ Reverse: 5’-TAGCCTCCGCAACATACTGG-3’
PDK4	Forward: 5’-GGTGGTGTTCCCCTGAGAAT-3’ Reverse: 5’-CAGACGAGAAATTGGCAAGC-3’

Supplemental Table 3.
Echocardiographic analysis of miR-195 transgenic mice and WT littermates

mice	AW	PW	LVEDD	LVESD	FS
WT	0.63±0.07	0.72+0.07	3.79+0.24	2.39+0.21	36.96%+2.60%
miR-195 overexpression	0.66±0.04	0.66+0.03	3.99±0.28	2.89±0.46	27.93%±6.67% *

* $p<0.05$ compared to WT

Supplemental Figures

Fig 1

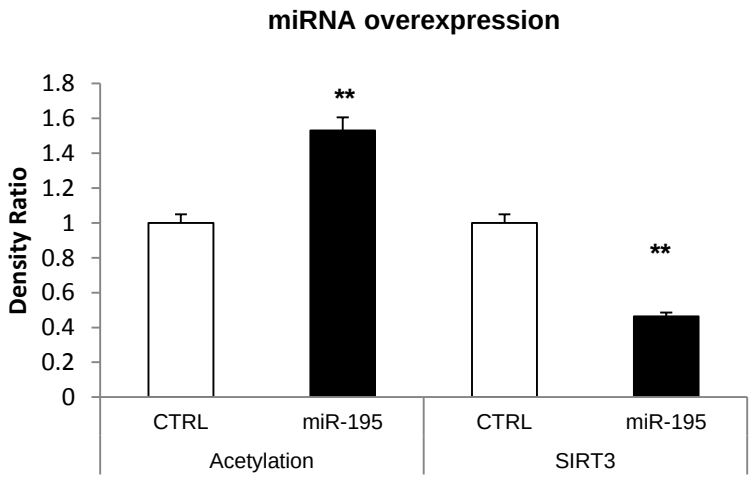


Fig 2

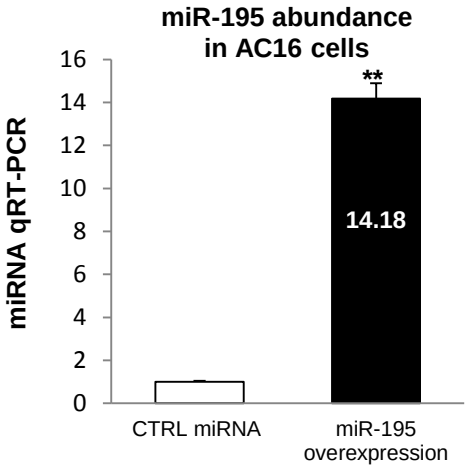


Fig 3

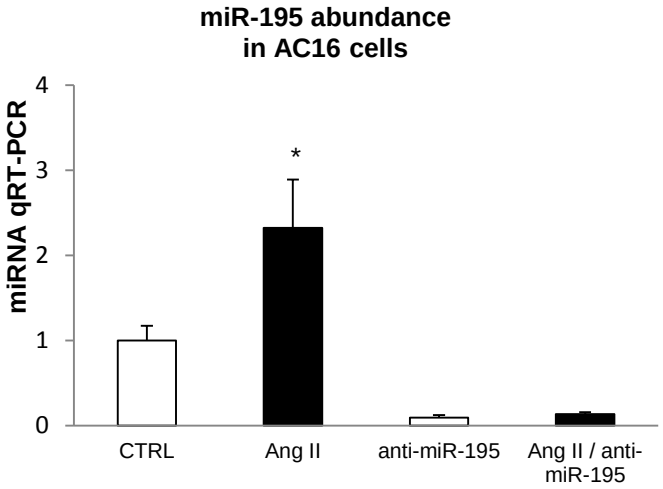
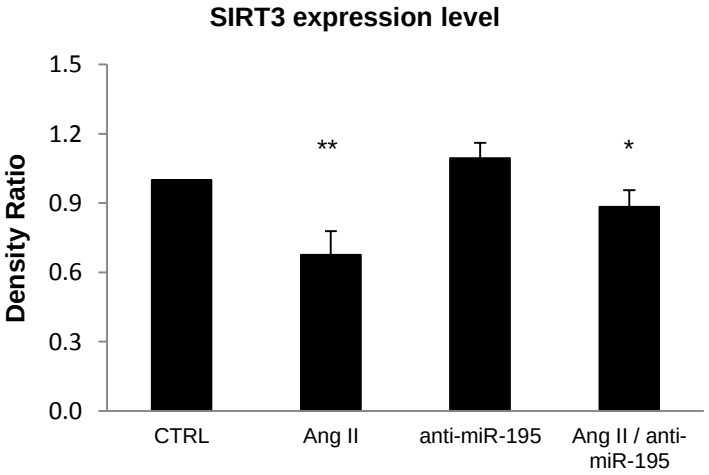


Fig 4



Supplemental Figures

Fig 5

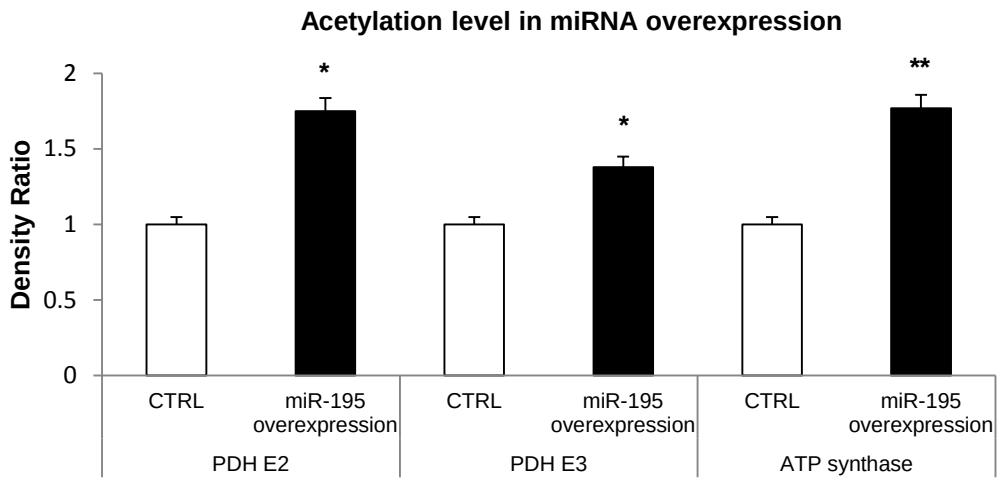


Fig 6

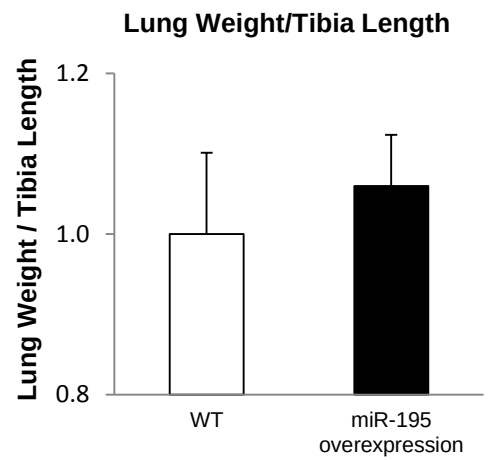


Fig 7

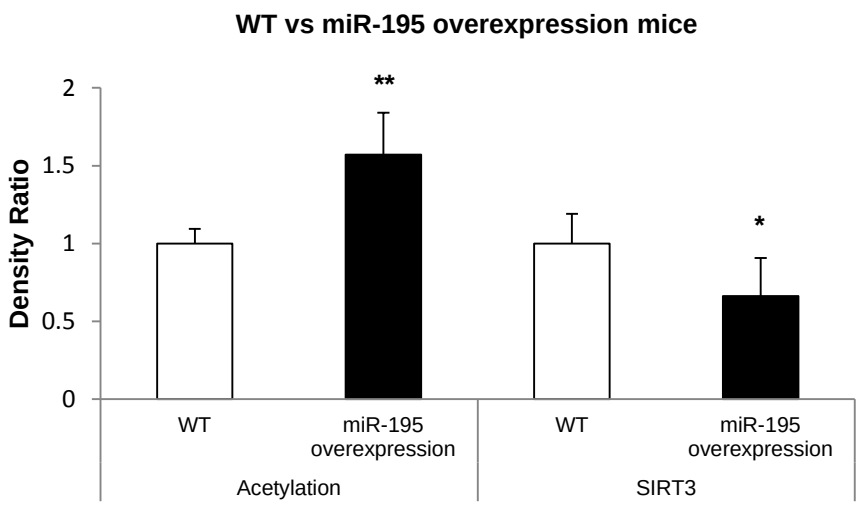
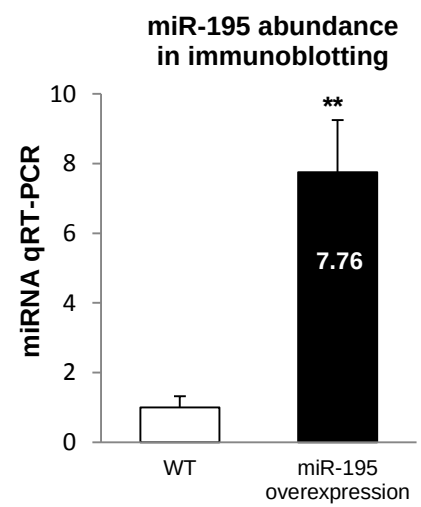


Fig 8



Supplemental Figures

Fig 9

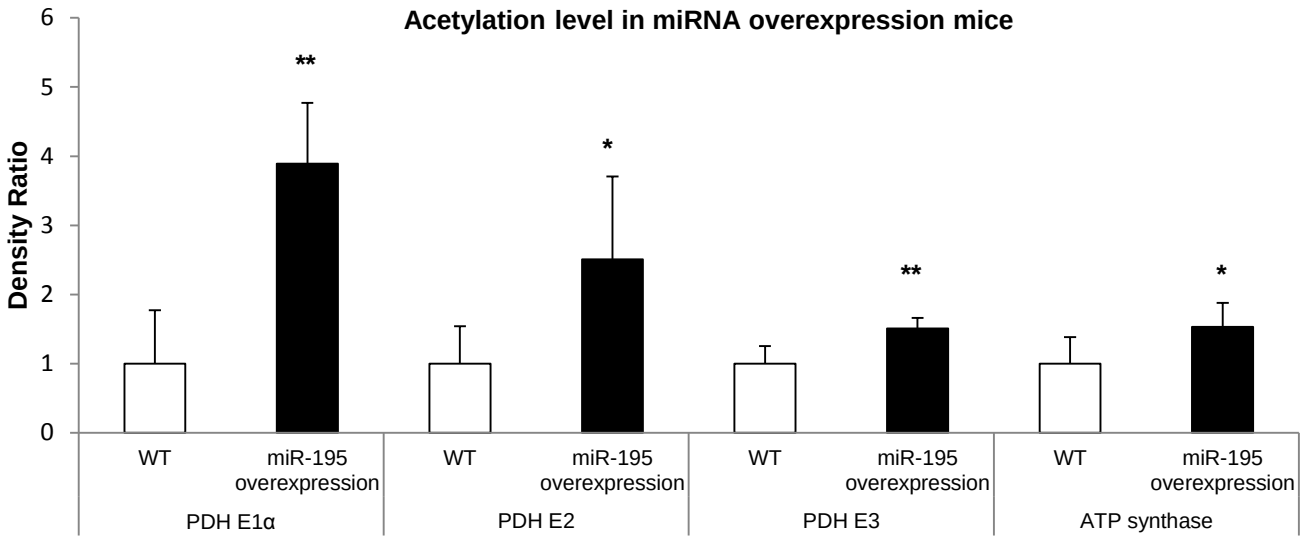


Fig 10

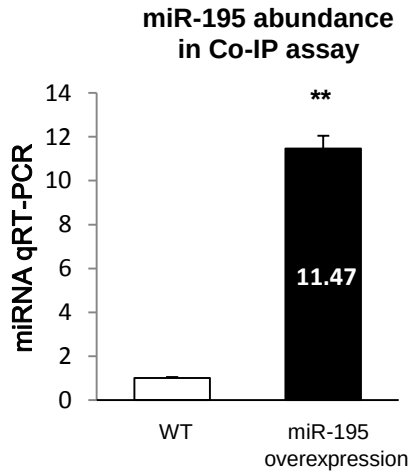


Fig 11

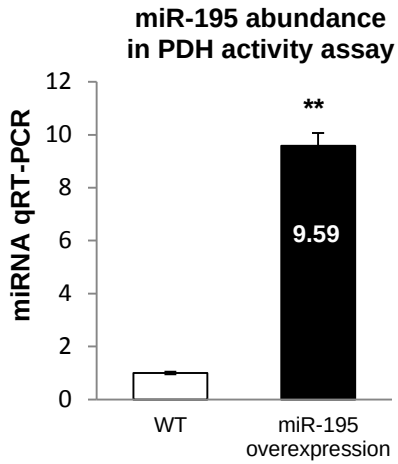
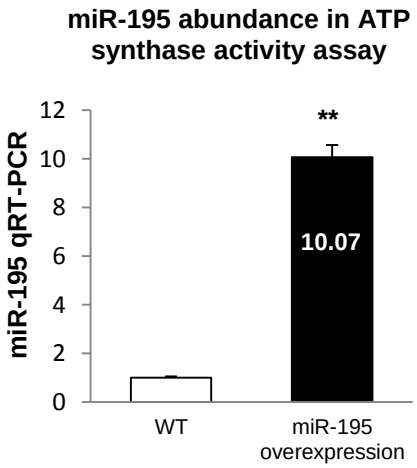


Fig 12



Supplemental Figures

Fig 13

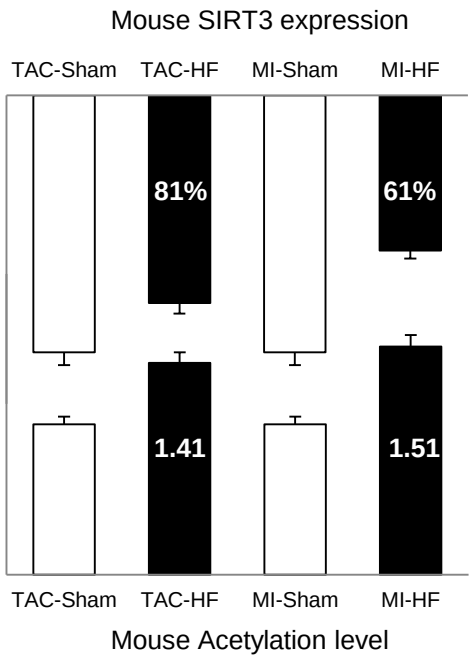


Fig 14

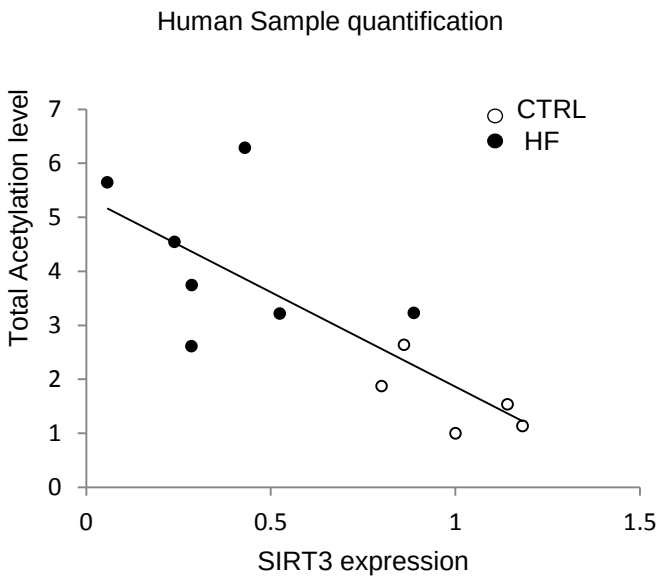


Fig 15

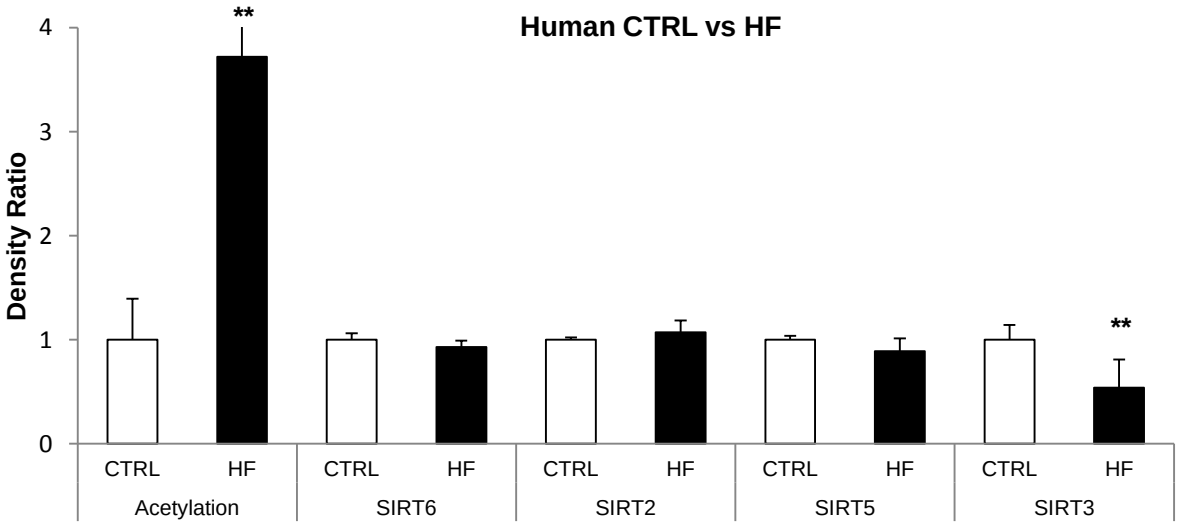
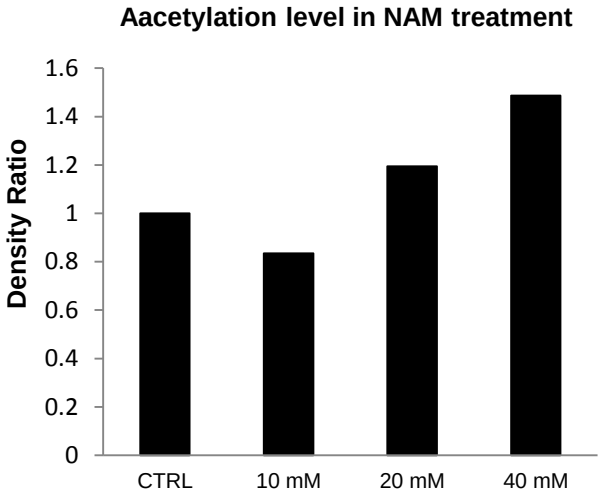


Fig 16



Supplemental Figures

Fig 17

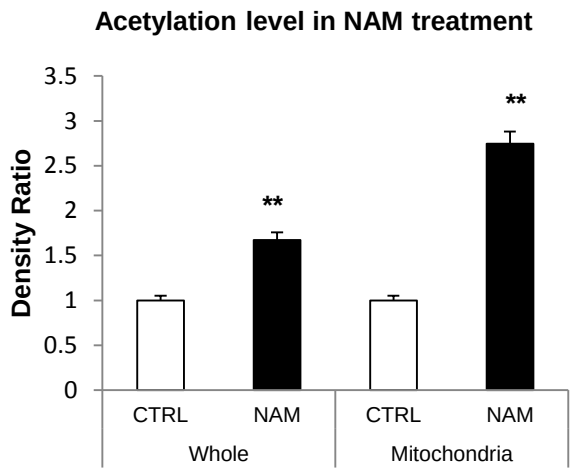


Fig 18

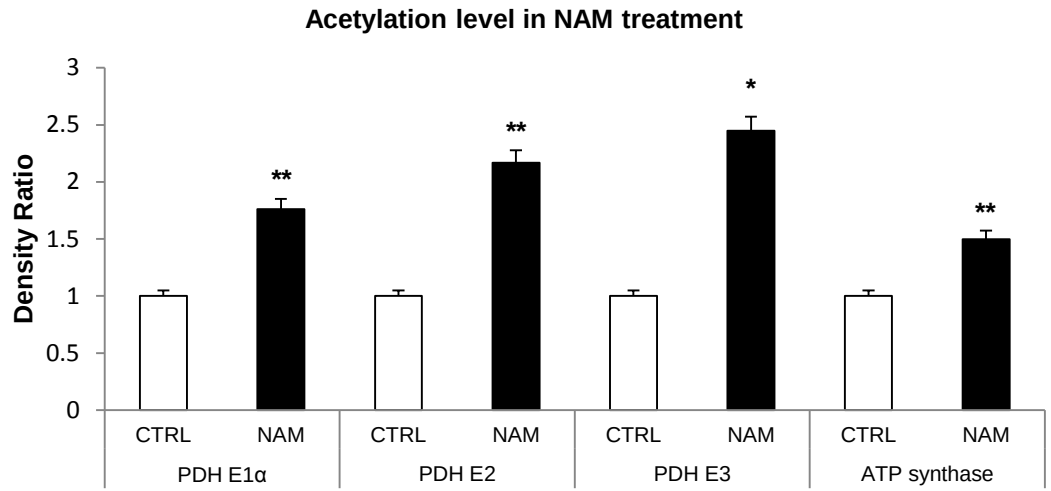


Fig 19

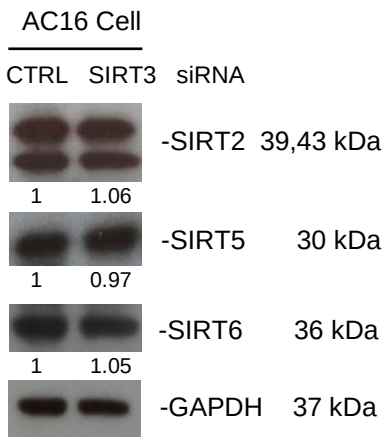
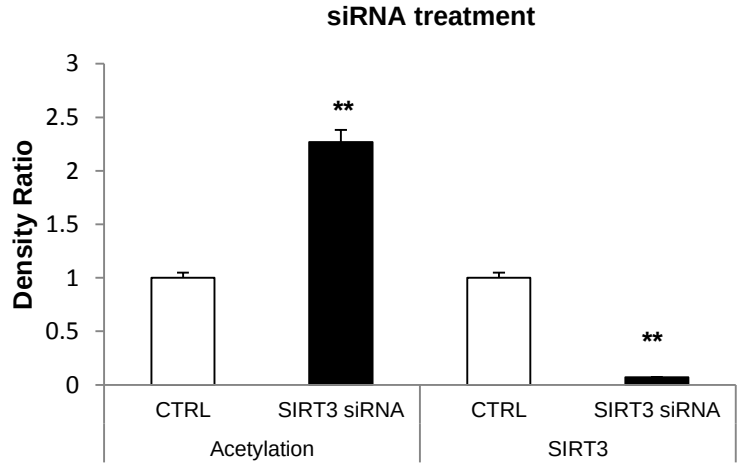


Fig 20



Supplemental Figures

Fig 21

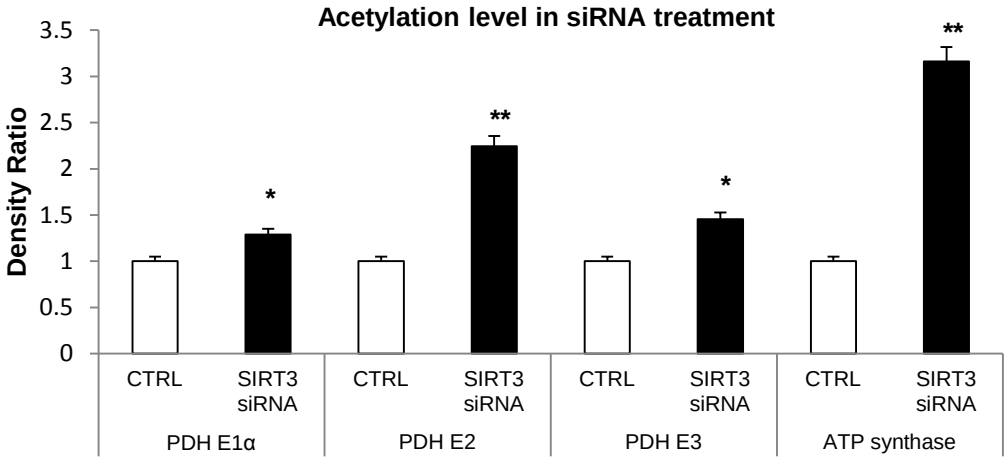


Fig 22

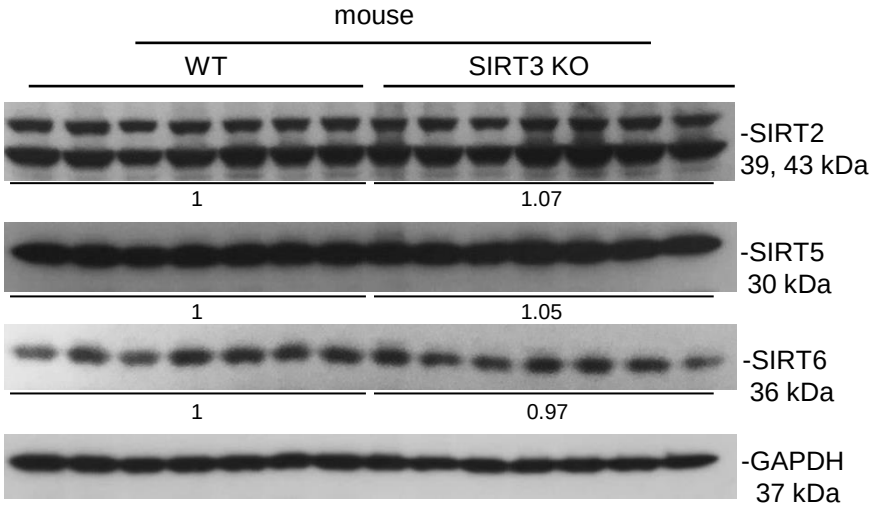


Fig 23

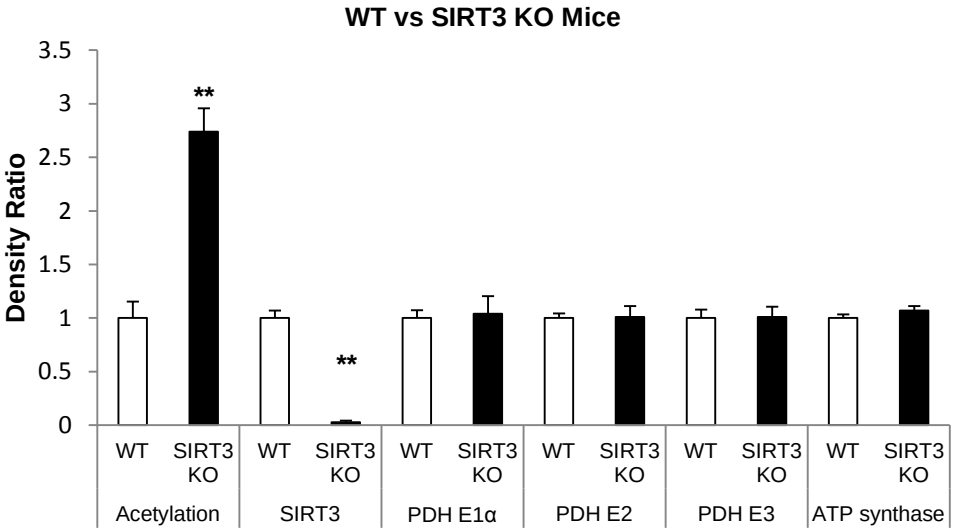
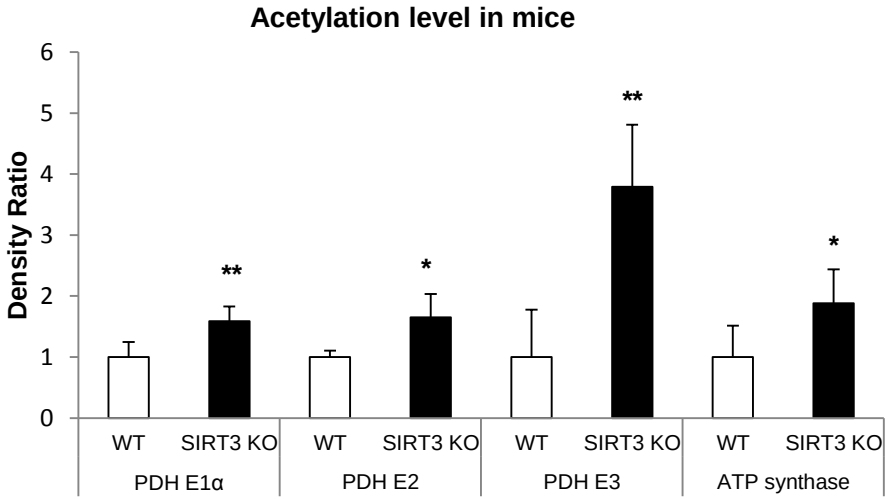


Fig 24



Supplemental Figures

Fig 25

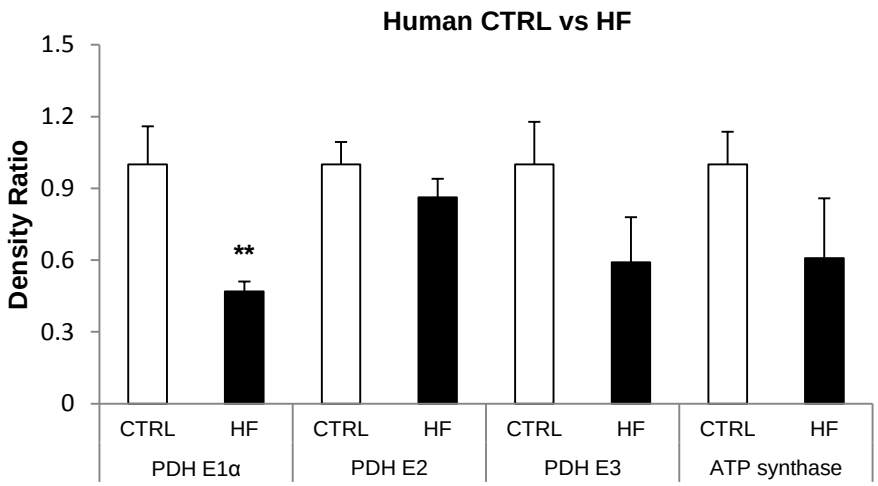


Fig 26

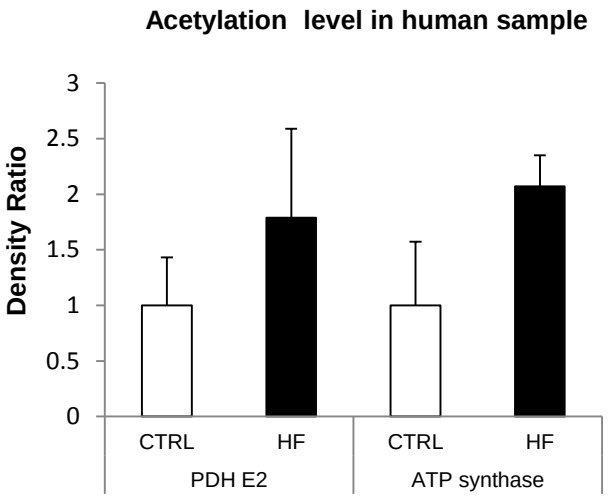
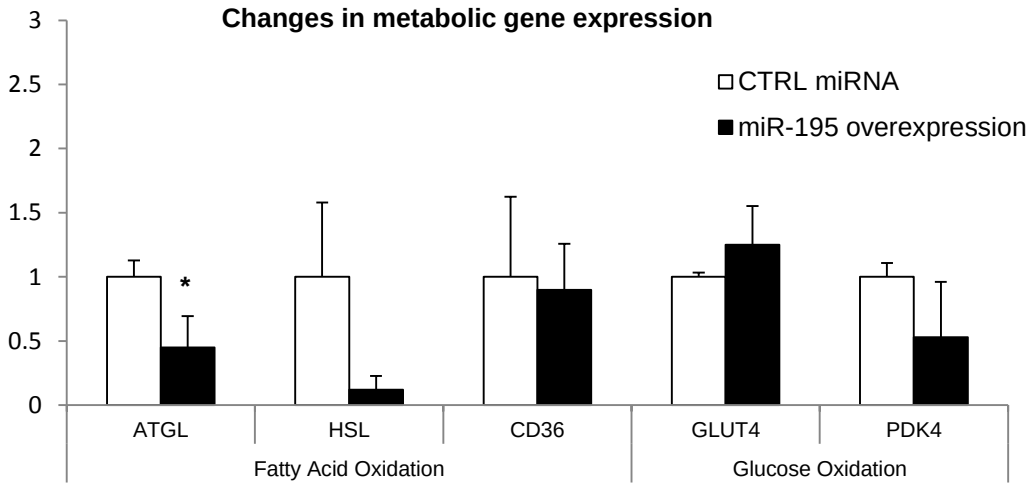


Fig 27



Supplemental Figure Legends

*** represents p<0.05, ** represents p<0.01.**

Figure 1. Densitometry analysis of total protein acetylation level and SIRT3 expression in miRNA overexpression in AC16 cells (Fig 1D).

Figure 2. miR-195 abundance was assessed by qRT-PCR, which detected a 14.18-fold elevation of the mature miR-195 in AC16 cells transfected with miR-195 precursor. miR-191 was used as normalization (Fig 1).

Figure 3. miR-195 abundance in AC16 cells in response to Ang II stimulation or/and anti-miR-195 (Fig 1E).

Figure 4. Densitometry analysis of SIRT3 expression in response to Ang II stimulation or/and anti-miR-195 (Fig 1E).

Figure 5. Densitometry analysis of acetylation in Co-IP assay using AC16 miRNA overexpression cells (Fig 1F).

Figure 6. A trend towards an increase in lung weight normalized by tibia length was observed in miR-195 overexpression mice compared to WT controls.

Figure 7. Densitometry analysis of total protein acetylation level and SIRT3 expression of cardiac tissue in miRNA overexpression mice (Fig 2B).

Figure 8. Cardiac miR-195 abundance in heart tissue used for immunoblotting (Fig 2B) was assessed by qRT-PCR, which detected a 7.78-fold elevation of the mature miR-195 in overexpression mice. miR-191 was used as normalization.

Figure 9. Densitometry analysis of acetylation in Co-IP assay using cardia tissue in transgenic mice (Fig 2C).

Figure 10. Cardiac miR-195 abundance in heart tissue used for Co-IP assay (Fig 2C) was assessed by qRT-PCR, which detected a 11.47-fold elevation of the mature miR-195 in overexpression mice. miR-191 was used as normalization.

Figure 11. Cardiac miR-195 abundance in heart tissue used for PDH actvity assay (Fig 2D) was assessed by qRT-PCR, which detected a 9.59-fold elevation of the mature miR-195 in overexpression mice. miR-191 was used as normalization.

Figure 12. Cardiac miR-195 abundance in heart tissue used for ATP synthase assay (Fig 2E) was assessed by qRT-PCR, which detected a 10.07-fold elevation of the mature miR-195 in overexpression mice. miR-191 was used as normalization.

Figure 13. Densitometry analysis of SIRT3 expression and total protein acetylation in animal model (Fig 3A).

Figure 14. Densitometry analysis of SIRT3 expression and total protein acetylation in human samples (Fig 3B).

Figure 15. Densitometry analysis of total protein acetylation level and key acetylases expression in human cardiac tissue (Fig 3B).

Figure 16. Densitometry analysis of total protein acetylation level in response to NAM treatment with increasing dose in AC16 cells (Fig 4A).

Figure 17. Densitometry analysis of total protein acetylation level in whole cell lysate and mitochondria fraction after NAM treatment in AC16 cells (Fig 4B).

Figure 18. Densitometry analysis of acetylation in Co-IP assay after NAM treatment in AC16 cells (Fig 4C).

Figure 19. siRNA mediated SIRT3 KD did not affect expression of other sirtuins. Cells were transfected with siRNAs targeting SIRT3 or non-specific sequence, and the whole cell lysates were prepared. Expression of SIRT2, SIRT5 and SIRT6 were analyzed by western blot and GAPDH was used as loading control.

Figure 20. Densitometry analysis of total protein acetylation level and SIRT3 expression in response to siRNA treatment in AC16 cells (Fig 5A).

Figure 21. Densitometry analysis of acetylation in Co-IP assay in AC16 cells treated with siRNA (Fig 5B).

Figure 22. The expression levels of other sirtuins were not affected in SIRT3 KO mice. Whole cell lysates were prepared from heart tissue of WT or SIRT3 KO mice. Expression of SIRT2, SIRT5 and SIRT6 were analyzed by western blot and GAPDH was used as loading control.

Figure 23. Densitometry analysis of total protein acetylation level and enzymes expression in WT and SIRT3 KO mice (Fig 6A).

Figure 24. Densitometry analysis of acetylation in Co-IP assay in WT and SIRT3 KO mice (Fig 6B).

Figure 25. Densitometry analysis of key enzymes expression in human cardiac tissue (Fig 7A).

Figure 26. Densitometry analysis of acetylation in Co-IP assay using human cardiac tissue (Fig 7B).

Figure 27. Changes of expression levels of human genes involved in fatty acid and glucose metabolism in response to miR-195 overexpression in AC16 cells.