Role of SIRT1 in Modulating Acetylation of the Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase in Heart Failure

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Rationale: **SERCA2a, sarco-endoplasmic reticulum Ca^{2+}-ATPase, is a critical determinant of cardiac function. Reduced level and activity of SERCA2a are major features of heart failure. Accordingly, intensive efforts have been made to develop efficient modalities for SERCA2a activation. We showed that the activity of SERCA2a is enhanced by post-translational modification with SUMO1 (small ubiquitin-like modifier 1). However, the roles of other post-translational modifications on SERCA2a are still unknown.**

Objective: **In this study, we aim to assess the role of lysine acetylation on SERCA2a function and determine whether inhibition of lysine acetylation can improve cardiac function in the setting of heart failure.**

Methods and Results: **The acetylation of SERCA2a was significantly increased in failing hearts of humans, mice, and pigs, which is associated with the reduced level of SIRT1 (sirtuin 1), a class III histone deacetylase. Downregulation of SIRT1 increased the SERCA2a acetylation, which in turn led to SERCA2a dysfunction and cardiac defects at baseline. In contrast, pharmacological activation of SIRT1 reduced the SERCA2a acetylation, which was accompanied by recovery of SERCA2a function and cardiac defects in failing hearts. Lysine 492 (K492) was of critical importance for the regulation of SERCA2a activity via acetylation. Acetylation at K492 significantly reduced the SERCA2a activity, presumably through interfering with the binding of ATP to SERCA2a. In failing hearts, acetylation at K492 appeared to be mediated by p300 (histone acetyltransferase p300), a histone acetyltransferase.**

Conclusions: **These results indicate that acetylation/deacetylation at K492, which is regulated by SIRT1 and p300, is critical for the regulation of SERCA2a activity in hearts. Pharmacological activation of SIRT1 can restore SERCA2a activity through deacetylation at K492. These findings might provide a novel strategy for the treatment of heart failure. (*Circ Res*. 2019;124:e63-e80. DOI: 10.1161/CIRCRESAHA.118.313865.)**

Key Words: acetylation ■ endoplasmic reticulum ■ heart failure ■ lysine ■ mice

One of the major features of heart failure (HF) is diminished sarcoplasmic reticulum Ca^{2+} uptake, associated with a decrease in the expression and enzymatic activity of the SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase).[1,2] In animal models of HF, increasing SERCA2a expression in cardiomyocytes normalizes intracellular Ca^{2+} handling, restores lusitropic and inotropic functions, and significantly improves cardiac function, energetics, and survival.[3–6] Phase 1 and 2a human trials, in which the SERCA2a gene was delivered to the myocardium of patients with advanced HF have also confirmed SERCA2a as an effective therapeutic target for HF.[7–10]

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During HF, besides changes in SERCA2a expression, there are changes in its activity, which are partially resulted from post-translational modifications (PTMs) to the protein. For example, we have previously shown that SUMO1 (small ubiquitin-like modifier 1) is conjugated to SERCA2a and that SUMO1

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Novelty and Significance

What Is Known?

- The SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) pump is dysregulated in the setting of heart failure.
- SERCA2a undergoes several post-translational modifications (PTMs), such as SUMOylation (a reversible PTM where SUMOs covalently attach to a target protein through a specific enzyme cascade reaction), glutathionylation, and nitration
- PTMs of SERCA2a are directly implicated in modulating its activity under normal and stress conditions.
- The roles of other PTMs on SERCA2a functions are still unknown.

What New Information Does This Article Contribute?

- SERCA2a is acetylated, particularly in the setting of heart failure.
- Acetylation of SERCA2a is mediated by p300 (histone acetyltransferase p300), which could be reversed by SIRT1 (sirtuin 1).
- Inhibition of SERCA2a acetylation by activating SIRT1 activity reverses contractile dysfunction in the setting of heart failure.
- Acetylation/deacetylation of SERCA2a at lysine position 492 (K492) is critical for regulation of SERCA2a activity in normal and diseased hearts.

Regulation of SERCA2a by PTMs has emerged as an important mechanism by which Ca^{2+} homeostasis is maintained in health and disrupted in disease. We report a novel regulatory mechanism whereby lysine acetylation of SERCA2a directly affects its function. We show that SERCA2a is a direct substrate of SIRT1 and p300. The beneficial effects of SERCA2a deacetylation on cardiac function via SIRT1 activation suggest that targeting SERCA2a's PTMs may provide a novel therapeutic strategy for the treatment of heart failure.

Nonstandard Abbreviations and Acronyms

β-gal	β-galactosidase
β-lap	β-lapachone
AAV9	adeno-associated virus serotype 9
ACM	adult cardiomyocyte
EF	ejection fraction
FOXO	Forkhead box O
HATs	histone acetyltransferases
HF	heart failure
LV	left ventricle
p300	histone acetyltransferase p300
PTM	post-translational modification
SERCA2a	sarco-endoplasmic reticulum Ca^{2+}-ATPase
Sir2	silence information regulator 2
SIRT1	sirtuin 1
SUMO1	small ubiquitin-related modifier 1
TAC	transverse aortic constriction

levels and SERCA2a SUMOylation, a reversible PTM where SUMOs covalently attach to a target protein through a specific enzyme cascade reaction, were simultaneously reduced in failing hearts.[11] Our data demonstrated that SUMOylation is essential for preserving SERCA2a ATPase activity and stability, such that reduced SUMOylation is thought to significantly contribute to SERCA2a dysfunction in failing hearts. Conversely, by increasing SUMOylation of SERCA2a, either through SUMO1 overexpression or activation of SUMOylation machinery, cardiac function in mice and porcine model with HF is markedly improved.[11–14] These data show that SUMOylation is a critical PTM that regulates SERCA2a activity. In addition, many other studies have demonstrated that SERCA is redox-regulated by thiol modifications.[15,16] For example, glutathionylation of SERCA at cysteine 674 resulted in increased activity; however, sulfonation of this residue caused a decrease in Ca^{2+} uptake. Nevertheless, the precise physiological implications of these modifications have not been proven, and roles of other PTMs for the regulation of SERCA2a activity are largely unknown.

Sirtuins are related to the yeast protein Sir2 (silence information regulator 2), which is an NAD^+-dependent class III HDAC (histone deacetylase) and mono-ADP-ribosyltransferase that plays a critical role in a variety of cellular processes, including gene silencing, DNA damage repair, and longevity.[17] SIRT1 activity has been implicated in the prevention against aging and oxidative stress[18] and also shown to be cardioprotective in animal models of HF by regulating oxidative stress and antioxidant enzymes.[19] Inhibition of SIRT1 activity induces cardiomyocyte apoptosis, accompanied by caspase-3 activation, whereas adenovirus-mediated overexpression of SIRT1 prevents apoptosis in response to serum starvation.[20] In transgenic mice, modest overexpression of SIRT1 protects the heart from oxidative stress through FOXO (Forkhead box O)-dependent mechanisms, whereas at higher levels (12.5-fold overexpression), SIRT1 increases oxidative stress in the heart.[18] SIRT1 was also shown to regulate autophagy by deacetylating several essential autophagy molecules, including Atg5 (autophagy related), Atg7, and Atg8.[21] Because constitutive autophagy is essential for maintaining cardiac structure and function, it is possible that SIRT1 exerts its cardioprotective roles at least partially through the activation of autophagy.

In this study, we show that acetylation is another essential PTM that modulates the activity of SERCA2a. Acetylation of SERCA2a was significantly elevated in human and animal failing hearts and correlated with the reduced SERCA2a activity. Moreover, our data provide clear evidence that SERCA2a is a direct substrate of SIRT1 and p300 (histone acetyltransferase p300). SIRT1 activation by β-lap (β-lapachone), a metabolic activator of SIRT1, significantly reduced acetylation and restored the SERCA2a function, resulting in beneficial outcomes under cardiac insults. This study provides a novel strategy for the restoration of contractile dysfunction of failing hearts.

Methods

All data have been made publicly available at figshare and can be accessed at https://doi.org/10.6084/m9.figshare.7739828 or from the corresponding author on request. Detailed Methods section is available in the Online Data Supplement.

Animal Models

All mice were housed and treated in accordance with National Institutes of Health and Institutional Animal Care and Use Committee guidelines and used protocols approved by the Icahn School of Medicine at Mount Sinai or the Gwangju Institute of Science and Technology Animal Care and Use Committees. Studies were conducted in male C57BL/6J mice aged 8 to 10 weeks (weight, 25–30 g) purchased from Jackson Laboratories. Cardiac-specific *Sirt1* knockout (SIRT1$^{-/-}$) mice were generated by crossing *Sirt1* $^{flox/flox}$ mice (Jackson Laboratory) with α-MHC (alpha-myosin heavy chain)-MerCreMer mice (αMHC-MerCreMer, Jackson Laboratory).[19] Conditional cardiomyocyte-specific *Serca2* knockout mouse model has been previously described.[14] All animal experiments were described in the Online Data Supplement.

Adult Cardiomyocyte Isolation and Physiology

Ventricular myocytes were isolated from mouse hearts using the method previously described.[11] The isolation process, analysis of mechanical property, and molecular analysis were described in the Online Data Supplement.

Production, Purification, and Administration of the Adenoviruses and Adeno-Associated Viruses

The production and purification and gene transfer of adenoviruses and adeno-associated viruses were described in the Online Data Supplement.

In Vitro Acetylation and Deacetylation of SERCA2a

For in vitro analysis of acetylation and deacetylation of SERCA2a, we performed cell-based and purified protein-based assays. Methods were described in detail in the Online Data Supplement.

SERCA2a Activity Assays

For analysis of in vitro activity of SERCA2a, we performed Ca^{2+} uptake assay and ATPase activity assay. Methods were described in detail in the Online Data Supplement.

Expression Plasmids

For expression in HEK293 (human embryonic kidney) cells, cDNAs encoding wild-type SERCA2a, K492Q SERCA2a, K492R SERCA2a, SIRT1, SIRT2, and p300 were cloned into a pcDNA vector. For expression in primary cardiomyocytes and mice, cDNAs encoding wild type (WT) SERCA2a, K492Q SERCA2a, and SIRT1 short hairpin RNA were cloned into adenoviral and adeno-associated viral vectors. Methods were described in detail in the Online Data Supplement.

Generation of the Antiacetylated-K492 of SERCA2a Antibody

A peptide encompassing K492 of SERCA2a, F^{488}SRDKSMSVYC^{498}, was synthesized, and the lysine residue was chemically acetylated (Anygen, Korea). Antibody for the acetylated peptide was generated in mice and purified by Abfrontier (Korea).

Statistical Analysis

Statistical analysis was described in the Online Data Supplement.

Results

SERCA2a Acetylation Is Elevated in HF and SERCA2a Interacts With SIRT1

A large-scale analysis of the acetylome in human cancer cell lines has revealed that SERCA2 undergoes acetylation.[22] Heart samples obtained from the left ventricles (LV) of patients with HF and normal human controls were analyzed by immunoblotting. In line with previous observations, SERCA2a levels were lower in failing hearts than in normal controls. Interestingly, the acetylation of SERCA2a was markedly higher in failing hearts than the normal controls (Figure 1A). In addition, this increase in SERCA2a acetylation was also observed in a murine model of HF induced by pressure overload (Figure 1B) and a porcine model of HF induced by myocardial infarction (Online Figure I), implying that an increase in acetylation is a general mechanism underlying the impaired function of SERCA2a in failing hearts.

Through proteomic analysis of the SERCA2a interactome, we identified and confirmed that SIRT1 interacts with SERCA2a in porcine hearts (Online Figure IIA through IIC). To verify this interaction, lysates of mouse adult cardiomyocytes (ACMs) were immunoprecipitated with an anti-SERCA2a antibody and probed with an anti-SIRT1 antibody. Inversely, the ACM lysates were also immunoprecipitated with an anti-SIRT1 antibody and probed with an anti-SERCA2a antibody. This reciprocal immunoprecipitation experiment showed that SERCA2a and SIRT1 are complexed in ACMs (Figure 1C). SIRT2 was not coimmunoprecipitated with SERCA2a (Online Figure IID) suggesting the specificity of the interaction between SERCA2a and SIRT1. Furthermore, HEK293 cells were transfected with expression plasmids for HA (hemagglutinin)-SIRT1 and Flag-SERCA2a and then cell lysates were subjected to reciprocal immunoprecipitation experiments. The results indicated that SERCA2a and SIRT1 constitute a complex in HEK293 cells (Figure 1D). In a similar experiment, SIRT2 was not coimmunoprecipitated with SERCA2a in HEK293 cells (Online Figure IIE). Confocal microscopy also revealed colocalization of SERCA2a and SIRT1 in ACMs (Figure 1E).

Taken together, these data showed that SIRT1 directly interacts with SERCA2a and raised a possibility that SIRT1 regulates the acetylation/deacetylation of SERCA2a.

SIRT1 Deacetylates SERCA2a

We next sought to determine the role of SIRT1 in acetylation/deacetylation of SERCA2a. In ACMs isolated from normal mice, acetylation of SERCA2a was significantly enhanced when treated simultaneously with EX-527 (SIRT1 inhibitor) and trichostatin A (class I and II HDAC inhibitor; data not shown), and the elevated acetylation of SERCA2a was significantly reduced by adenovirus-mediated overexpression of SIRT1 but not a control β-gal (β-galactosidase; Figure 2A). ACMs isolated from mice harboring cardiomyocyte-specific deletion of *Sirt1* (SIRT1$^{-/-}$) exhibited significantly elevated acetylation of SERCA2a compared with normal ACMs (Online Figure III), and the elevated acetylation of SERCA2a was normalized by reintroduction of SIRT1 but not by overexpression of β-gal (Figure 2B).

Flag-SERCA2a expressed in HEK293 cells was acetylated as determined by immunoprecipitation with an anti-Flag antibody followed by probing with antiacetyl-lysine antibody. Acetylation of Flag-SERCA2a was significantly reduced when HA-SIRT1 was coexpressed (Figure 2C), whereas it was significantly increased in cells where SIRT1 expression was knocked-down by transfection of a short hairpin RNA directed against SIRT1 (sh-SIRT1; Figure 2D). The level of SERCA2a was unaltered. The reciprocal immunoprecipitation experiments consistently showed that acetylation of SERCA2a was regulated by SIRT1.

To further verify the role of SIRT1, acetylated Flag-SERCA2a was partially purified from the transfected HEK293 cells treated with nicotinamide (SIRT1 inhibitor)

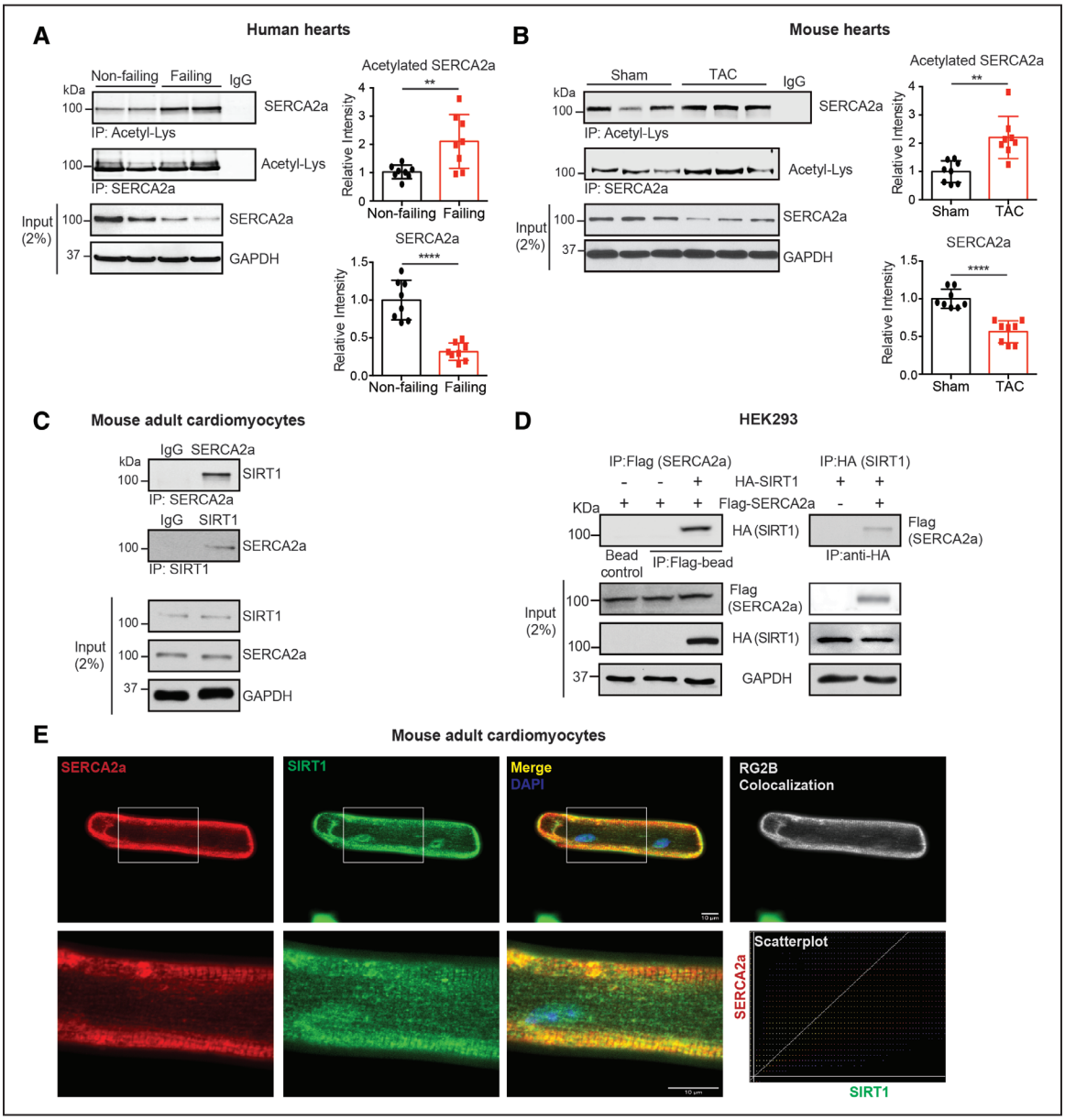

Figure 1. SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) acetylation is increased in failing hearts. A, SERCA2a acetylation was increased in human failing hearts. The human heart homogenates were immunoprecipitated with antiacetyl-lysine antibody (reverse IP with anti-SERCA2a) and probed with anti-SERCA2a antibody (reverse blot with antiacetyl-lysine). IgG was used as a negative control, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Graphs show means±SD, with each data point representing 1 heart sample. Donors, n=8; failing patients n=8. $^{**}P<0.01$; $^{****}P<0.0001$ vs nonfailing by unpaired *t* test. **B**, SERCA2a acetylation was increased in transverse aortic constriction (TAC)-induced failing mouse hearts. Graphs show means±SD, with each data point representing 1 heart sample; n=8 of Sham and n=8 of TAC mice; $^{**}P<0.01$; $^{****}P<0.0001$ vs Sham by unpaired *t* test. **C**, SERCA2a interacts with SIRT1 (sirtuin 1) in adult mouse cardiomyocytes (ACMs). ACM lysates were immunoprecipitated with anti-SERCA2a or anti-SIRT1 antibodies and probed with anti-SERCA2a or anti-SIRT1 antibodies. **D**, SERCA2a directly interacts with SIRT1 in HEK293 (human embryonic kidney) cells. Flag-agarose beads or HA agarose beads were incubated with lysates of HEK293 cells transfected with either Flag-tagged SERCA2a or together with HA-tagged SIRT1. Immunoprecipitates were probed with anti-Flag or anti-HA antibodies. **E**, Immunofluorescence images showing colocalization of SERCA2a and SIRT1 (merge, yellow) in ACMs. SERCA2a staining is shown in red, SIRT1 staining in green, and DAPI staining in blue. Pixel colocalization analyzed using the JACop plugin for Image J is shown in white. Scatter plots correspond to the colocalization between SERCA2a and SIRT1 (scale bar, 10 μm). All data shown are representative of 3 independent experiments. DAPI indicates 4',6-diamidino-2-phenylindole; HA, hemagglutinin; and IP, immunoprecipitation.

and trichostatin A. Purified acetylated Flag-SERCA2a was incubated with recombinant human SIRT1 in the presence or absence of nicotinamide adenine dinucleotide (NAD^+, SIRT1 cofactor) or nicotinamide. Immunoblotting with an antiacetyl-lysine antibody revealed that recombinant human SIRT1 deacetylated Flag-SERCA2a in the presence of NAD^+ and that this modification was inhibited completely by nicotinamide (Online Figure IV).

Taken together, these data demonstrated that SIRT1 directly deacetylates SERCA2a.

Acetylation Suppresses SERCA2a Activity

SIRT1 supports and promotes a wide variety of cellular processes, such as apoptosis/cell survival, endocrine signaling, and gene transcription. In addition, SIRT1 regulates the function of many transcription factors and cofactors through the

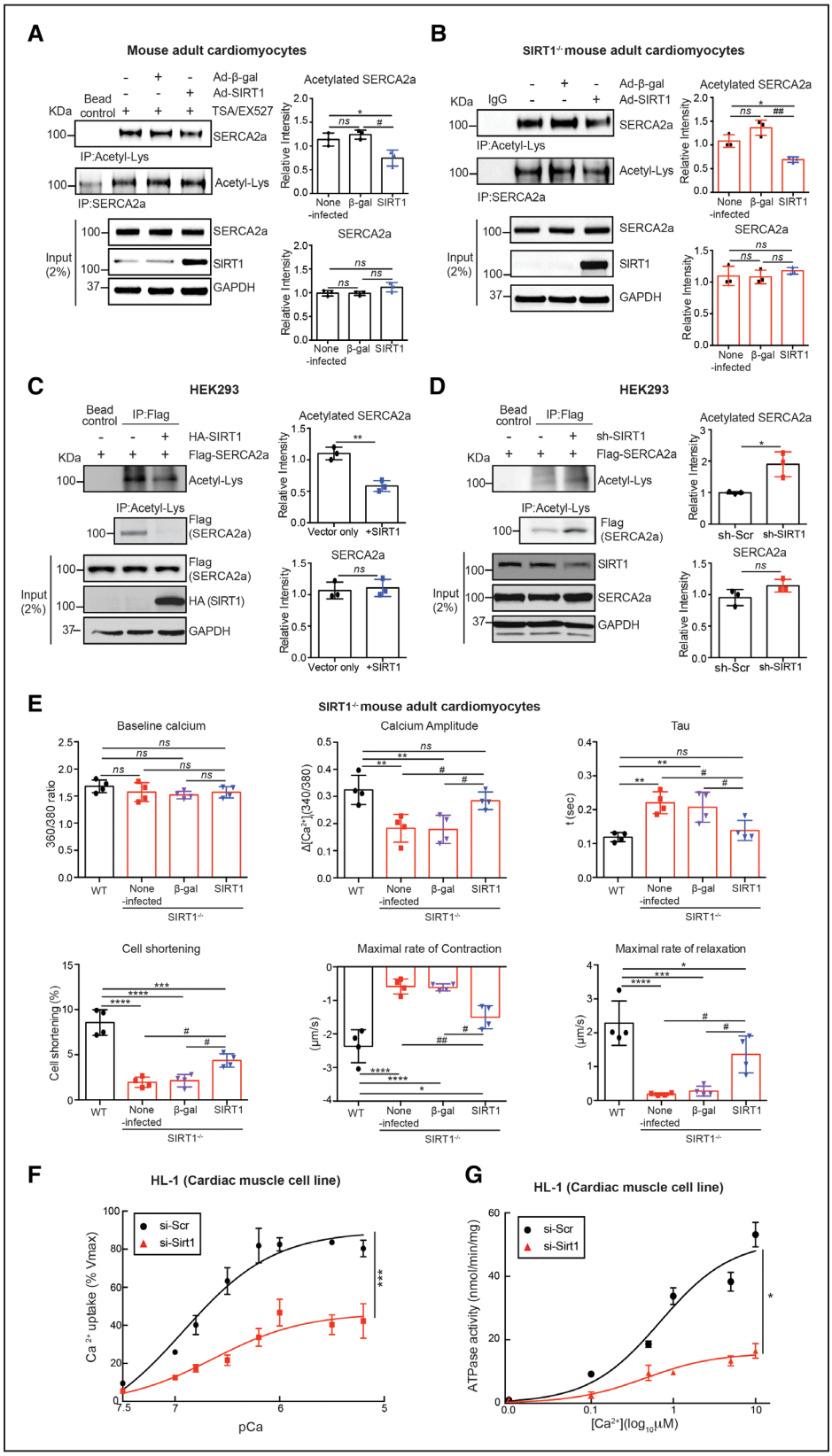

Figure 2. SIRT1 (sirtuin 1) deacetylates SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) and regulates its activity. **A**, SIRT1 overexpression reduced acetylation of SERCA2a in normal adult mouse cardiomyocytes (ACMs). ACMs were isolated from 8 wk old male C57BL/6J mice and infected with the indicated adenoviruses (50 MOI). 24 h after adenovirus infection, ACMs were treated with trichostatin A (TSA) and nicotinamide (NAM, as SIRT1 inhibitor) for 4 h to inhibit deacetylation of SERCA2a. ACM lysates were used for immunoprecipitation with antiacetyl-lysine or anti-SERCA2a antibodies. Graphs show means±SD, with each data point representing 1 sample. *P<0.05 vs noninfected ACMs and #P<0.05 vs Ad-SIRT1 by 1-way ANOVA. **B**, Knockout of SIRT1 elevated acetylation of SERCA2a in ACMs. ACMs were isolated from 8 wk old male $SIRT1^{-/-}$ mice. $SIRT1^{-/-}$ ACMs were infected (*Continued*)

mechanism of deacetylation.[17] In failing hearts, SIRT1 levels are significantly reduced, and the reduction seems to correlate with the increased SERCA2a acetylation. Thus, the increase in SERCA2a acetylation caused by the reduced SIRT1 level may contribute to SERCA2a dysfunction in failing hearts.

To test this hypothesis, we measured Ca^{2+} transient profiles and mechanical properties of ACMs isolated from SIRT1$^{-/-}$ mice using video-based edge-detection system. The SIRT1$^{-/-}$ ACMs exhibited significantly reduced Ca^{2+} handling as demonstrated by decreased Ca^{2+} amplitude and increased tau and reduced contractility as demonstrated by decreased cell shortening and maximal rates of contraction and relaxation in comparison to WT ACMs (Figure 2E). However, the dysregulated Ca^{2+} handling and contractility in SIRT1$^{-/-}$ ACMs were significantly normalized by reintroduction of SIRT1 (Figure 2E). These data suggest that increased acetylation of SERCA2a caused by knockdown of SIRT1 may impair its functions.

To explore the effects of SIRT1-mediated deacetylation on the enzyme activity of SERCA2a, HL-1 cardiac muscle cells were transfected with a siRNA (small interfering RNA) directed against SIRT1(si-SIRT1). Immunoblotting showed that SIRT1 levels were markedly reduced in si-SIRT1–transfected cells, but those of SERCA2a and its endogenous regulator PLN (phospholamban) were not affected. Acetylation of p53, a well-studied substrate of SIRT1[17], was significantly increased in si-SIRT1–transfected cells compared with si-scramble-transfected cells, indicating successful knockdown of SIRT1 in si-SIRT1–transfected cells. As expected, SIRT1 knockdown significantly elevated levels of acetylated SERCA2a, indicating that SIRT1 deacetylates SERCA2a in HL-1 cells (Online Figure V). Microsomal fractions were isolated and their Ca^{2+} uptake and ATPase activities, which are contributed mainly by SERCA2a, were determined. Knockdown of SIRT1 significantly reduced Ca^{2+} uptake activity (Vmax=89.53±3.81 and Km=6.92±0.14 of si-Scramble-transfected cells versus Vmax=46.41±4.09 and Km=6.63±0.24 of si-Sirt1, Figure 2F) and ATPase activity (Vmax=51.33±3.16 and Km=0.69±0.16 of si-Scramble-transfected cells versus Vmax=15.89±1.34 and Km=0.46±0.16 of si-Sirt1; Figure 2G). These data showed that the enzymatic activity of SERCA2a is regulated by its acetylation.

To further evaluate the role of SIRT1-mediated deacetylation of SERCA2a in vivo, mice were injected with a recombinant adeno-associated virus serotype 9 designed to express either a SIRT1-specific short hairpin RNA (rAAV9-sh-SIRT1) or a scrambled sequence (rAAV9-Scr [recombinant adeno-associated virus serotype 9 containing scrambled sequence]; Figure 3A). A rAAV9-sh-SIRT1–mediated reduction in SIRT1 level was evident as early as 4 weeks after viral injection and persisted for up to several months (data not shown). Immunoblotting and immunoprecipitation analyses were performed 6 weeks after the injection of the viruses. Knockdown of SIRT1 significantly increased acetylation of SERCA2a and slightly decreased the SERCA2a level (Figure 3B). The increased acetylation of SERCA2a was associated reduced Ca^{2+} uptake (Vmax=73.67±2.86 and Km=6.88±0.13 of sh-Scr [scramble shRNA] versus Vmax=48.95±5.51 and Km=6.42±0.25 of sh-Sirt1, Figure 3C) and ATPase activities (Vmax=68.68±2.78 and Km=0.52±0.09 of sh-Scr versus Vmax=50.35±2.78 and Km=0.73±0.15 of sh-Sirt1, Figure 3D) in the microsomal fractions. In addition, systolic function was significantly decreased in rAAV9-sh-SIRT1-injected mice as demonstrated by decreased LV fractional shortening and ejection fraction. Adverse LV remodeling was also evident in mice that received rAAV9-sh-SIRT1 compared with mice that received rAAV9-Scr, as demonstrated by reduced interventricular septal thickness at systole and increased LV internal dimensions at systole (Figure 3E and Online Table I). These data showed that SIRT1-mediated deacetylation plays an important role in SERCA2a activity in vivo.

SIRT1 Activation Reduces SERCA2a Acetylation and Restores Its Activity in HF

We have previously shown that β-lap, a natural quinine compound, indirectly activates SIRT1 by elevating intracellular levels of NAD^+, a critical regulator of SIRT1 activity.[23,24] In mice, transverse aortic constriction (TAC) caused a significant reduction in the intracellular NAD^+/NADH ratio and SIRT1 protein expression levels (Online Figure VIA and Figure 4B), which was consistent with an increase in SERCA2a acetylation (Figures 1B and 4B). In parallel, increased acetylation of known SIRT1 substrates (p53, PARP-1 [poly-ADP-ribose polymerase 1], and Hif-2α [hypoxia-inducible factor-2 alpha]) in TAC mouse hearts was observed,[25] further indicating a reduction in the levels of SIRT1 expression and activity (Online Figure VIB).

Figure 2 Continued. with the indicated adenoviruses (50 MOI) for 24 h. Antiacetyl-lysine or anti-SERCA2a was incubated with ACM lysates. Immunoprecipitates were probed with anti-SERCA2a or antiacetyl-lysine antibodies. Graphs show means±SD, with each data point representing 1 sample. *P<0.05 vs noninfected ACMs and ##P<0.01 vs Ad-SIRT1 by 1-way ANOVA. **C**, SIRT1 overexpression reduces acetylation of SERCA2a in HEK293 (human embryonic kidney) cells. Antiacetyl-lysine or anti-Flag agarose beads were incubated with lysates of HEK293 cells transfected with either Flag-tagged SERCA2a alone or together with HA (hemagglutinin)-tagged SIRT1. Acetylation of SERCA2a was triggered by treatment with TSA and NAM (as SIRT1 inhibitor). Immunoprecipitates were probed with anti-Flag or antiacetyl-lysine antibody. Graphs show means±SD, with each data point representing 1 sample. **P<0.01 vs Vector only by unpaired t test. **D**, Knockdown of SIRT1 elevates acetylation of SERCA2a in HEK293 cells. Lysates of HEK293 cells expressing either Flag-SERCA2a alone or together with a SIRT1-specific shRNA were immunoprecipitated with antiacetyl-lysine agarose beads or anti-Flag beads. Immunoprecipitates were probed with anti-Flag or antiacetyl-lysine antibody. Graphs show means±SD, with each data point representing 1 sample. *P<0.05 vs sh-scrambled control by unpaired t test. **E**, SIRT1 knockout showed decreased cardiomyocyte function. ACMs were isolated from SIRT1$^{-/-}$ mice and infected with Ad-SIRT1 or Ad-β-gal (β-galactosidase; as a negative control). ACMs isolated from wild-type (WT) mice served as control. 24 h after infection with adenovirus (50 MOI of each virus), the contractile response of SIRT1$^{-/-}$ ACMs was assessed by calcium amplitude, decay time constant (tau), peak shortening, maximal rate of contraction, and maximal rate of relaxation using a video-based edge-detection system (IonOptix, Inc. Milton, MA). Fifteen cardiomyocytes were measured per mouse, n=4. Graphs show means±SD, with each data point was represented a mean average of 15 cardiomyocytes isolated from 1 heart sample. *P<0.05; ***P<0.001; ****P<0.0001 vs WT and #P<0.05; ##P<0.01 vs SIRT1$^{-/-}$ +Ad-SIRT1 by 1-way ANOVA. Ad-β-gal indicates adenovirus containing beta-galactosidase gene; Acetyl-lys, anti-acetylated lysine antibody; Ad-SIRT1, adenovirus containing Sirt1 gene; IP, immunoprecipitation; MOI, multiplicity of infection; ns, not significant; pCa, calcium concentration; sh-SIRT1, short hairpin RNA directed against SIRT1; si Scr, si-Scramble-transfected cells; and si-SIRT, siRNA (small interfering RNA) directed against SIRT1.

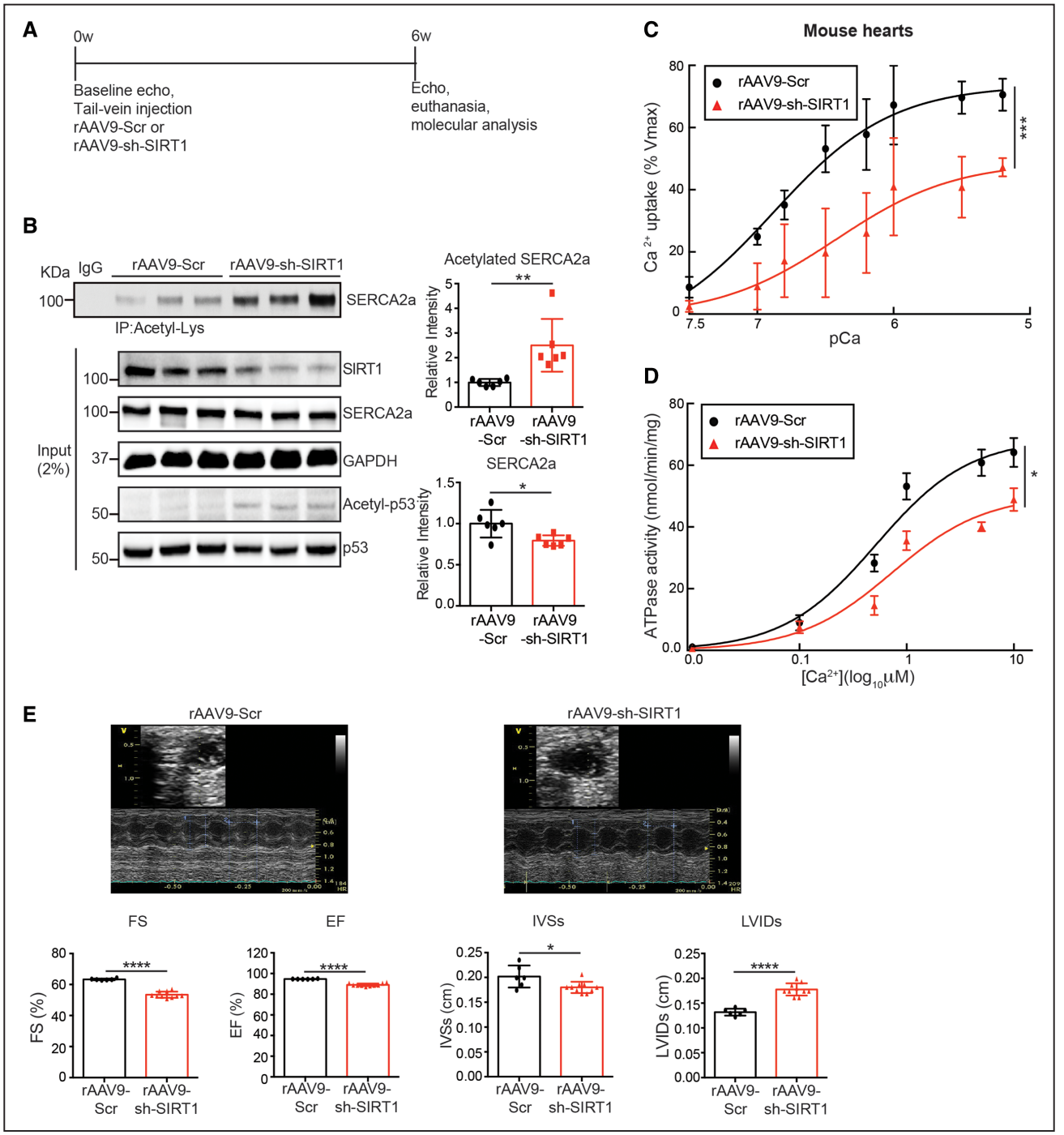

Figure 3. Knockdown of SIRT1 (sirtuin 1) elevates SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) acetylation in vivo. A, Protocol for adeno-associated virus serotype 9 designed to express either a SIRT1-specific short hairpin RNA(AAV9-sh-SIRT1)-mediated SIRT1 knockdown in normal mice. **B**, Knockdown of SIRT1 elevated levels of SERCA2a acetylation in normal mouse hearts. Eight-week-old male mice were injected with 1×10^{11} copies of rAAV9-scrambled (Scr) or recombinant AAV9-shRNA-SIRT1 (rAAV9-shRNA-SIRT1; sh-SIRT1). Six weeks later, hearts were harvested and homogenized. The heart homogenates were immunoprecipitated with antiacetyl-lysine agarose beads and probed with anti-SERCA2a antibody. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=6 per each group. *P<0.05; **P<0.01 vs sh-scrambled control by unpaired t test. **C** and **D**, Knockdown of SIRT1 reduced SERCA2a activity. **C**, Calcium uptake and **D**, ATPase activity were measured using microsomal fractions isolated from mouse hearts injected with rAAV9-Scr (recombinant adeno-associated virus serotype 9 containing scrambled sequence) or rAAV9-sh-SIRT1 (normalized to expression levels of SERCA2a). Data are represented as the mean±SD of n=3 hearts. *P<0.05; ***P<0.001 vs scrambled control by paired t test. **E**, Knockdown of SIRT1 induced cardiac dysfunction. Echocardiographic M-mode images show that mice injected with rAAV9-shRNA-SIRT1 underwent functional deterioration. Ejection fraction (EF), fractional shortening (FS), LV internal diameter at systole (LVIDs), and interventricular septal thickness at systole (IVSs) were determined in mice injected with rAAV9-sh-scrambled control (n=6) and mice injected with rAAV9-shRNA-SIRT1 (n=10). Graphs show means±SD, with each data point representing 1 mouse. * P<0.05; *** P<0.001 vs sh-scrambled control by unpaired t-test. pCa indicates calcium concentration; and sh, small hairpin.

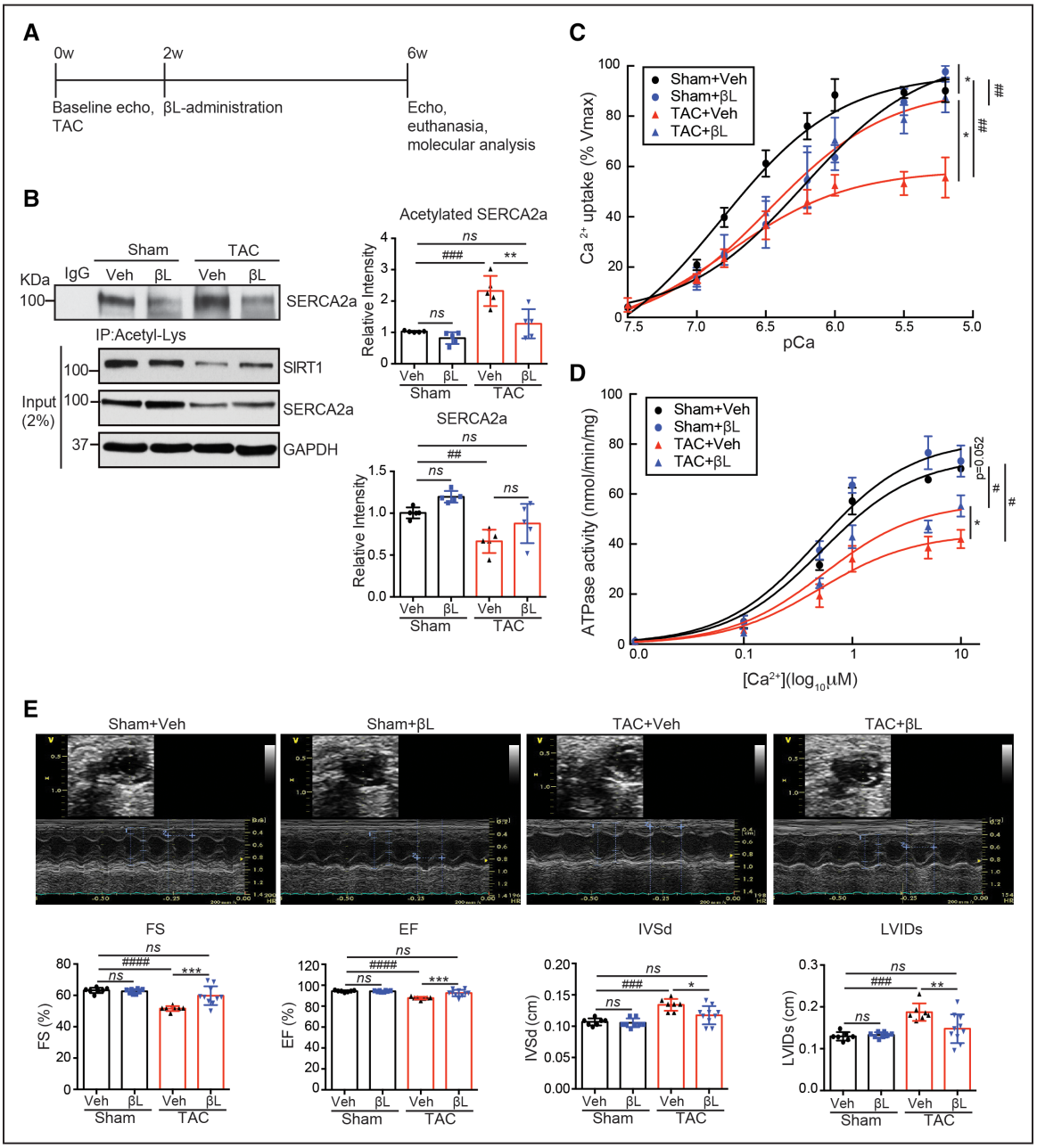

Figure 4. βL (β-lapachone) treatment diminishes acetylation and restores the activity of SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) in a pressure-overload mouse model. **A**, Protocol for βL administration in transverse aortic constriction (TAC) mice. **B**, βL treatment reduced levels of SERCA2a acetylation in mouse hearts. Eight-week-old male C57BL/6J mice were subjected to TAC operation. Vehicle (Veh) or 150 mg/(kg·day) of βL was administered to mice 2 wk post sham or TAC surgery. Four weeks later, hearts were harvested, and heart homogenates were immunoprecipitated with antiacetyl-lysine agarose beads and probed with anti-SERCA2a antibody. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=5 sham mice treated with vehicle (Sham+Veh), n=5 sham mice treated with βL (Sham+βL), n=5 TAC mice treated with vehicle (TAC+Veh), and n=5 TAC mice treated with βL (TAC+ βL). *P<0.05; **P<0.01; ***P<0.001 vs βL treatment and ##P<0.01; ###P<0.001 vs Sham+Veh by 1-way ANOVA. **C** and **D**, βL administration restored SERCA2a activities in TAC mouse hearts. **C**, Calcium uptake and (**D**) ATPase activity were measured in mouse hearts (normalized to expression levels of SERCA2a). Data are represented as the mean±SD of n=3 experiments. *P<0.05 vs βL treatment and #P<0.05; ##P<0.01 vs Sham+Veh by paired t test. **E**, βL administration restored cardiac function in TAC-operated mice. Echocardiographic M-mode images show that βL administration restored cardiac function in TAC-operated mice. Left ventricular (LV) chamber dimensions and LV systolic function were measured 6 wk post sham or TAC surgery. Data are represented as the mean±SD, with each data point representing 1 heart sample. Sham mice treated with vehicle (Sham+Veh, n=7), sham mice treated with βL (Sham+ βL, n=8), TAC mice treated with vehicle (TAC+ Veh, n=7), and TAC mice treated with βL (TAC+βL, n=10). *P<0.05; **P<0.01; ***P<0.001 vs βL treatment and ###P<0.001; ####P<0.0001 vs Sham+Veh by 1-way ANOVA. EF indicates ejection fraction; FS, fractional shortening; IVSd, interventricular septal thickness at diastole; LVIDs, LV internal diameter at systole; ns, not significant; and pCa, calcium concentration.

At 2 weeks post-TAC surgery, β-lap administration was initiated to induce SIRT1 activation (Figure 4A). As expected, daily oral administration of β-lap for 4 consecutive weeks corrected these metabolic abnormalities and inhibited acetylation of known SIRT1 substrates in failing mouse hearts (Online Figure VI). TAC-induced acetylation of SERCA2a was also reduced by β-lap treatment (Figure 4B). As expected, the TAC-induced acetylation of SERCA2a caused a significant

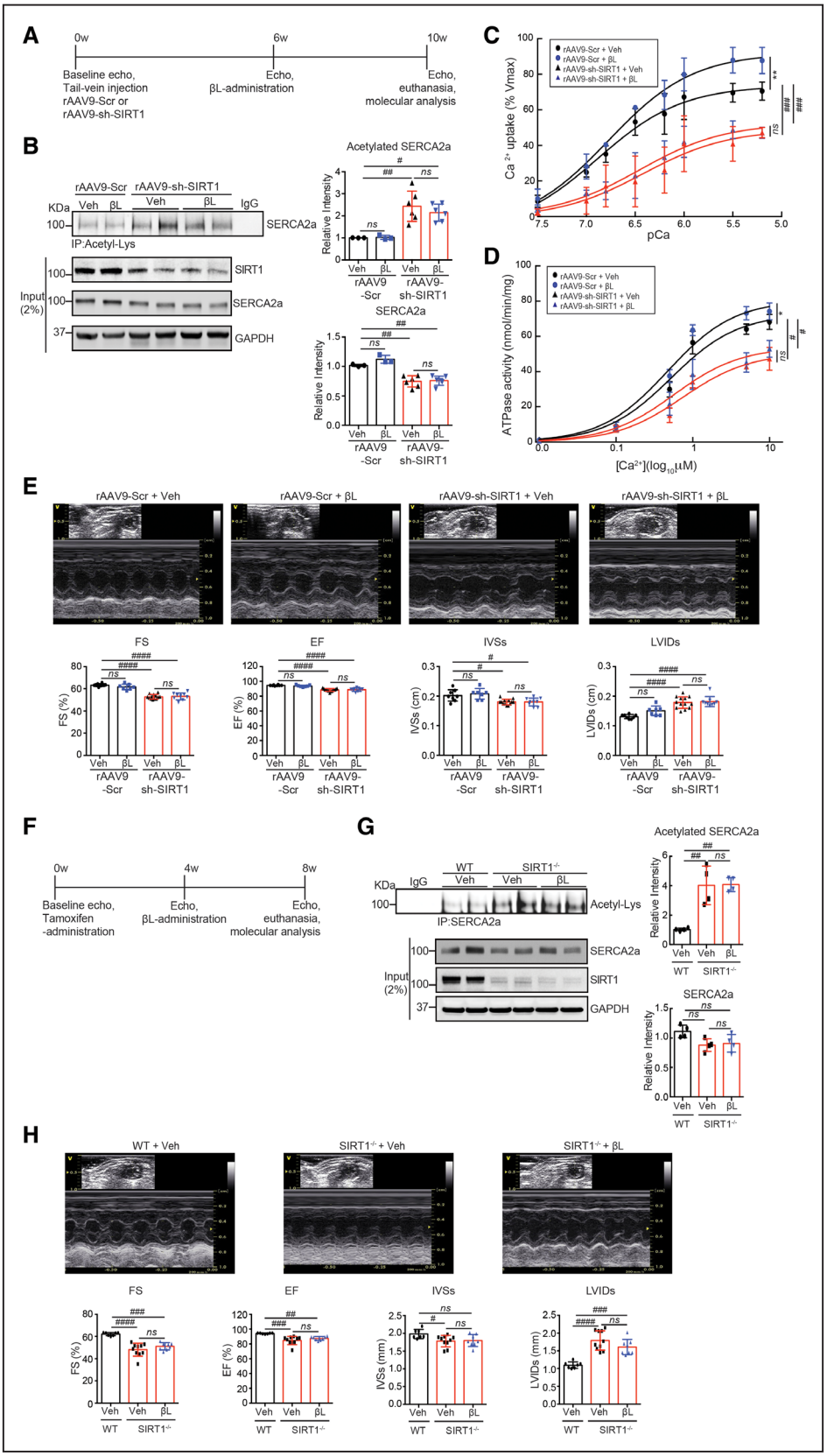

Figure 5. **βL (β-lapachone) treatment does not affect SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) function in SIRT1 (sirtuin 1) knockdown hearts. A**, Protocol for βL administration in adeno-associated virus serotype 9 designed to express either a SIRT1-specific short hairpin RNA (AAV9-sh-SIRT1)-mediated SIRT1 knockdown mice. **B**, βL did not restore levels of SERCA2a acetylation in SIRT1 knockdown mice. 8 wk old male C57BL/6J mice were injected with 1×10^{11} copies of recombinant AAV9 (rAAV9)-Scr or rAAV9-sh-SIRT1. Six weeks later, vehicle (Veh) or 150 mg/(kg·day) of βL was administered orally for 4 wk. Heart homogenates were immunoprecipitated with antiacetyl-lysine agarose beads and probed with anti-SERCA2a (*Continued*)

decrease in SERCA2a activity compared with Sham mice. The maximum rate of Ca^{2+} uptake (Vmax=96.95±2.38 and Km=6.82±0.07 of Sham+vehicle versus Vmax=58.59±2.03 and Km=6.77±0.10 of TAC+Veh) was reduced by ≈40% in TAC heart tissues, as compared to Sham hearts. However, this SERCA2a dysfunction was almost completely restored in β-lap–treated TAC-operated mice (Vmax=104.30±3.65 and Km=6.23±0.07 of Sham+ βL and Vmax=91.14±3.42 and Km=6.48±0.08 of TAC+βL; Figure 4C). The ATPase activity of SERCA2a was also reduced in TAC-operated mice (Vmax=74.75±2.61 and Km=0.52±0.08 of Sham+vehicle versus Vmax=44.26±2.13 and Km=0.50±0.10 of TAC+vehicle), and this defect was significantly normalized by β-lap (Vmax=81.66±3.29 and Km=0.48±0.08 of Sham+ βL and Vmax=56.76±2.51 and Km=0.55±0.10 of TAC+βL; Figure 4D). The levels of PLN phosphorylation and expression of SIRT1 were not altered by β-lap (Online Figure VII), suggesting that the observed changes in the microsomal Ca^{2+} uptake and ATPase activities were the result of decreased SERCA2a acetylation because of activation of SIRT1. β-lap also restored TAC-induced contractile dysfunction and adverse cardiac remodeling, as determined by echocardiography (Figure 4E and Online Table II).

Taken together, these results indicate that pharmacological activation of SIRT1 protects cardiac function by reducing SERCA2a acetylation during pressure overload.

Effects of β-lap on SERCA2a Are SIRT1-Dependent

To confirm that the observed beneficial effect of β-lap on SERCA2a function is SIRT1-dependent, we utilized 2 independent SIRT1 deficient mouse models. First, WT mice were intravenously injected with rAAV9-sh-SIRT1 or rAAV9-Scr for 6 weeks and then exposed to β-lap for 4 weeks (Figure 5A). In rAAV9-sh-SIRT1-injected mice, SERCA2a acetylation was significantly elevated, which was not restored by β-lap (Figure 5B). In these mice, Ca^{2+} uptake and ATPase activities of SERCA2a were also significantly impaired, and these defects were not normalized by β-lap (Figure 5C and 5D and Online Table VII). The contractile dysfunction and adverse cardiac remodeling in rAAV9-sh-SIRT1-treated mice were not rescued by β-lap (Figure 5E and Online Table III).

Second, $SIRT1^{-/-}$ mice[19] were fed with β-lap for 4 weeks (Figure 5F). Although SIRT1 level was prominently reduced, the acetylation of p53, a well-known substrate of SIRT1[17], was significantly elevated (Online Figure VIIIA). SIRT1 deficiency did not alter the expression levels of SIRT2 and SIRT3 nor the acetylation of tubulin, a well-known target of SIRT2. However, overall lysine acetylation levels were significantly elevated in these mice (Online Figure VIIIb). In $SIRT1^{-/-}$ mice, elevated SERCA2a acetylation was not restored by β-lap (Figure 5G). The contractile dysfunction and adverse cardiac remodeling in $SIRT1^{-/-}$ mice were not rescued by β-lap (Figure 5H and Online Table IV). It is of note that both *Serca2a* mRNA and protein levels were not significantly altered by β-lap in $SIRT1^{-/-}$ mice (Figure 5G and Online Figure IX).

To further address the critical link between SIRT1 activity and SERCA2a function, we set out to determine the effects of β-lap on cardiac function of mice harboring cardiac-specific deletion of *Serca2* ($SERCA2^{-/-}$). Two weeks after induction of *Serca2* gene excision, the $SERCA2^{-/-}$ mice were subjected to β-lap administration for 4 consecutive weeks (Online Figure XA). These mice displayed significantly decreased cardiac function in terms of ejection fraction, fractional shortening, interventricular septal thickness at systole, and LV internal dimensions at systole. β-lap-mediated activation of SIRT1 did not rescue the cardiac dysfunction in $SERCA2^{-/-}$ mice (Online Figure XB). The endogenous SERCA2a level was barely detectable, and the SERCA2a acetylation was not significantly affected by β-lap in $SERCA2^{-/-}$ mice (Online Figure XC). ACMs isolated from $SERCA2^{-/-}$ mice exhibited defective Ca^{2+} handling and contractility. The cardioprotective effects of β-lap were not observed in these ACMs (Online Figure XD through XH). These data suggest that β-lap exerts its beneficial effects through SIRT1-mediated deacetylation of SERCA2a.

Taken together, these results demonstrate that SIRT1 plays a critical role in modulating SERCA2a activity through deacetylation in the heart.

Identification and Characterization of Acetylated Lysine Residues in SERCA2a

To identify critical acetylation sites on SERCA2a, a series of 1dimensional SDS-PAGE and LC-MS/MS (liquid chromatography with tandem mass spectrometry) analyses were performed on SERCA2a acetylated in vitro and in vivo. Using this approach, a total of 12 acetylation sites on SERCA2a were

Figure 5 Continued. antibody. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=3 rAAV9-Scr (recombinant adeno-associated virus serotype 9 containing scrambled sequence) injected mice treated with Veh (rAAV9-Scr+Veh), n=3 rAAV9-Scr injected mice treated with βL (rAAV9-Scr+βL), n=6 rAAV9-sh-SIRT1 injected mice treated with vehicle (rAAV9-sh-SIRT1+Veh), and n=6 rAAV9-sh-SIRT1 injected mice treated with βL (rAAV9-sh-SIRT1+ βL). not significant (ns) vs βL treatment and #*P*<0.05; ##*P*<0.01 vs rAAV9-Scr +Veh by 1-way ANOVA. **C**, βL administration did not restore SERCA2a activity in the hearts of mice treated with rAAV9-sh-SIRT1. **C**, Calcium uptake and (**D**) ATPase activity were measured in mouse hearts. Data are represented as the mean±SD of n=3 experiments. **P*<0.05; ***P*<0.01 vs βL treatment and #*P*<0.05; ###*P*<0.001 vs rAAV9-Scr+Veh by paired *t* test. **E**, βL administration did not recover cardiac function in mice treated with rAAV9-sh-SIRT1. Echocardiographic M-mode images show that βL administration did not restore normal cardiac function in SIRT1 knockdown mice. LV chamber dimensions and LV systolic function were measured. Data are represented as the mean±SD, with each data point representing 1 heart sample. rAAV9-Scr+Veh (n=8), rAAV9-Scr+βL (n=7), rAAV9-sh-SIRT1+ Veh (n=13) and rAAV9-sh-SIRT1+ βL (n=9). *ns*, not significant vs βL treatment and #*P*<0.05; ####*P*<0.0001 vs rAAV9-Scr +Veh by 1-way ANOVA. **F**, Protocol for βL administration in cardiac-specific SIRT1 knockout ($SIRT1^{-/-}$) mice. **G**, βL administration did not affect levels of SERCA2a acetylation in $SIRT1^{-/-}$ mice. 8 wk old male $SIRT1^{-/-}$ mice were injected intraperitoneally with tamoxifen to induce S*irt1* gene disruption. Four weeks after tamoxifen administration vehicle or 1 mg/(g·d) of βL was administered orally for 4 wk. Heart homogenates were immunoprecipitated with anti-SERCA2a antibody and probed with antiacetyl-lysine antibody. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=4 per each group. ns vs βL treatment and ##*P*<0.01 vs wild-type (WT)+Veh by 1-way ANOVA. **H**, βL administration did not recover cardiac function in $SIRT1^{-/-}$ mice. Echocardiographic M-mode, LV chamber dimensions, and LV systolic function were measured. Data are represented as the mean±SD, with each data point representing 1 heart sample. WT+Veh (n=7), $SIRT1^{-/-}$+Veh (n=10) and $SIRT1^{-/-}$+βL (n=8). ns vs βL treatment and #*P*<0.05; ##*P*<0.01; ###*P*<0.001; ####*P*<0.0001 vs WT+Veh by 1-way ANOVA. EF indicates ejection fraction; FS, fractional shortening, IVSs, interventricular septal thickness at systole; LVIDs, LV internal diameter at systole; pCa, calcium concentration; and sh, small hairpin.

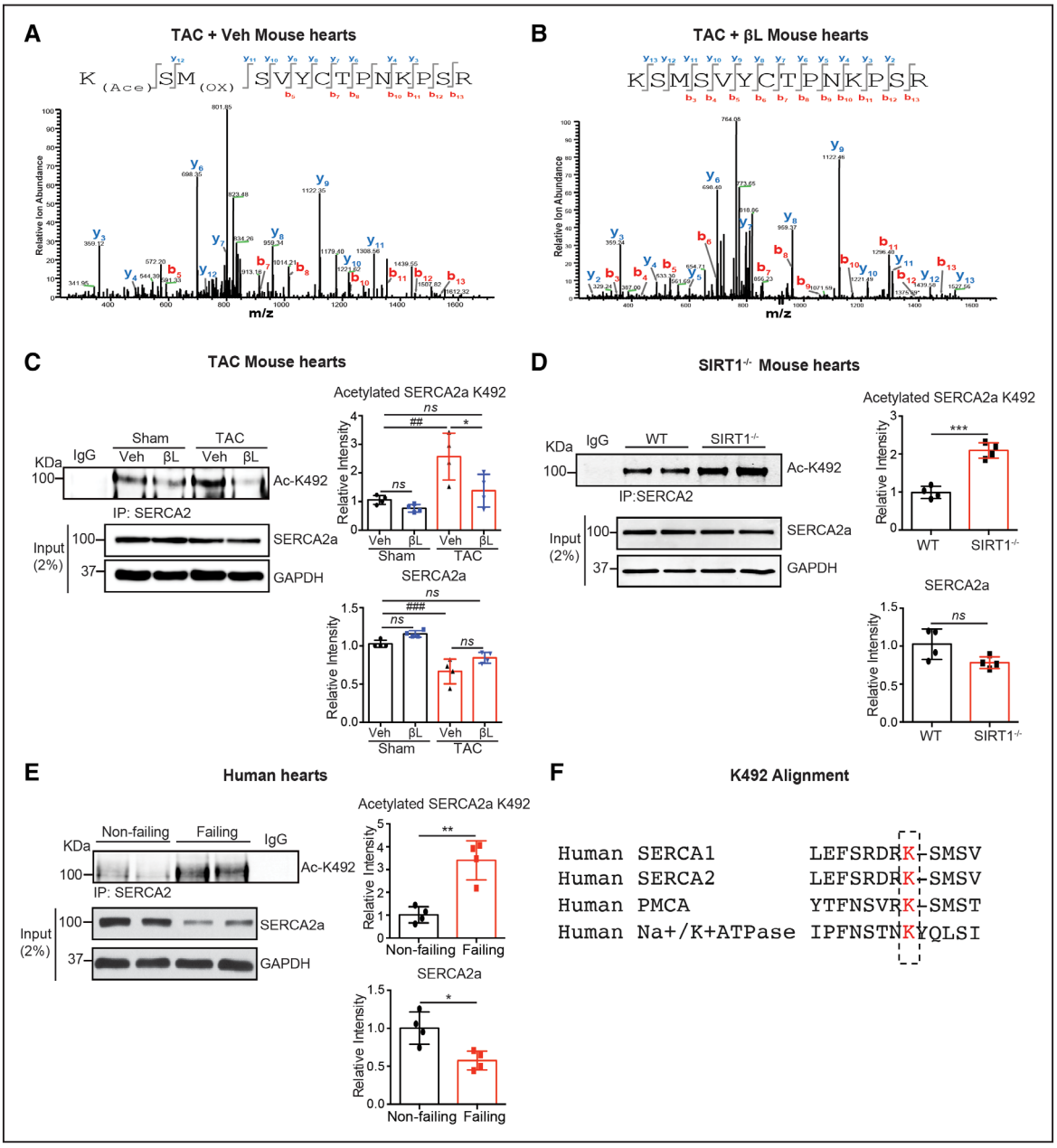

Figure 6. Acetylation of K492 on SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) is increased in failing and SIRT1 (sirtuin 1) knockout hearts. **A** and **B**, Representative MS/MS spectra of tryptic peptides of SERCA2a at K492 identified in transverse aortic constriction (TAC) mouse hearts treated with vehicle (Veh) or βL (β-lapachone). **A**, Acetyl-Lys peptide $K_{AC}SM_{OX}SVYCTPNKPSR$ (residues 492–505) was detected in heart extracts from TAC mice. **B**, Lys peptide KSMSVYCTPNKPSR (residues 492–505) was detected in heart extracts from TAC mice treated with β-lap (TAC+βL). K_{Ac}: acetyl-lysine; M_{Ox}: oxidized methionine. **C**, Levels of K492 acetylation of SERCA2a were elevated in TAC-induced failing mouse hearts, as determined by a specific antiacetyl-K492 antibody. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=4 per each group. *P<0.05 vs β-lap treatment and ##P<0.01; ###P<0.001 vs Sham+Veh by 1-way ANOVA. **D**, Acetylation levels of SERCA2a at K492 were elevated in SIRT1$^{-/-}$ mouse hearts. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=4 per each group. ***P<0.001 vs wild-type (WT) by unpaired t test. **E**, Acetylation levels of SERCA2a at K492 were increased in human failing hearts. The representative immunoblots were presented. Graphs show means±SD, with each data point representing 1 heart sample. n=4 per each group. *P<0.05; **P<0.01 vs nonfailing by unpaired t test. **F**, Amino acid sequence alignment of different members of the human P-ATPase family of proteins showing high degree of conservation of K492 (K492 and homologous lysine residues are highlighted in red). Ac-K492 indicates acetylated K492; Acetyl-lys, anti-acetylated lysine antibody; IP, immunoprecipitation; MS/MS, tandem mass spectrometry; ns indicates not significant; and PMCA, plasma membrane Ca^{2+} ATPase.

identified (data not shown). Among these sites, we recognized a previously identified acetylation site on SERCA, Lys464[22]. To identify SIRT1-specific deacetylation sites in SERCA2a, semi-quantitative LC-MS/MS analyses were performed on SERCA2a isolated from mouse hearts in which SIRT1 was either inhibited or activated. Among the 12 putative lysine residues, K492 was highly acetylated in TAC-operated mice, which was normalized by β-lap (Figure 6A and 6B). In addition, a peptide containing acetylated K492 was identified in hearts of SIRT1$^{-/-}$ mice (Online Figure XI).

To confirm these results, an antibody specific for Ac-K492 (acetyl-K492 of SERCA2a) was generated. To test

the specificity of Ac-K492 antibody against acetyl-K492 of SERCA2a, Flag-tagged WT SERCA2a (Flag-SERCA2a) or K492R SERCA2a (nonacetylated K492) was expressed in HEK293 cells. To induce SERCA2a acetylation, the transfected cells were treated simultaneously with Ex-527 and trichostatin A. Immunoblotting with Ac-K492 antibody detected a strong SERCA2a band in WT SERCA2a-transfected cells, but not in cells transfected with the K492R mutant of SERCA2a (Online Figure XII). Next, immunoblotting with this antibody revealed that the level of acetylation at K492 was significantly elevated in the TAC-induced HF mice and was normalized on SIRT1 activation by β-lap (Figure 6C). Upregulation of K492 acetylation was also found in SIRT1$^{-/-}$ mice (Figure 6D) and failing human hearts (Figure 6E). K492 is highly conserved among the family of human P-type ATPases supporting its essential role in the regulation of ATPase activity (Figure 6F).

Taken together, these results suggest that acetylation at K492 may be associated with the reduced SERCA2a activity in failing hearts and its deacetylation may be mediated by SIRT1.

Effects of Acetylated K492 on SERCA2a Activity

To explore the functional significance of acetylation of SERCA2a at K492, we generated Flag-tagged acetylation mimicking (K492Q) and nonacetylated (K492R) mutants of SERCA2a. HEK293 cells were transfected with plasmids expressing these recombinant proteins. The protein levels of both K492Q and K492R SERCA2a were comparable to WT SERCA2a level. Total lysine acetylation levels in these mutants were slightly lower than WT (Online Figure XIIIA). Ca^{2+} uptake, ATPase activity, and ATP-binding affinity were measured, and the values were normalized by the protein levels of SERCA2a. K492Q SERCA2a exhibited significantly decreased Ca^{2+} uptake and ATPase activities compared with WT SERCA2a, which is consistent with our view that acetylation at 492 significantly reduces the SERCA2a activity (Figure 7A and 7B). However, K492R SERCA2a also exhibited significantly reduced Ca^{2+} uptake and ATPase activities, although the reduction was lesser than K492Q SERCA2a (Figure 7A and 7B). ATP-binding affinity was completely abolished in K492Q and significantly reduced in K492R SERCA2a, which suggest that K492 might play a key role in ATP-binding activity of SERCA2a (Figure 7C). We perfomed molecular modeling of SERCA2a based on the crystal structure of the rabbit SERCA1a complexed with ATP.[26] The deduced model suggested that acetylation at K492 might alter the ATP-binding pocket to render the ATP-binding of SERCA2a less favorable (Figure 7D). Notably, previous mutagenesis studies of SERCA1a, the major SERCA isoform in skeletal muscles, showed that K492 plays a critical role in ATP binding.[26,27] In addition, our modeling revealed that the K492R mutant might exert steric hindrance to the ATP-binding pocket that is composed of R677, V678, and E679 in the phosphorylation domain of the SERCA2a. Thus, K492R, like K492Q, seems to interfere with the binding of ATP to SERCA2a. Therefore, it is likely that K492 is highly vulnerable to modification (ie, acetylation) or substitution so that any alteration at K492 can negatively influence the ATP-binding properties of ultimately leading to decreased activity of SERCA2a.

To further verify the functional consequences of acetylation of SERCA2a at K492 on contractile function in vitro, adenoviruses expressing WT or K492Q SERCA2a were infected to ACMs isolated from SERCA2$^{+/-}$ mice. In these mice, the SERCA2a level was reduced to ≈30% of the normal level, and infection with the viruses restored the expression level of SERCA2a to ≈70% (Online Figure XIIIB). WT SERCA2a significantly restored the defective Ca^{2+} handling and contractility of SERCA2$^{+/-}$ ACMs. However, K492Q SERCA2a had no effects on these properties, supporting the importance of K492 for the function of SERCA2a (Figure 7E through 7I). We further extended these findings to in vivo studies. WT or K492Q SERCA2a was reintroduced via AAV-mediated gene delivery to SERCA2$^{+/-}$ mice (Online Figure XIVA). SERCA2$^{+/-}$ mice showed significantly decreased cardiac function compared with WT mice (Online Figure XIVB and Online Table VI). Reintroduction of WT SERCA2a restored the contractile function, whereas reintroduction of K492Q SERCA2a did not rescue the impaired cardiac function (Online Figure XIVB and Online Table VI). AAV-mediated reintroduction of both WT and K492Q SERCA2a in SERCA2$^{+/-}$ mice restored the total SERCA2a protein level to the range of WT mice (Online Figure XIVC). ACMs were isolated from SERCA2$^{+/-}$ mice injected with rAAV9-WT or K492Q SERCA2a. The defective Ca^{2+} handling and contractility were completely restored by WT, but not by K492Q SERCA2a (Online Figure XIVD through XIVDH).

Taken together, these results suggest that acetylation of SERCA2a at K492 profoundly impairs the SERCA2a activities.

p300 Acetyltransferase Acetylates SERCA2a

We finally pursued to identify the acetyltransferase that is involved in the SERCA2a acetylation in failing hearts. Expression levels of acetyltransferases, p300, GCN5 (histone acetyltransferases), and PCAF (P300/CBP-associated factor), were elevated in human failing hearts compared with normal donors (Online Figure XV).

Flag-tagged SERCA2a was coexpressed either with or without Myc (MYC proto-oncogene)-tagged p300. Cell lysates were immunoprecipitated with an anti-Flag antibody, and the resulting precipitates were probed with antiacetyl-lysine antibody (Figure 8A). Reverse immunoprecipitation experiment was also performed where cell lysates were immunoprecipitated with an antiacetyl-lysine antibody and probed with anti-Flag antibody. Results from both experiments showed that p300 directly interacts with SERCA2a and that p300 acetylates SERCA2a.

To further confirm acetylation of SERCA2a by p300 in vitro, SERCA2a purified from normal porcine hearts was incubated in the presence or absence of hp300 (human recombinant p300). Coimmunoblotting with an antiacetyl-lysine and anti-SERCA2a antibodies revealed that SERCA2a is acetylated when p300 is coincubated (Figure 8B upper). This SERCA2a acetylation was inhibited by C646 (selective p300 inhibitor; Figure 8B, bottom). MS-based analysis and coimmunoblotting experiment revealed that the acetylation at K492 was prominently elevated by p300 treatment, which is prevented by coincubation with recombinant human SIRT1 (Figure 8C

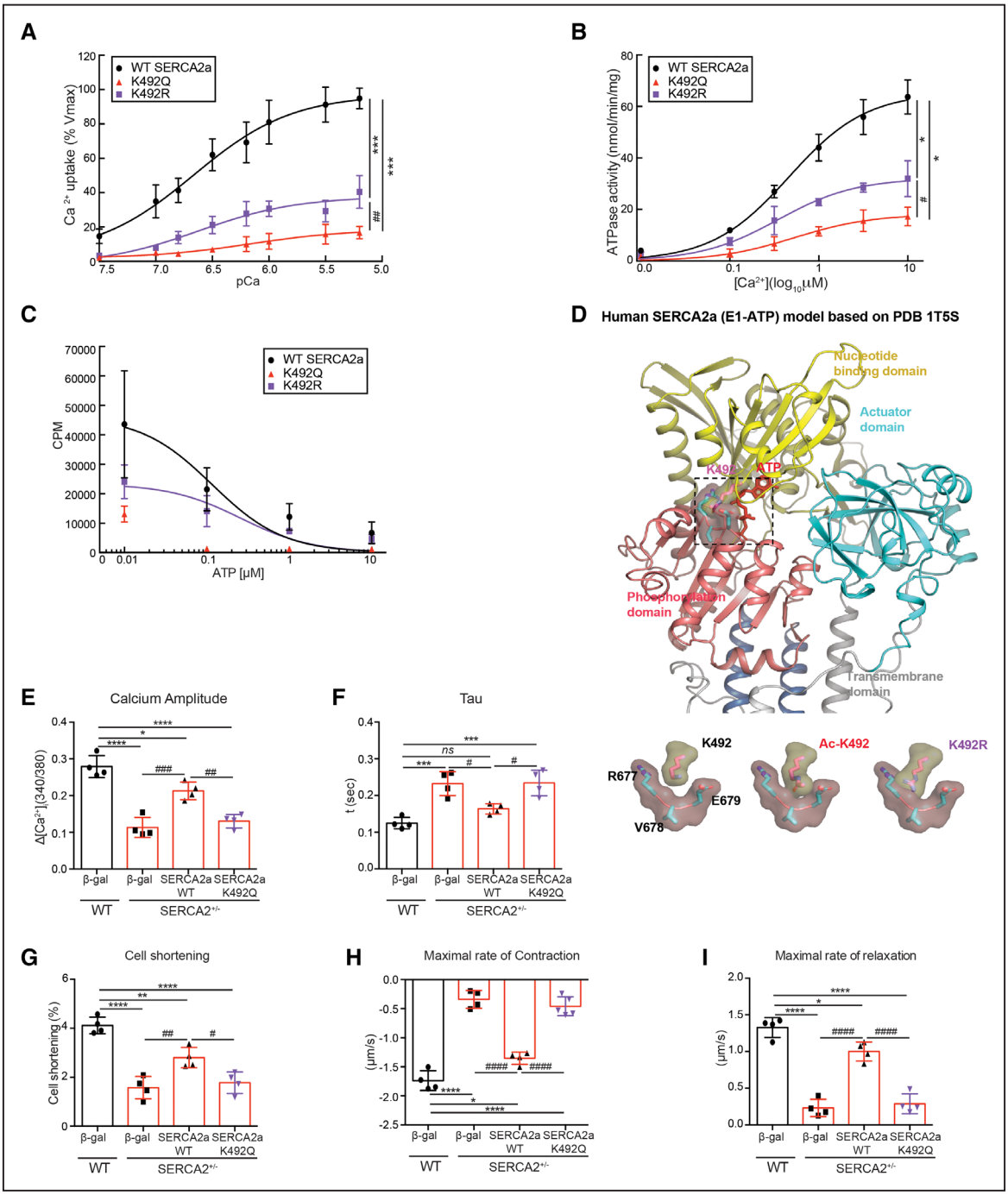

Figure 7. Acetylation at K492 plays a critical role in regulating SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) activity. A, The calcium uptake, (**B**) ATPase activity, and (**C**) ATP-binding assays of SERCA2a in microsomal fractions isolated from HEK293 (human embryonic kidney) cells transfected with wild-type (WT) or mutant (K492Q or K492R) forms of SERCA2a (normalized to expression levels of SERCA2a). Data are represented as the mean±SD of n=3 experiments. **P*<0.05; ****P*<0.001 vs WT SERCA2a and #*P*<0.05; ##*P*<0.01 vs K492R SERCA2a by paired *t* test. **D**, K492 acetylation site is located in the ATP-binding pocket of SERCA2a. Cartoon representation of SERCA2a based on the crystal structure of SERCA1a (PDB: 1T5S). The phosphorylation domain, nucleotide-binding domain, and actuator domain are shown in salmon, yellow, and cyan, respectively. The transmembrane domain is shown in grey. K492 (pink) is located within the nucleotide-binding domain adjacent to the ATP molecule (red). Close-up views of K492, acetylated K492 (Ac-K492), and nonacetylated K492 mutant (K492R) relative to the phosphorylation domain are shown on the bottom. **E** through **I**, Overexpression of the acetyl-mimicking mutant (K492Q) of SERCA2a showed contractile dysfunction in adult cardiomyocytes isolated from $SERCA2^{+/-}$ mice. Adult ventricular cardiomyocytes were isolated from $SERCA2^{+/-}$ mice and infected with Ad-WT SERCA2a, Ad- K492Q SERCA2a, or Ad-β-gal (β-galactosidase as a negative control). Adult mouse cardiomyocytes (ACMs) isolated from WT mice infected with Ad-β-gal served as control. The contractile response of SERCA2a restoration in $SERCA2^{+/-}$ ACMs was assessed by calcium amplitude (**E**), decay time constant (tau; **F**), peak shortening (**G**), maximal rate of contraction (**H**), and maximal rate of relaxation (**I**) using a video-based edge-detection system (IonOptix, Inc. Milton, MA) 24 h after infection of ACMs with adenovirus (50 MOI of each virus). n=4. Graphs show means±SD, with each data point was represented a mean average of 15 ACMs isolated from 1 heart sample. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 vs WT+β-gal and #*P*<0.05; ###*P*<0.001; ####*P*<0.0001 vs $SERCA2^{+/-}$+SERCA2a-WT by 1-way ANOVA. Ad indicates adenovirus; CPM, counts per minute; MOI, multiplicity of infection; ns, not significant pCa, calcium concentration; and PDB, Protein Data Bank.

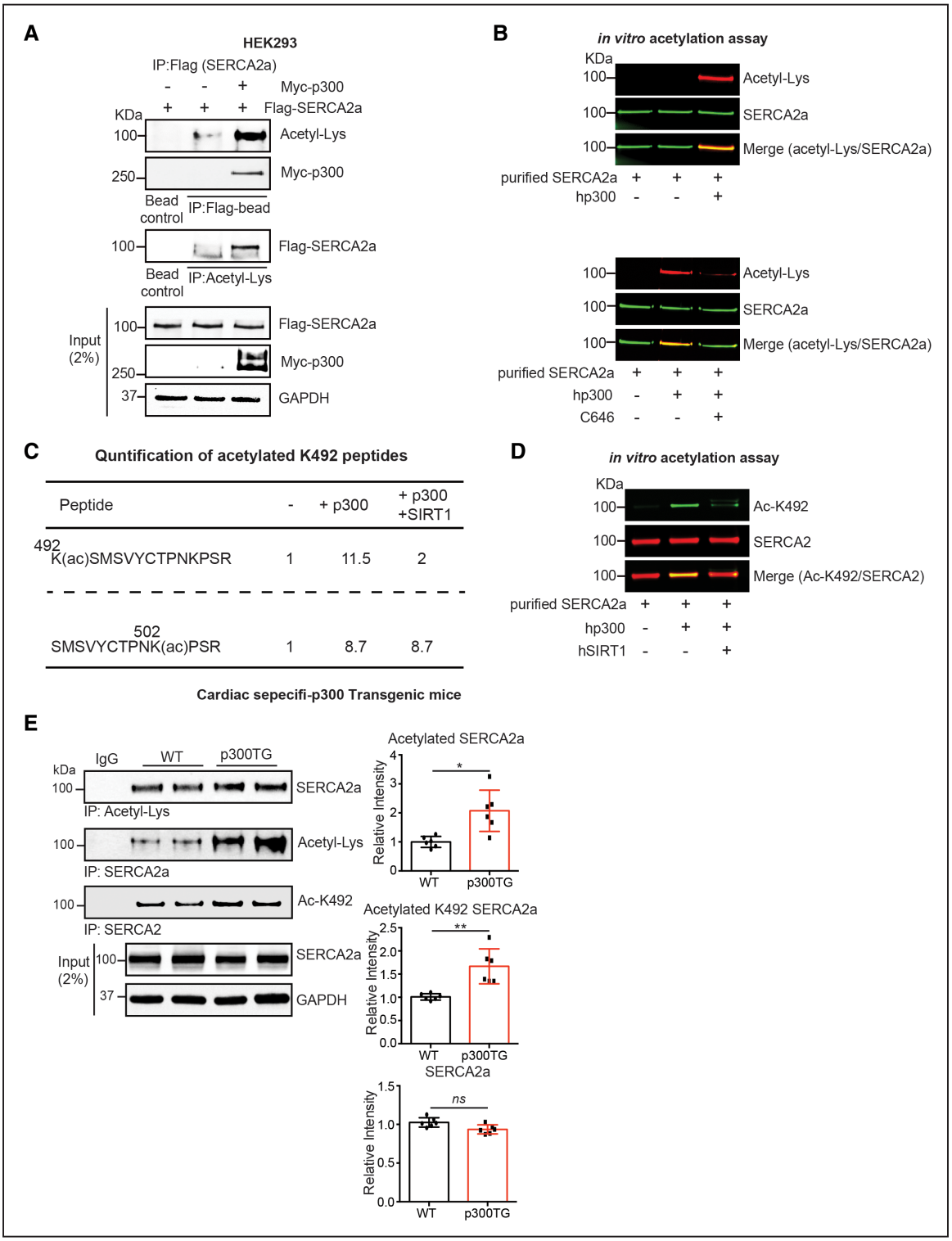

Figure 8. SERCA2a (sarco-endoplasmic reticulum Ca^{2+}-ATPase) is acetylated by p300 (histone acetyltransferase p300) acetyltransferase. **A**, p300 directly acetylates and interacts with SERCA2a in HEK293 (human embryonic kidney) cells. HEK293 cells were cotransfected with Flag-SERCA2a alone or with Myc-p300. Interaction between p300 and SERCA2a as well as acetylation levels of SERCA2a were determined by immunoprecipitation followed by probing with appropriate antibodies. The experiments shown are representative of 3 independent experiments. **B**, SERCA2a was acetylated by p300 in vitro. Purified porcine SERCA2a protein was incubated with 10 μM acetyl-CoA (coenzyme A) in the presence or absence of hp300 (human recombinant p300; **upper**) protein. p300-mediated acetylation of SERCA2a was prevented by treatment with C646, a selective inhibitor of p300 (**lower**). **C**, K492 acetylation on SERCA2a was regulated by p300 and SIRT1 (sirtuin 1). Purified porcine SERCA2a protein was incubated with 10 μM acetyl-CoA and hp300 followed by incubation in the presence or absence of recombinant SIRT1. After incubation, the samples were subjected to analysis by mass spectrometry which showed that p300-mediated acetylation at K492 was deacetylation by SIRT1. Comparison of acetylated lysine 492 levels using multiple reaction monitoring analysis. Acetyl-Lys 492 peptide K_{AC}SMSVYCTPNKPSR (residues 492–505) was acetylated by p300 and deacetylated by SIRT1. Acetyl-Lys 502 peptide SMSVYCTPNK_{AC}PSR (residues 493–505) was not perturbed in the presence of SIRT1. **D**, Acetylation status of K492 in purified porcine SERCA2a protein was confirmed by immunoblotting with antiacetyl-K492 antibody. Graphs show means±SD, with each data point representing 1 heart sample. n=6 per each group. *P<0.05 vs wild-type (WT) mice by unpaired t test. Acetyl-lys indicates anti-acetylated lysine antibody; IP, immunoprecipitation; hSIRT, recombinant human hSIRT1; Myc, MYC proto-oncogene; and ns, not significant.

and 8D). Acetylation at several other lysine residues was also increased by p300, but not prevented by coincubation with recombinant human SIRT1 (data not shown). Acetylation at K502 was shown as such an example (Figure 8C).

It was previously shown that the transgenic mice harboring cardiac-specific overexpression of p300 displayed severe contractile dysfunction.[51,52] Total levels of SERCA2a acetylation and acetylation at K492 were significantly increased in the hearts of p300 transgenic mice compared with WT mice (Figure 8E).

Taken together, our data suggest that p300 directly acetylates SERCA2a and that acetylation/deacetylation of SERCA2a at K492 is determined by SIRT1 and p300.

Discussion

SERCA2a is SUMOylated by SUMO1, and this modification is essential for preserving SERCA2a activity and stability.[11] Elevation of SERCA2a expression or SUMOylation has been suggested as an effective therapeutic strategy for the treatment of HF.[11–14] The results presented here show that SERCA2a also undergoes acetylation, which is increased in failing human hearts and animal models of HF. Moreover, acetylation of SERCA2a is mediated by p300, and this modification is reversed by SIRT1. SIRT1 deficiency elevated acetylation of SERCA2a and promoted its enzymatic dysfunction. In contrast, deacetylating SERCA2a by activating SIRT1 with SIRT1 activating compound, β-lap, effectively restored SERCA2a function. However, knockdown of SERCA2a abolished the inotropic effects of SIRT1 activation. Mass spectrometry analysis revealed a SIRT1-regulated SERCA2a deacetylation at K492. The level of acetylation of SERCA2a at K492 was shown to be significantly increased in HF and K492 is critical in modulating SERCA2a activity, suggesting that acetylation of SERCA2a at K492 may be a marker of pathophysiological condition in HF.

Over the years, several PTMs of SERCA2a, such as glutathionylation and nitration, have been directly implicated in modulating its activity under normal and stress conditions.[15,16] In our previous work, we found that covalent attachment of SUMO1 to K480 and K585 of SERCA2a enhances its stability and enzymatic activity.[11] Cardiac-specific overexpression of SUMO1 by AAV-mediated gene delivery significantly improved cardiac contractility and protected SERCA2a from oxidative stress in mouse and porcine models of HF.[11–13] Recently, we found that SUMO1 is negatively regulated by microRNA-146a, which results in the inhibition of SERCA2a SUMOylation.[28] In addition, we have shown that SUMOylation of SERCA2a can be upregulated by enhancing the activity of a SUMO-activating enzyme (E1) using specific pharmacological molecules, resulting in improved contractile function and further supporting the cardioprotective properties of SUMOylation in the heart.[14]

Because lysine residues can undergo multiple PTMs, such as ubiquitination, SUMOylation, acetylation, methylation, and glycation, it is likely that cross-talk between different PTMs of SERCA2a exists and modulates its function in the heart. Cross-talk between different PTMs is likely to be important for signal-dependent regulation of protein activity.[29] At the organism level, acetylation plays an important role in immunity, circadian rhythmicity, and memory formation.[30] Protein acetylation is enzymatically mediated by HATs (histone acetyltransferases) and can be reversed by HDACs and is becoming an important target in drug design for numerous disease conditions.[31,32] Many HDACs have been identified with impacts on cardioprotection. Specifically, Sirtuins (silent mating type information regulation 2 homolog, HDAC class III) are important in ischemia/reperfusion studies owing to their impact on aging, apoptosis, metabolic homeostasis, and stress responses.[19,33,34] In addition, both class I and class II HDACs have been closely linked to cardiac hypertrophy.[35] Mitochondrial acetylation has been shown to be important in the pathophysiology of HF.[36] Functional interplay between acetylation and SUMOylation have been reported to regulate the transcriptional activity of myocyte enhancer factor-2, histones, p53, and tumor suppressor HIC1 (HIC ZBTB transcriptional repressor 1) in multiple cancers.[37–40] Furthermore, several HATs and HDACs undergo SUMOylation, and its SUMOylation regulates localization or activity of some of these enzymes.[41,42] Aberrant acetylation or deacetylation has been implicated in human diseases such as cancer and neurodegenerative diseases.[43] Despite the increasing number of studies related to protein acetylation, our understanding of the exact role of acetyltransferases and deacetylases in cardiac function is controversial as these enzymes have numerous protein targets. A large-scale analysis of human acetylome in cancer cell lines suggested that acetylation of SERCA2 plays a role in changes in the cell phenotype.[22] A recent study has identified 3 acetylated lysine residues (K464, K510, K533) in SERCA2a from guinea pig hearts, raising the possibility that acetylation of SERCA2a may also influence intracellular Ca^{2+} cycling in the heart.[44]

This study clearly indicated that SERCA2a is acetylated and that this acetylation is more prominent in the setting of HF. In addition, our proteomic analysis of SERCA2a interactome indicated that there is indeed interaction between SERCA2a and SIRT1, which has known antiaging and stress-resistance effects.[17,18] Activation of SIRT1 exerts several beneficial effects in failing hearts, including normalization of metabolic deficits.[45] SIRT1-mediated deacetylation of SERCA2a may contribute to the cardioprotective effects of SIRT1 activating compounds, such as β-lap.

β-lap is a quinone-containing natural compound that is obtained from the bark of the South American Lapacho tree (*Tabebuia avellandedae*).[46] This compound is an antitumor agent with strong cytotoxic activity against a variety of cancer cell lines.[47] A recent study showed that oral administration of β-lap prevents obesity and obesity-related metabolic phenotypes in mice,[23] whereas another study demonstrated that β-lap prevents arterial restenosis in rats by activating AMPK (5' AMP-activated protein kinase).[25] The pharmacological activity of β-lap is dependent on a FAD (flavin adenine dinucleotide)-containing enzyme NQO1 (NADH:quinone oxidoreductase). NQO1 mediates reduction of β-lap using NADH as an electron source.[48] Thus, β-lap treatment promotes oxidation of NADH to NAD^+ resulting in increased intracellular levels of NAD^+. Because SIRT1 activity strictly requires NAD^+ as a cofactor, β-lap was supposed to increase SIRT1 activity. Previous reports showed that β-lap indeed increased SIRT1 activity

through elevation of intracellular NAD^+/NADH ratio in neuronal cells and cardiomyocytes.[25] Therefore, in this study, β-lap (as a SIRT1 activating compound) exerts cardioprotective effects at least partially through the SIRT1-mediated deacetylation of SERCA2a. Further studies examining the long-term effects of targeting SERCA2a acetylation are warranted to evaluate the potential of this therapeutic strategy.

p300 is a transcriptional coactivator that functions as an integrator of numerous signaling pathways, and it regulates many DNA-binding proteins to facilitate transcriptional activation.[42] p300 has been implicated in numerous disease processes, including several forms of cancer and neurodegenerative diseases.[49] Several lines of evidence suggest that p300 plays an important role in the growth of cardiac myocytes during development.[50] p300 knockout mice presented cardiac defects, such as impaired expression of cardiac genes and reduced ventricular trabeculation.[50] In adult mouse hearts, p300 overexpression exhibits symptoms representative of HF accompanied by acetylation of hypertrophy responsive transcription factors.[51] Cardiac-specific p300 transgenic mice showed significantly more ventricular dilations and diminished systolic function after myocardial infarction than wild-type mice.[52] Genetic reduction of p300 limited both hypertrophy and the attendant risk of HF.[53] Together, these findings imply that in some cases a therapeutic benefit may be obtained through inhibition of p300. The present study demonstrates that p300 expression is increased in failing hearts, and p300 induces SERCA2a acetylation in vitro and in vivo.

AAV-mediated transfer of SERCA2a to patients with advanced HF yielded highly promising results in phase 1 and 2a clinical studies.[8,9] However, the SERCA2a gene therapy failed to meet the primary goals in a recent phase 2b clinical trial that encompassed 250 HF patients.[10] This disappointing result can be explained by several factors such as doses and qualities of the infused AAV. In addition, we need to consider that SERCA2a activity, as well as its level, is regulated at multiple levels. Previous and present studies showed that SUMOylation and acetylation are critical PTMs which regulate SERCA2a activity. Any modalities that enhance SUMOylation or deacetylation of SERCA2a can be valid therapeutic strategies for the treatment of HF. We previously showed that SUMO1 gene transfer indeed led to restoration of SERCA2a levels, improved hemodynamic performance and reduced mortality in a mouse model of pressure overload-induced HF[11]. We further demonstrated that gene transfer of SUMO1 in combination with SERCA2a led to reversal of HF in a porcine model of ischemic HF[12]. Because the 2 lysine residues (K480 and K585) of SERCA2a that are subject to SUMOylation are not acetylated, there should be no direct competitions between SUMOylation and acetylation. Rather these 2 modifications seem to be regulated through independent mechanisms but toward opposite directions. Yet, SUMOylation, due to the relatively large size of SUMO1, may sterically hinder the acetylation at near lysine residues. In the TAC-induced HF mouse hearts, SERCA2a SUMOylation was decreased, whereas its acetylation was increased. Increased SUMOylation of SERCA2a by SUMO1 gene transfer resulted in normalization of SERCA2a acetylation in TAC mice, whereas reduced SUMO1 expression by AAV9.sh-Sumo1 gene transfer increased acetylation of SERCA2a accompanied by a reduction of SERCA2a SUMOylation in vivo (data not shown). In this study, we first reported the functional and physiological effects of acetylation at K492 on SERCA2a. Acetylation at K492 directly regulates the access of ATP to its binding site thus regulates the activities of SERCA2a. K492 is one of a group of residues forming the ATP-binding pocket of SERC2a and was previously shown to have a critical role in assuring proper binding of ATP to SERCA1a.[26] Therefore, we conclude that K492 is highly vulnerable to modification (ie, acetylation) or substitution, and any alteration in this site can significantly alter the ATP-binding capacity of SERCA2a in a less favorable way. However, in this study, we did not find definite evidence to support the possibility that acetylation and SUMOylation regulate each other directly in the heart.

In this study, we demonstrate a novel regulatory mechanism for cardiac function whereby lysine acetylation influences the activity of SERCA2a, a key molecule involved in the regulation of cardiac contractility. In addition, the beneficial effects of SERCA2a deacetylation on cardiac function via SIRT1 activation suggest that targeting SERCA2a's PTMs may provide a novel therapeutic strategy for the treatment of HF.

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Disclosures

None.

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