

Normalization of NAD⁺ Redox Balance as a Therapy for Heart Failure

BACKGROUND: Impairments of mitochondrial function in the heart are linked intricately to the development of heart failure, but there is no therapy for mitochondrial dysfunction.

METHODS: We assessed the reduced/oxidized ratio of nicotinamide adenine dinucleotide (NADH/NAD⁺ ratio) and protein acetylation in the failing heart. Proteome and acetylome analyses were followed by docking calculation, mutagenesis, and mitochondrial calcium uptake assays to determine the functional role of specific acetylation sites. The therapeutic effects of normalizing mitochondrial protein acetylation by expanding the NAD⁺ pool also were tested.

RESULTS: Increased NADH/NAD⁺ and protein hyperacetylation, previously observed in genetic models of defective mitochondrial function, also are present in human failing hearts as well as in mouse hearts with pathologic hypertrophy. Elevation of NAD⁺ levels by stimulating the NAD⁺ salvage pathway suppressed mitochondrial protein hyperacetylation and cardiac hypertrophy, and improved cardiac function in responses to stresses. Acetylome analysis identified a subpopulation of mitochondrial proteins that was sensitive to changes in the NADH/NAD⁺ ratio. Hyperacetylation of mitochondrial malate-aspartate shuttle proteins impaired the transport and oxidation of cytosolic NADH in the mitochondria, resulting in altered cytosolic redox state and energy deficiency. Furthermore, acetylation of oligomycin-sensitive conferring protein at lysine-70 in adenosine triphosphate synthase complex promoted its interaction with cyclophilin D, and sensitized the opening of mitochondrial permeability transition pore. Both could be alleviated by normalizing the NAD⁺ redox balance either genetically or pharmacologically.

CONCLUSIONS: We show that mitochondrial protein hyperacetylation due to NAD⁺ redox imbalance contributes to the pathologic remodeling of the heart via 2 distinct mechanisms. Our preclinical data demonstrate a clear benefit of normalizing NADH/NAD⁺ imbalance in the failing hearts. These findings have a high translational potential as the pharmacologic strategy of increasing NAD⁺ precursors are feasible in humans.

Chi Fung Lee, PhD
Juan D. Chavez, PhD
Lorena Garcia-Menendez, DVM
Yongseon Choi, MD
Nathan D. Roe, PhD
Ying Ann Chiao, PhD
John S. Edgar, PhD
Young Ah Goo, PhD
David R. Goodlett, PhD
James E. Bruce, PhD
Rong Tian, MD, PhD

Correspondence to: Rong Tian, MD, PhD, Mitochondria and Metabolism Center, University of Washington, Room N130, 850 Republican Street, Seattle, WA 98109-8057. E-mail rongtian@u.washington.edu

Sources of Funding, see page 893

Key Words: cardiac metabolism ■ heart failure ■ hypertrophy ■ mitochondria ■ permeability transition pore

© 2016 American Heart Association, Inc.

Clinical Perspective

What Is New?

- We show that mitochondrial dysfunction increases NADH/NAD⁺ ratio and protein hyperacetylation resulting in a greater sensitivity of the heart to chronic stress.
- Mechanistically, we have identified that hyperacetylation of the regulators of mitochondrial permeability transition pore and malate aspartate shuttle mediates the increased susceptibility to stresses.
- Increased acetylation levels of cyclophilin D and oligomycin sensitive conferring protein on the adenosine triphosphate synthase complex promote the interaction between oligomycin sensitive conferring protein and cyclophilin D and increase mitochondrial permeability transition pore sensitivity. Moreover, hyperacetylation and inhibition of malate aspartate shuttle limits the mitochondrial import and oxidation of NADH generated in the cytosol resulting in cytosolic redox imbalance.

What are the Clinical Implications?

- The identified mechanisms described above are observed in animal models and human failing hearts.
- Our preclinical results show that expanding the cardiac NAD⁺ pool via pharmacologic or genetic approaches normalizes the NADH/NAD⁺ ratio and protein acetylation in hypertrophied and failing hearts.
- Importantly, these measures improve cardiac function and reduce pathologic hypertrophy in mice. Thus, the study identifies NADH/NAD⁺ ratio a viable therapeutic target for mitochondrial dysfunction and heart failure.

Cardiovascular disease is a leading cause of death worldwide.¹ As the life expectancy increases and the mortality of acute ischemic events decreases, the incidence of heart failure is mounting at a pace of 900 000 per year.² However, medical therapy for heart failure has been stalled for almost 2 decades. Novel concepts and strategies in the treatment of heart failure are needed urgently.

The heart is a high energy-consuming organ. Mitochondrion is the powerhouse of the cell and mitochondrial dysfunction is a well-recognized maladaptive mechanism during the development of heart failure.^{3,4} Targeting mitochondria for heart failure therapy has long been sought; however, previous work focusing on improving mitochondrial energy production and reducing reactive oxygen species yielded few successful clinical applications.⁵ In recent years, protein lysine acetylation emerged as an important mechanism linking mitochondrial metabolism to cellular pathologies.^{6–8} The level of protein acetylation reflects the balance of acetylation and deacetylation. Although the former is dependent on the abundance of acetyl-CoA and the activity of acetyl-

transferase, the later is determined by the deacetylase activity, and primarily sirtuins in the mitochondria. The sirtuin deacetylases consume NAD⁺ as a cosubstrate⁹; mitochondrial function is critical for setting the NADH/NAD⁺ balance thus the NAD⁺ available for sirtuin activity.

Using a mouse model with primary mitochondrial dysfunction (cardiac-specific deletion of a Complex-I protein, *Ndufs4*: cKO), we recently found that elevation in NADH/NAD⁺ ratio induce mitochondrial protein hyperacetylation and renders the heart highly susceptible to stresses.¹⁰ In this study we defined the molecular intermediaries linking specific NAD⁺-sensitive hyperacetylation targets to the development of heart failure and demonstrated the relevance of these mechanisms in human heart failure. Furthermore, we showed that restoring the NADH/NAD⁺ ratio by genetic and pharmacologic approaches is an effective and potentially translatable strategy for the treatment of heart failure in clinical practice.

METHODS

Animal Care, Surgical Procedures, and Echocardiography

All procedures involving animal use were performed with the approval of Institutional Animal Care and Use Committee of the University of Washington. Procedures for animal care, surgeries, and echocardiography were in the [online-only Data Supplement](#).

Ex Vivo Measurements of Cardiac Function and Energetics

Langendorff perfused mouse hearts were isolated as described in the [online-only Data Supplement](#).¹¹

Mitochondrial Isolation, Proteome, and Acetylome Analyses

Mitochondria were isolated as described.¹² Peptide generation and mass spectrometric protocols were in the [online-only Data Supplement](#).

Acquired tandem mass spectra were searched for sequence matches against the International Protein Index mouse database using SEQUEST. The following modifications were set as search parameters: peptide mass tolerance at 500 ppm, trypsin digestion cleavage after K or R (except when followed by P), one allowed missed cleavage site, carboxymethylated cysteines (static modification), and oxidized methionines or acetylation on K (variable modification). PeptideProphet¹³ and ProteinProphet¹⁴ were used to assign confidence in the identified spectra resulting from the SEQUEST search. It relies on probability models and an empirical Bayesian approach to model fitting. First a score is produced to reflect the quality of each spectrum. Then a probability-based model is produced for the distribution of correctly and incorrectly identified spectra and fit to the scores of all identified spectra. The confidence in individual spectra are evaluated using the posterior probability. A cutoff is applied on the scores for the set of correctly identified spectra to control the false discovery rate, defined as the percentage of

false positives which pass the cutoff. This method produces a similar estimation of the false discovery rate as the commonly used target-decoy search strategy.¹⁵ For more details on the statistics used in the Prophets we refer readers to Ma et al.¹⁶ We used a PeptideProphet probability ≥ 0.9 and ProteinProphet probability ≥ 0.9 for positive identification at an error rate of $>1\%$. Differences in relative expression of proteins were calculated using peptide spectral counting algorithm.¹⁷

Comet (v2013.02 rev1)¹⁸ was used to search the mass spectral data against the UniProt protein database for *Mus musculus* containing forward and reverse sequences (33224 total protein sequences). Comet search parameters included a precursor mass tolerance of 25 ppm, allowing for up to 3 ¹³C offset. Trypsin was selected as the digesting enzyme allowing for up to 2 missed cleavage sites. Variable modifications included oxidation of Met (15.9949 Da) and acetylation on Lys or protein n-termini (42.010565 Da). Static modifications included carbamidomethylation of Cys (57.021464 Da). Fragment ion mass tolerance was set to 0.02 Da. Resulting peptide spectrum matches were filtered to $<1\%$ false discovery rate using a forward/reverse sequence strategy.

Molecular Docking Calculation

Detailed methods of molecular docking of cyclophilin D (CypD) and oligomycin-sensitive conferring protein (OSCP) were in the [online-only Data Supplement](#).

Mitochondrial Calcium Uptake Assay and Biochemical Assays

Methods of mitochondrial calcium uptake assays and all biochemical assays used in this study were in the [online-only Data Supplement](#).

Antibodies, Western Blot, and Immunoprecipitation

Methods of antibodies, Western blot, and immunoprecipitation were in the [online-only Data Supplement](#).

Statistical Analysis

Comparisons among the multiple groups were performed by 1-way analysis of variance, followed by Newman-Keuls multiple comparison test. For comparisons only involving 2 groups, unpaired 2-tailed t-tests were used. For repeated measurements of multiple groups, 2-way repeated measure analysis of variance was performed. All analyses were performed using GraphPad Prism 6.0. All data are expressed as mean \pm SEM and a $P<0.05$ was considered significant. All analyses were validated with permutation test versions, which are not dependent on any assumption of data distribution.¹⁹

RESULTS

Protein Hyperacetylation in the Failing Heart Was Reversed by Expanding the NAD⁺ Pool

We showed previously that increased NADH/NAD⁺ ratio and inhibition of NAD⁺-dependent protein deacetylation

caused by mitochondrial Complex-I deficiency (cKO) increased cardiac susceptibility to stress.¹⁰ We sought to test whether protein hyperacetylation occurs during the development of heart failure. In cardiac tissues of patients with heart failure with ischemic or dilated cardiomyopathy ([online-only Data Supplement Table I](#)), we observed higher acetylation levels compared with non-failing human hearts (Figure 1A). In addition, pressure overload generated by transverse aortic constriction (TAC) elevated cardiac NADH/NAD⁺ ratio and mitochondrial protein hyperacetylation (Figure 1B-C, [Figure IA-B in the online-only Data Supplement](#)), further supporting a positive correlation of NAD⁺-sensitive protein acetylation and heart failure development. Next, we tested whether restoring NADH/NAD⁺ balance by either a genetic or pharmacologic approach can normalize protein acetylation. We elevated NAD⁺ synthesis via the NAD⁺ salvage pathway by supplementing NAD⁺ precursor nicotinamide mononucleotide (NMN) or overexpressing the rate-limiting enzyme, nicotinamide phosphoribosyltransferase, in the mouse hearts (cNAMPT, [Figure 1D](#); [Figure IC in the online-only Data Supplement](#)).²⁰ Both measures increased NAD⁺ level^{10,20} without affecting cardiac function in unstressed mice ([Figure ID-H in the online-only Data Supplement](#)). NMN administration normalized the NADH/NAD⁺ ratio and importantly, reversed mitochondrial protein hyperacetylation in control mice after TAC (Figure 1B-C, [Figure IA-B in the online-only Data Supplement](#)). Similarly, NMN administration also attenuated the NAD⁺ redox imbalance and protein hyperacetylation induced by primary mitochondrial dysfunction in cKO hearts (Figure 1E-F, [Figure II-J in the online-only Data Supplement](#)). The data from multiple models collectively indicate that the NAD⁺-dependent protein hyperacetylation is present in failing hearts and hearts with mitochondrial dysfunction, which can be abrogated by elevation of NAD⁺ levels and restoration of the NADH/NAD⁺ balance.

Normalization of Protein Acetylation Blunted the Development of Heart Failure During Chronic Stresses

Next, we sought to determine if the reversal of protein hyperacetylation by NMN would improve cardiac function and reduce pathologic hypertrophy induced by pressure overload. TAC caused significant cardiac hypertrophy, left ventricular dilation and decline in fractional shortening in control mice (Figure 2A-D, [Figure IIA in the online-only Data Supplement](#)). NMN administration improved fractional shortening (Figure 2A), left ventricular dilation and hypertrophy (Figure 2B-D) in TAC-stressed mice. In addition, the accelerated course of heart failure in cKO mice after TAC ([Figure IIB in the online-only Data Supplement](#))¹⁰ also was abrogated by NMN administration. NMN treatment preserved contractile function (Figure 2E), reduced cardiac hypertrophy, left ventricular dilation, and

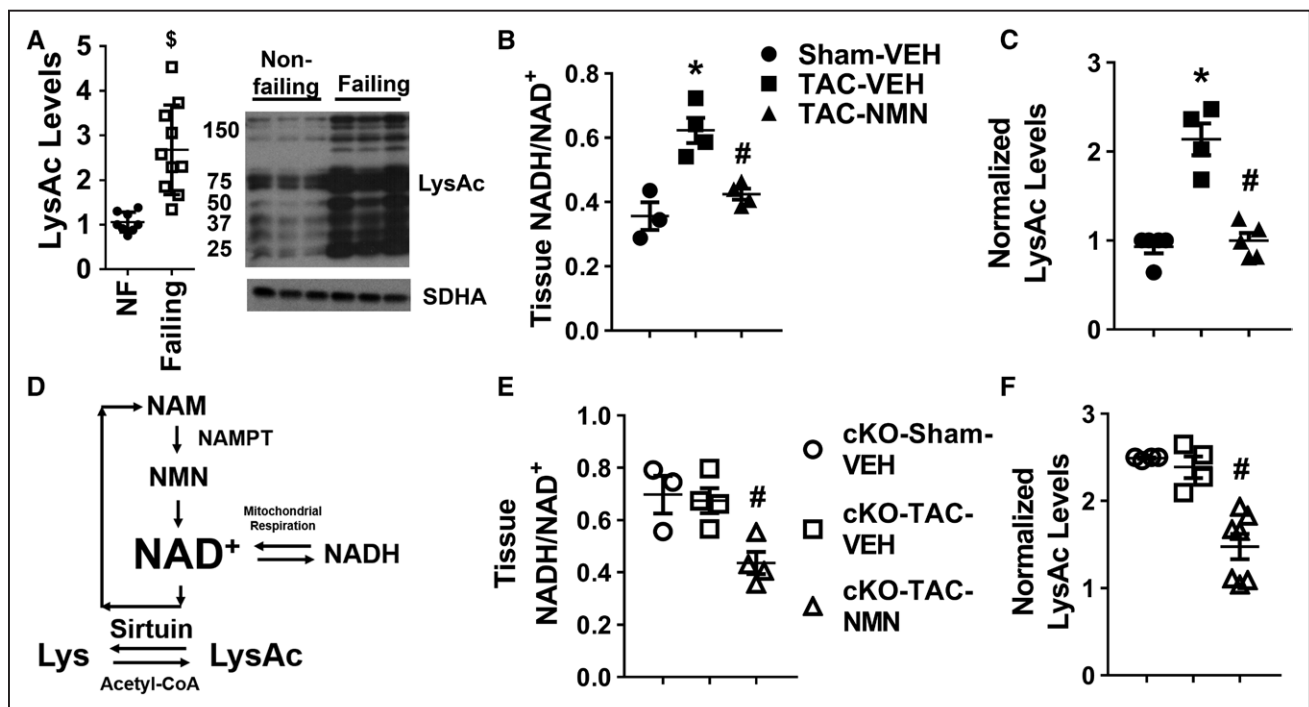


Figure 1. Protein hyperacetylation in the failing hearts was normalized by restoring the NADH/NAD⁺ ratio.

A, Acetylation of human failing hearts were estimated by Western blotting. $N=8$ to 10 . The ratio of heart failure patients with ischemic versus dilated cardiomyopathy is $1:1$. **B**, Tissue NADH/NAD⁺ ratio and **(C)** mitochondrial protein acetylation levels by Western blotting of control mice 4 weeks after sham or transverse aortic constriction (TAC) surgeries with and without nicotinamide mononucleotide (NMN) treatment were measured. $N=3$ to 4 . **D**, Sirtuin deacetylase reaction requires NAD⁺. Mitochondrial NAD⁺ level is determined by the NADH/NAD⁺ ratio and the rate of NAD⁺ synthesis via salvage pathway. Nicotinamide phosphoribosyltransferase (NAMPT), the rate limiting enzyme of this pathway, catalyzes the conversion of nicotinamide (NAM) into NMN. Overexpression of NAMPT and supplementation of NMN were used to elevate NAD⁺ levels in this study. **E**, Tissue NADH/NAD⁺ ratio and **(F)** mitochondrial protein acetylation in cardiac tissues of the cKO mice were measured. $N=3$ to 4 . * $P<0.05$ compared with sham-vehicle; # $P<0.05$ compared with TAC-VEH; and \$ $P<0.05$ compared with nonfailing. All data are expressed as means \pm SEM.

lung edema (Figure 2F-H, Figure IIC-D in the online-only Data Supplement). The data strongly supported the efficacy of NMN administration in delaying the development of heart failure in mice with mitochondrial dysfunction, either primary or acquired.

We also tested whether elevation of NAD⁺ levels specifically in heart by increasing NAMPT activity also would be effective to prevent cardiac dysfunction and hypertrophy induced by another stressor with distinct mechanism, isoproterenol (ISO) stimulation. Transgenic mice overexpressing NAMPT only in the hearts (cNAMPT)²⁰ were crossed with control and cKO mice to obtain mice with cNAMPT expression. We stressed the mice by chronic β -adrenergic stimulation with isoproterenol, which has been shown to cause cardiac myocyte death and pathologic hypertrophy.²¹ Isoproterenol (30 mg/kg/d) delivered by osmotic minipump for 2 weeks (Figure IIIA in the online-only Data Supplement) induced significant cardiac dysfunction and hypertrophy in both control (Figure 3A-D, Figure IIIB in the online-only Data Supplement) and cKO mice (Figure 3E-H, Figure IIIC in the online-only Data Supplement) while cKO mice pre-

sented worse phenotypes. Elevation of cardiac NAD⁺ levels by cNAMPT protected both mice from isoproterenol-induced cardiac dysfunction, LV dilation, and hypertrophy (Figure 3, Figure III in the online-only Data Supplement). The data also supported that targeting the NAD⁺ salvage pathway to elevate cellular NAD⁺ levels represents a viable intervention strategy to improve cardiac function in response to chronic stresses. Moreover, the cNAMPT mice provide a useful tool to dissect the mechanistic roles of NADH/NAD⁺-sensitive protein acetylation in heart failure propensity.

Acetylome Analyses Identified NADH/NAD⁺-Sensitive Changes in Acetylation Landscape

To test the hypothesis that protein acetylation that is sensitive to NADH/NAD⁺ ratio modulates cardiac sensitivity to stress, we performed acetylome analysis of cKO hearts with and without cNAMPT. We first compared the mitochondrial proteomes from control and cKO hearts to rule out the possibility that increases in acetylated proteins in cKO were due to increases in the total pro-

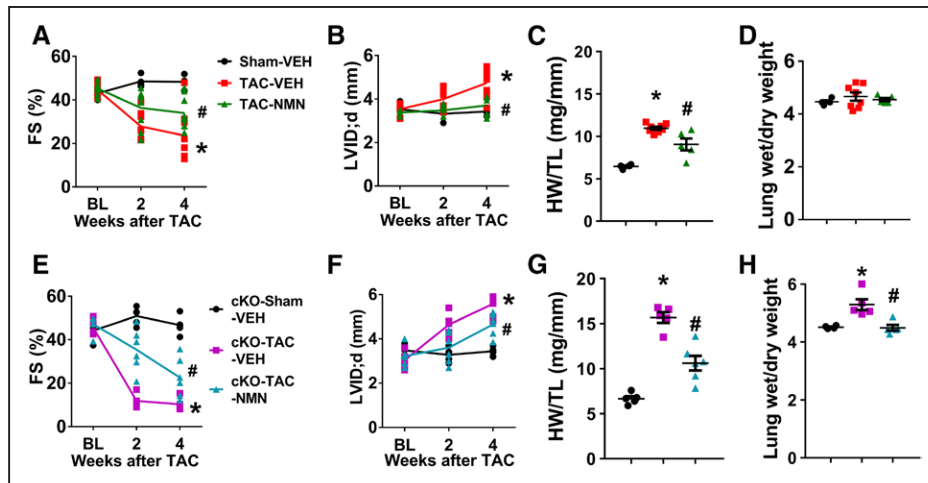


Figure 2. Nicotinamide mononucleotide (NMN) protected hearts from pathologic hypertrophy and contractile dysfunction induced by chronic pressure overload.

A, Fractional shortening (FS), **(B)** left ventricular (LV) dilation (left ventricular internal dimension at diastole [LVID;d]), **(C)** cardiac hypertrophy (heart weight/tibia length, [HW/TL] at 4-week end point), and **(D)** lung edema (wet/dry lung weight at 4-week end point) of control mice after sham or transverse aortic constriction (TAC) surgeries with or without NMN treatment were assessed. N=5 to 8. **E**, FS, **(F)** LV dilation, **(G)** cardiac hypertrophy, and **(H)** lung edema of cKO mice after indicated treatments were assessed. N=5 to 6. * $P<0.05$ compared with corresponding sham; and # $P<0.05$ compared with corresponding TAC-vehicle. All data are expressed as means \pm SEM.

tein amount. We did not observe significant up-regulation of mitochondrial protein levels while multiple proteins in mitochondrial complex-I were downregulated (Table II-III and Figure IVA-B in the online-only Data Supplement). This is consistent with the observation that deletion of *Ndusf4* resulted in poor assembly of complex-I and hence degradation of complex-I proteins.¹⁰ Despite the minimal changes in protein levels, the number of acety-

lated proteins (Figure 4A) and acetylation levels of peptides increased in cKO hearts (Figure 4B), consistent with increased acetylation shown by Western blot (Figure 4C, Figure IVC in the online-only Data Supplement). Hyperacetylation of some known protein targets were validated by Western blots (Figure IVD-E in the online-only Data Supplement). Increased acetylation levels in cKO hearts were not attributable to alterations in the levels of

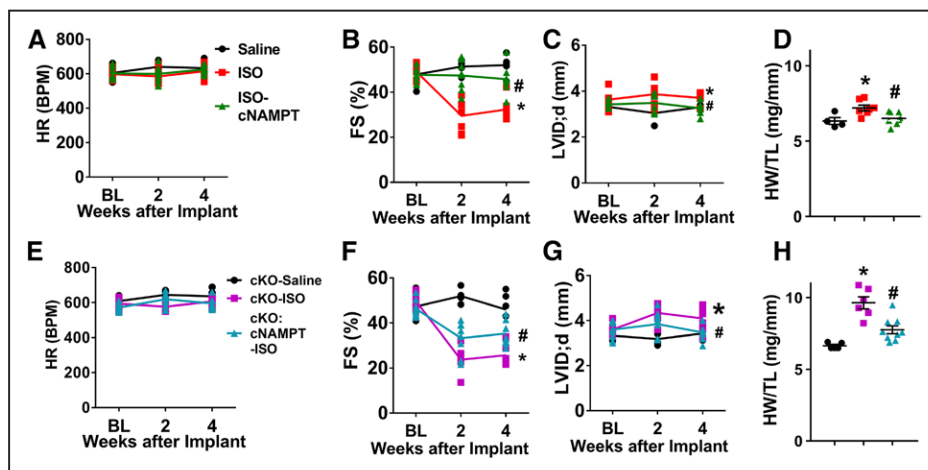


Figure 3. Cardiac nicotinamide phosphoribosyltransferase (NAMPT) expression reversed isoproterenol- (ISO) induced cardiac dysfunction and hypertrophy.

A, Heart rate (HR), **(B)** fractional shortening (FS), and **(C)** left ventricular internal dimension at diastole (LVID; d), and **(D)** cardiac hypertrophy (heart weight/tibia length [HW/TL]) of control or control+cNAMPT mice challenged with saline or isoproterenol (ISO, 30 mg/kg/d) for 2 weeks were measured. N=5 to 9. **E**, HR, **(F)** FS, **(G)** LVID; d, ratio of **(H)** HW/TL of complex-I protein (cKO) or cKO+cNAMPT mice challenged with saline or ISO were recorded. N=5 to 8. * $P<0.05$ compared with corresponding saline; # $P<0.05$ compared with corresponding ISO. Data are expressed as means \pm SEM.

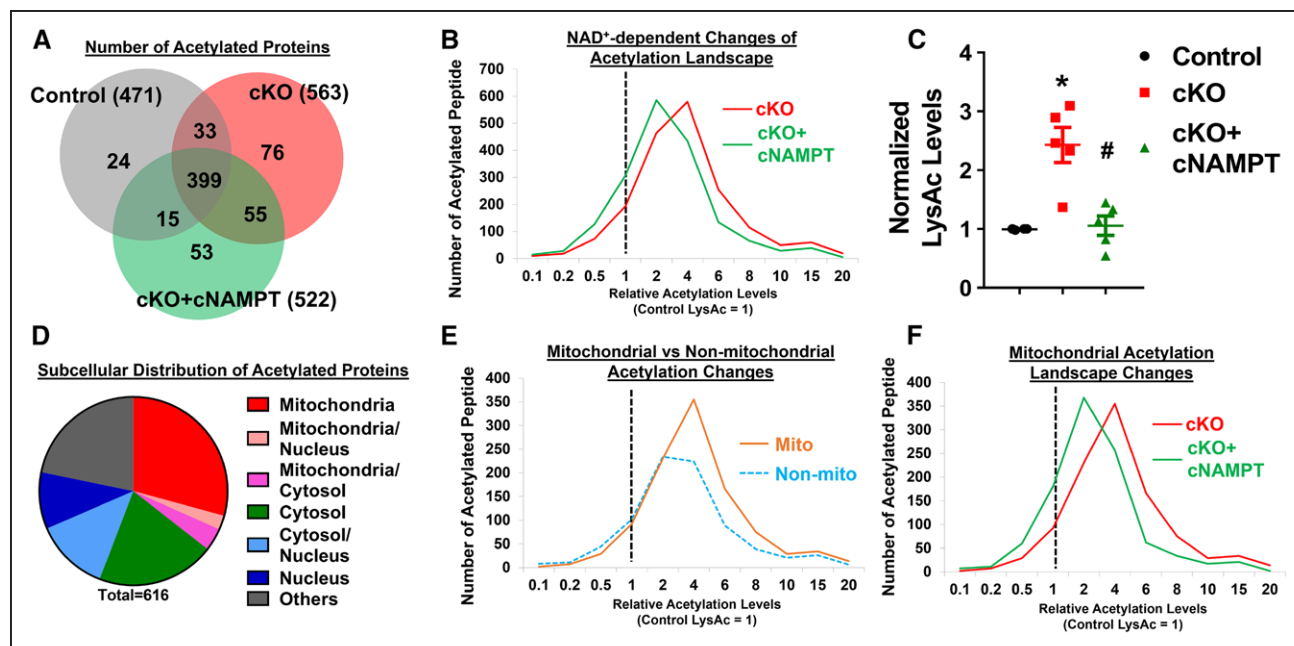


Figure 4. Acetylome analysis identified NADH/NAD⁺-sensitive changes in acetylation landscape.

A, Venn diagram showing the overlapping of acetylated proteins identified in control, complex-I protein (cKO), and cKO+cNAMPT hearts. Numbers in bracket represent the total number of acetylated proteins identified in each group. **B**, Acetylated peptides of cKO and cKO+cNAMPT hearts were quantified by liquid chromatography–mass spectrometry and normalized to control values. Distribution of the acetylation changes was plotted. Dotted line at 1 was set to the mean of control peptide levels. Distribution shift to the right of 1 represented increased acetylation levels compared to control. **C**, Acetylation levels from indicated hearts were assessed by Western blot. * $P < 0.05$ compared with control. # $P < 0.05$ compared with cKO. **D**, Pie chart showing subcellular localization of acetylation proteins identified in cKO hearts. **E**, Changes of acetylation in mitochondrial (mito) versus nonmitochondrial (nonmito) compartments. **F**, Mitochondrial acetylation landscape changes of cKO and cKO+cNAMPT hearts were compared.

acetyl-CoA, mitochondrial acetyltransferase GCN5L1, or sirtuin expressions (Figure IVF-G in the online-only Data Supplement). As expected, cNAMPT overexpression normalized the NADH/NAD⁺ ratio and hyperacetylation in cKO hearts (Figure 4C, Figure IVC, H in the online-only Data Supplement). The number of acetylation proteins as well as the levels of acetylated peptides in cKO hearts were reduced by cNAMPT (Figure 4A-B, left-shift from cKO). Although mitochondrial proteins only constituted about a third of the 616 acetylated proteins identified (Figure 4D), a disproportionately high fraction of hyperacetylated peptides were originated from mitochondrial proteins in cKO (Figure 4E), and they responded robustly to the normalization of NADH/NAD⁺ ratio by cNAMPT (Figure 4F, left-shift from cKO). These observations also support a critical role of NADH/NAD⁺ ratio in connecting mitochondrial function and acetylation landscape. Interestingly, acetylation levels of proteins from nonmitochondrial compartments also were elevated in cKO hearts (Figure 4E, right-shift from 1). Expression of cNAMPT moderately reduced the acetylation of nonmitochondrial proteins (Figure IVI in the online-only Data Supplement). This observation raised an intriguing possibility that the NAD⁺ redox imbalance caused by mitochondrial dysfunction could affect other cellular compartments, and as such contributed to pathologic remodeling of the heart

by altering cytosolic redox state and whole cell hyperacetylation.

Acetylation of Malate Aspartate Shuttle Regulated Cytosolic NAD⁺ Redox Balance and Cardiac Energetics

Among the subpopulation of proteins whose acetylation responded robustly to NADH/NAD⁺ ratio, hyperacetylation of the mitochondrial isoforms of malate aspartate shuttle (MAS) proteins (Figure 5A, Table IV in the online-only Data Supplement) is of interest. MAS is a key player in the communication of cytosolic and mitochondrial NAD⁺ redox states,²² carrying electrons from cytosolic NADH into mitochondria for oxidative phosphorylation.²³ Decreased MAS activity was found in cKO and isoproterenol-treated hypertrophic hearts, and in both cases could be restored by overexpressing cNAMPT (Figure 5B-C). Acetylation of a number of lysines in the MAS proteins was reduced toward normal level by overexpressing cNAMPT (Table IV in the online-only Data Supplement). Moreover, incubation of mitochondrial proteins with acetyl-coA promoted acetylation of mitochondrial GOT2 (glutamate oxaloacetate transaminase) and inhibited its activity (Figure VA-C in the online-only Data Supplement), also supporting the notion that protein hyperacetylation

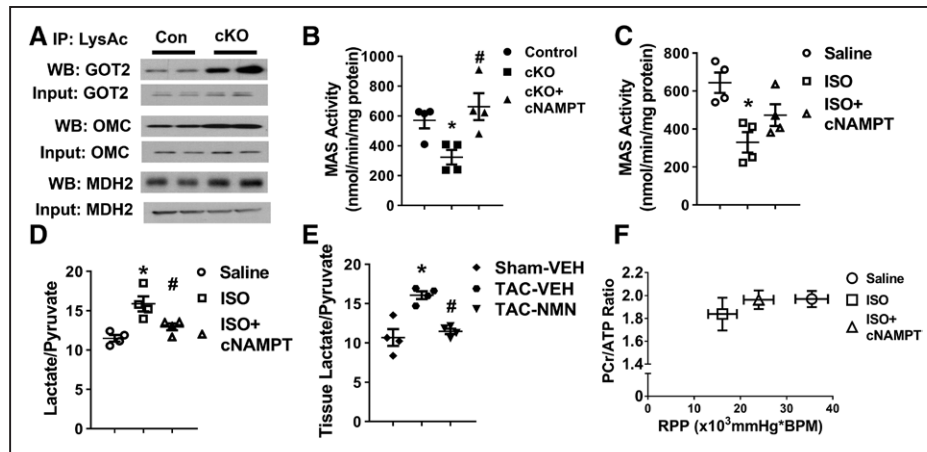


Figure 5. Inhibition of malate aspartate shuttle (MAS) by acetylation altered cytosolic redox state and cardiac energetics.

A, Acetylation levels of mitochondrial isoforms of MAS proteins were assessed by immunoprecipitation/Western blot (IP-WB) analysis. N=4. MAS activity of mitochondria isolated from **(B)** control, cKO and cKO+cNAMPT and **(C)** isoproterenol- (ISO) treated hearts were measured. N=4. Tissue lactate/pyruvate ratio of **(D)** ISO-treated or **(E)** transverse aortic constriction- (TAC) stressed hearts were measured. N=4. **F**, The relationship between cardiac energetics, estimated by phosphocreatine to adenosine triphosphate ratio (PCr/ATP) and contractile function, estimated by rate pressure product (RPP), measured simultaneously in isolated perfused hearts by ³¹P NMR spectroscopy. N=4. **P*<0.05 compared with corresponding control/saline/sham-vehicle; and #*P*<0.05 compared with corresponding cKO/ISO/TAC-VEH. All data are expressed as means±SEM.

suppressed the shuttle activity. The lactate/pyruvate ratio, a marker for cytosolic NADH/NAD⁺ ratio, was elevated in isoproterenol-treated and TAC-stressed hearts and was lowered by cNAMPT or NMN (Figure 5D-E). The data collectively suggest that hyperacetylation of MAS decreases the import of cytosolic NADH into mitochondria for oxidation hence altered cytosolic NADH/NAD⁺ ratio.

Upregulation of glycolysis is a hallmark of metabolic remodeling in pathologic hypertrophy.^{24,25} Elevation of MAS flux under these conditions would facilitate aerobic glycolysis for adenosine triphosphate (ATP) production.^{24–28} It was shown previously that elevated MAS flux at early stage of pathological hypertrophy was attenuated as the heart transitions into energetic and contractile failure, even though the MAS protein levels were unaltered.²⁸ To test whether inhibition of MAS by acetylation is partially responsible for the impaired energetics in pathologic hypertrophy, we performed ³¹P NMR (31 phosphorus nuclear magnetic resonance) spectroscopy of isolated perfused hearts to measure myocardial high energy phosphate content and contractile function simultaneously. We observed a downward-left shift in the relationship of myocardial energetic status assessed by phosphocreatine to ATP ratio (PCr/ATP) and contractile function assessed by the rate pressure product in isoproterenol-treated hearts (Figure 5F). The impairments were improved by partially restored MAS activity via overexpressing cNAMPT (Figure 5C, F, upward-right shift in plot). These results suggest that acetylation of MAS proteins is a key mechanism through which mitochondria

dysfunction impacts energetics via redox-sensitive regulations during pathological remodeling.

Acetylation of OSCP and CypD Increased mPTP Sensitivity in the Failing Heart

We previously showed that increased cardiac susceptibility to stresses in cKO hearts partly is attributable to the hypersensitivity of calcium-triggered mitochondrial permeability transition pore (mPTP) opening.¹⁰ Here we found that similar to cKO, mitochondria isolated from TAC hearts demonstrated a lower calcium retention capacity, indicating increased sensitivity of mPTP opening, which were normalized by elevation of NAD⁺ levels (Figure 6A, Figure VIA in the online-only Data Supplement). Several hyperacetylated proteins identified by acetylome analysis participate in mPTP regulation (Table V in the online-only Data Supplement) such as CypD and OSCP, and the majority of the hyperacetylated sites on these proteins were responsive to NADH/NAD⁺ ratio (Table V in the online-only Data Supplement). Although the physical identity of the mPTP remains elusive, CypD is an undisputed regulator of the mPTP.^{29,30} Recent studies suggest that mitochondrial ATP synthase forms the mPTP³¹, and the opening of mPTP is regulated through the interaction of CypD with OSCP subunit of ATP synthase.^{31,32} We observed increased acetylation of both CypD and OSCP in cKO mitochondria and in human failing hearts (Figure 6B-C and Figure VIB, Table V in the online-only Data Supplement). Furthermore, increased acetylation was associated with increased interaction of OSCP-CypD

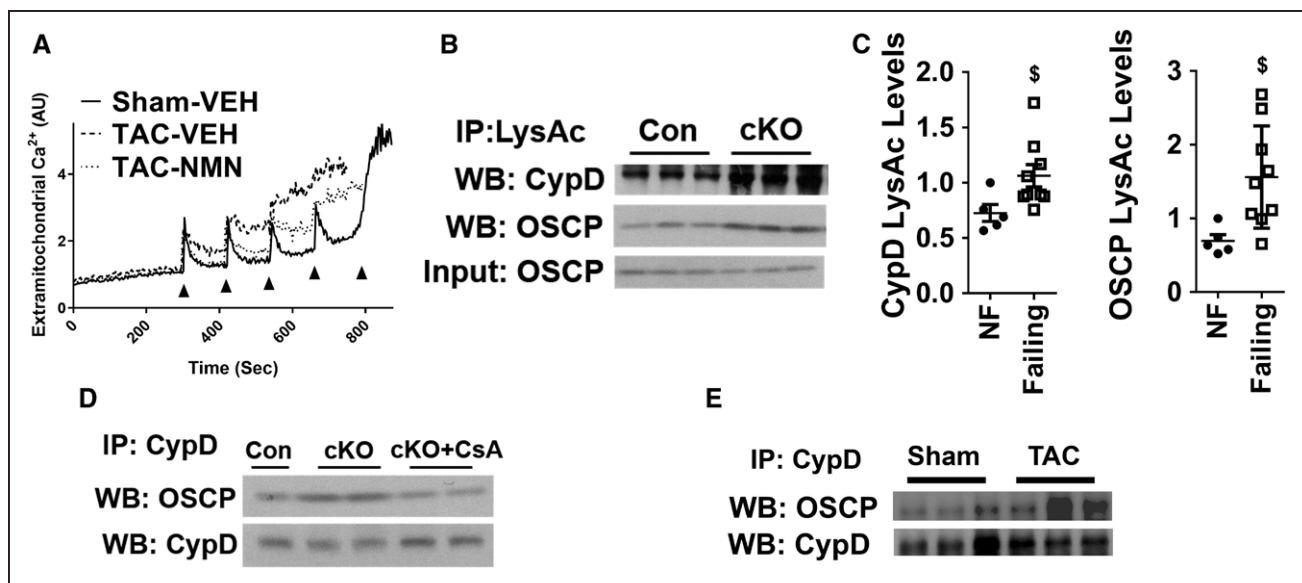


Figure 6. Acetylation promoted the interaction between oligomycin-sensitive conferring protein (OSCP) and cyclophilin D (CypD) and increased the sensitivity of mitochondrial permeability transition pore (mPTP).

A, Representative experiment of calcium-stimulated mPTP opening in mitochondria isolated from sham or transverse aortic constriction (TAC) hearts with and without nicotinamide mononucleotide (NMN) treatment. $N=3$ to 4. Arrows represent each calcium pulse. **B**, Acetylation levels of CypD and OSCP in control (Con) and cKO mitochondria determined by immunoprecipitation/Western blot (IP-WB). $N=3$. **C**, Acetylation levels of CypD and OSCP from nonfailing and failing human hearts were quantified. $N=5$ to 9. $^{\$}P<0.05$ compared with nonfailing. **D**, Interaction of CypD with OSCP were determined by IP-WB in mitochondria isolated from cKO hearts. $N=3$ to 4. Cyclosporine A (CsA) was added to mitochondria at a final concentration of 1 $\mu\text{mol/L}$. **E**, Interaction of CypD with OSCP in heart tissues after sham/TAC surgeries was assessed by IP-WB.

in cKO, TAC-stressed, or isoproterenol-treated hearts (Figure 6D-E, Figure VIC in the online-only Data Supplement). The increased interaction of OSCP-CypD in cKO was alleviated by cyclosporine A, an established mPTP inhibitor (CsA; Figure 6D), indicating the importance of such an interaction in regulating mPTP sensitivity. The data strongly suggest that NAD^+ -sensitive acetylation modulates the OSCP-CypD interaction and thus the mPTP sensitivity in hearts with pathologic hypertrophy.

Acetylation of OSCP-K70 Promoted OSCP-CypD Interaction and the mPTP Opening

To identify specific acetylation sites on OSCP and CypD responsible for regulating the protein-protein interaction, crystal structures of CypD³³ and OSCP of ATP synthase³⁴ were subjected to molecular docking.³⁵ Docking results from the highest scoring solutions consistently identified an interaction interface of OSCP with variable CypD orientations (Figure VIIA in the online-only Data Supplement). The conserved interface coincided with an empty space next to OSCP in $\text{F}_1\text{-ATP}$ synthase, which serves as a potential interaction interface for CypD binding on the intact $\text{F}_1\text{-ATP}$ complex (Figure VIIA-C in the online-only Data Supplement). Acetylated lysine residues identified in the 2 proteins from the acetylome analysis were mapped to the docked models (Figure 7A, left panel magenta spheres, Table V in the online-only Data Supplement).

Analysis of the top-10 scoring docking solutions revealed that OSCP-K70 always was present in the putative interaction interface. Acetylation of OSCP-K70 (OSCP-K70Ac) was normalized by cNAMPT expression in cKO (Table V in the online-only Data Supplement). In addition, OSCP-K70 is highly conserved among species surveyed (Figure VIID in the online-only Data Supplement, red asterisk) while other lysines (16 out of 21 lysines) are not. Seven out of the top-10 docking solutions showed that several different lysine residues of CypD could be in close contact with OSCP-K70 (data not shown). In 2 of these solutions, OSCP-K70 was in close proximity (3.1 Å) of CypD-K66, which would result in charge repulsion, destabilizing the OSCP-CypD interaction (Figure 7A, right panel, magenta sticks). Previous studies suggest that hyperacetylation of CypD-K166 modulate mPTP sensitivity.^{36,37} Although CypD-K166Ac was not detected in our acetylome analysis (Table V in the online-only Data Supplement), it was one of the lysines from CypD found within the interaction interface out of the highest scoring docking solutions. This suggests that CypD-K166 may cause repulsion with OSCP-K70 and regulate mPTP opening.

Electrostatic interaction was suggested in regulating the OSCP-CypD interaction.³¹ Together with the structural information (Figure 7A, Figure VIIA-C in the online-only Data Supplement), we hypothesized that OSCP-K70Ac promotes the OSCP-CypD interaction and sensitizes mPTP opening by alleviating electrostatic repulsion. To

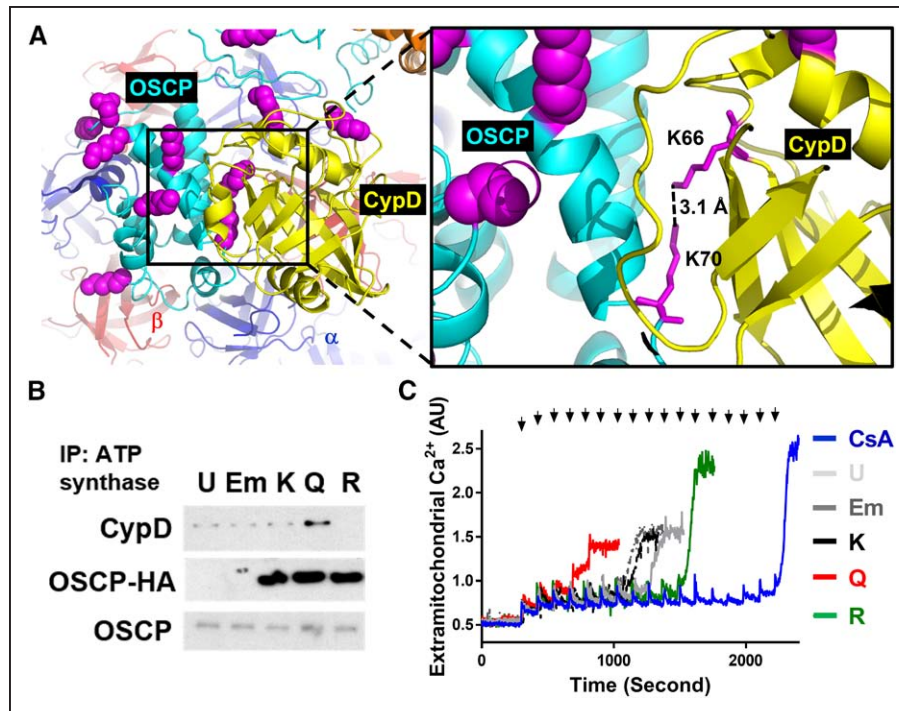


Figure 7. Acetylation of oligomycin-sensitive conferring protein- (OSCP) K70 was a critical determinant of mitochondrial permeability transition pore (mPTP) sensitivity.

A, Docking of C-V adenosine triphosphate (ATP) synthase (PDB: 2WSS) and cyclophilin D (CypD; yellow, PDB: 2BIU) with Patch-Dock and predefined constraint (see Methods). Left: overall view of OSCP and CypD interface with acetylated lysine residues highlighted as magenta spheres. Two lysines are located at the predicted interaction interface (black box). Right: magnified image of the putative interaction interface depicting the proposed regulation of interaction by acetylation on K70 of OSCP and K66 of CypD (magenta sticks). The distance between the 2 ϵ -nitrogen atoms of K70 of OSCP and K66 of CypD is measured by PyMOL (dotted line). **B**, Expression vectors indicated were transfected into HEK293 cells. Cell lysates were immunoprecipitated by ATP synthase antibodies and the presence of OSCP, HA-tagged OSCP and CypD was assessed by Western blot. Em indicates empty pcDNA3.1 vector transfected; K, wild-type HA-tagged OSCP; Q, K70Q mutant of OSCP; R, K70R of OSCP; and U: untransfected. Representative blots from at least 3 independent experiments were shown in **B**. **C**, Representative calcium-stimulated mPTP opening of permeabilized HEK293 cells after transfections were shown. CsA (1 μ M) was added after permeabilization of untransfected HEK293 cells. Arrows represent each 5 nmol calcium pulse.

determine the significance of OSCP-K70Ac, wild-type (K), K70Q (Q), and K70R (R) mutants of OSCP-K70 were expressed in HEK293 cells (Figure VII E in the online-only Data Supplement). Expression of acetylation-mimetic K70Q mutant showed increased interaction with CypD and sensitized mPTP opening (lowered calcium retention capacity), while expression of acetylation-insensitive K70R showed the opposite effects (Figure 7B-C, Figure VII F-G in the online-only Data Supplement). The data collectively indicate a critical role of OSCP-K70Ac in determining mPTP sensitivity via its interaction with CypD.

DISCUSSION

In this study we demonstrated that protein hyperacetylation induced by mitochondrial dysfunction is a positive regulator of pathologic remodeling in mouse hearts with primary or acquired mitochondrial dysfunction as well as in human failing hearts. Our study identified 2 distinct mechanisms that hyper-

acetylated protein targets, ie, the MAS and the regulators of mPTP, mediate increased propensity to heart failure. Importantly, we demonstrated that normalization of NADH/NAD⁺ ratio in the heart by genetic or pharmacologic approaches alleviated hyperacetylation, abrogated the 2 pathogenic mechanisms, and blunted the course of heart failure in mouse models, thus suggesting a novel therapy for heart failure.

Mitochondrial dysfunction has been recognized as a maladaptive mechanism during the development of heart failure.^{3,4} However, there has been no effective therapy in the clinic.⁵ Based on findings obtained from a genetic model of defective mitochondrial function, the present study identified and targeted pathogenic mechanisms caused by the imbalance of NADH production and oxidation in the mitochondria of hypertrophied and failing heart. We found that expanding the NAD⁺ pool could normalize NADH/NAD⁺ ratio, restore protein acetylation, and mitigate the development of heart failure in multiple mouse models of pathologic hypertrophy and

failure. The strategy represents a novel therapeutic approach that targets the modification of protein functions consequent of mitochondrial dysfunction. Indeed, the NAD⁺ precursors such as NMN or nicotinamide riboside have yielded beneficial outcomes in animal models of diabetes mellitus,³⁸ obesity,³⁹ and aging.⁴⁰ By comparing mouse models and human failing hearts in this study, we provide strong evidence that a similar mechanism applies to human heart failure, and indicate a highly translatable therapeutic strategy. Although NMN has poor oral bioavailability, oral administration of nicotinamide riboside, a recently approved nutritional supplement, is effective in rising blood NAD⁺ levels in healthy volunteers (Airhart et al, unpublished). Additional study of the safety and tolerability of expanding NAD⁺ pool in patient population is thus highly warranted.

Previous studies have shown that the level of protein acetylation is related closely to the metabolic state of the cell, and it fluctuates with nutritional status.⁴¹ Persistent hyperacetylation in the heart, such as in mice with deletion of Sirt3 or Ndufs4, resulted in increased sensitivity to stress while the unstressed heart is normal.^{10,36} We previously observed that increased NADH/NAD⁺ ratio in the Ndufs4 deficient heart inhibited Sirt3 resulting in protein hyperacetylation.¹⁰ Restoring Sirt3 activity through normalization of NADH/NAD⁺ ratio in this study reverses mitochondrial protein hyperacetylation in cKO hearts as well as in hearts with pathologic hypertrophy by pressure overload or isoproterenol stimulation. Acetylome analyses from studies of Sirt3-null mice^{42–44} and failing hearts⁴⁵ have identified thousands of mitochondrial acetylation sites, but only a handful of their functions have been biochemically characterized. The specific mechanisms connecting protein acetylation and increased sensitivity to stress remain to be poorly understood. Here we identified a subgroup of mitochondrial proteins that are highly sensitive to NADH/NAD⁺ ratio, among which hyperacetylation of malate aspartate shuttle and regulators of mPTP were shown to be linked causally to the development of heart failure. Furthermore, by expanding the NAD⁺ pool we were able to normalize but not overcorrect the NADH/NAD⁺ ratio, suggesting that such an approach is desirable from bioenergetics point of view.

It is conceivable that the disease mechanisms mediated by protein hyperacetylation involve other protein targets beyond the 2 groups reported here. Previous studies have identified other molecular targets of acetylation which are sensitive to NAD⁺ precursor supplementation in diabetes and mitochondrial diseases.^{38–40} Furthermore, we found that downregulation of malate aspartate shuttle (MAS) activity resulted in significant changes of cytosolic redox state. The MAS transfers the electron from NADH generated by glycolysis into mitochondria. Inhibition of MAS by acetylation serves as a feedback mechanism to protect mitochondrial compartment from further increases in NADH/NAD⁺ ratio but at the cost

of altering cytosolic redox environment and sirtuin activities in the nonmitochondrial compartment. In addition, multiple redox dependent regulatory mechanisms, such as cysteine oxidation, glutathionylation, redox-mediated phosphorylation, have been shown to play important roles in the development of cardiac dysfunction.^{46,47} Of note, ATP citrate lyase, an enzyme that catalyzes the cytoplasmic conversion of mitochondrial citrate into the acetyl-coA in the cytosol, is activated at reduced state,⁴⁸ thus increasing substrate supply for protein acetylation. Consistently, a significant number of hyperacetylated proteins are found in the nonmitochondrial compartments of the complex-I deficient hearts, suggesting that mitochondrial dysfunction ultimately could affect whole cell acetylation. Although the scope of this study did not allow us to determine the specific contribution by each of these mechanisms in the progression of heart failure, it provides compelling evidence for targeting NADH/NAD⁺ ratio for therapy. Moreover, observations made here open a new avenue for investigating mitochondrial-cytosolic redox communications in chronic diseases involving mitochondrial dysfunction.

Cell death caused by the opening of mitochondrial permeability transition pore (mPTP) is an important mechanism in the development of heart failure. We and others have shown previously that increased mitochondrial protein acetylation sensitizes the mPTP,^{10,36} but the specific molecular targets are unknown due to the lack of physical identity of the mPTP. By combining the computational and mutagenesis approaches, we identified the acetylation of K70 on OSCP as a key determinant of mPTP sensitivity via its interaction of CypD. This finding also reconciles with prior reports suggesting that acetylation of CypD at K166 could increase the sensitivity of mPTP.³⁶ Our computation models propose that acetylation of several lysine residues on CypD, including K66 that is at the closest proximity and K166 that is present at the putative interface of one top scoring model, could further reduce the repulsion between the 2 proteins. OSCP is located at the matrix side of the F₁ subcomplex of the ATP synthase, making stable interaction with proteins of both F₁ and the peripheral stalk (Figure VIB-C in the online-only Data Supplement). Recent studies suggest that either the dimer of F₁F₀-ATP synthase³¹ or F₀ sub-complex^{49,50} forms the mPTP under specific conditions. Our results, although in line with this model, do not confirm the specific identity of the physical pore. They, nevertheless, provide a novel target to manipulate the mPTP sensitivity for therapy.

In summary, using a mouse model of mitochondrial complex-I deficiency as the discovery tool we have unveiled novel mechanisms by which mitochondrial dysfunction modulates cellular stress response through NADH/NAD⁺-sensitive protein acetylation. The findings were validated in multiple mouse models of pathological hypertrophy, as well as in human failing hearts. Our pre-

clinical data not only demonstrate a clear benefit of expanding NAD⁺ pool in heart failure therapy, the currently available NAD⁺ precursor compounds, but also make our findings immediately translatable.

ACKNOWLEDGMENTS

We thank Dr Michael Sack for GCN5L1 antibody and Dr Junichi Sadoshima for providing cardiac-specific NAMPT mice.

SOURCES OF FUNDING

This study is supported by grants from the National Institutes of Health (HL110349 and HL067970 to R.T.; R01GM086688 to J.E.B.; 2T32DK007247 to N.D.R.; T32AG000057 to Y.A.C.; S10RR029021 to 14T High Resolution Imaging Facility), the American Heart Association (postdoctoral fellowship 13POST16200007 to C.F.L.), the Ellison/American Federation for Aging Research (Postdoctoral Fellowship to Y.A.C.) and the Glenn/AFAR Postdoctoral Fellowship for Translational Research on Aging (to Y.A.C.).

DISCLOSURES

None.

AFFILIATIONS

From Mitochondria and Metabolism Center (C.F.L., L.G.-M., Y.C., N.D.R., R.T.), Department of Anesthesiology and Pain Medicine (C.F.L., L.G.-M., Y.C., N.D.R., R.T.), Department of Genome Sciences (J.D.C., J.E.B.), Department of Pathology (Y.A.C.), and Department of Medicinal Chemistry (J.S.E., Y.A.G., D.R.G.), University of Washington, Seattle, WA.

FOOTNOTES

Received March 15, 2016; accepted July 8, 2016.

The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.116.022495/-/DC1>.

Circulation is available at <http://circ.ahajournals.org>.

REFERENCES

- Roth GA, Forouzanfar MH, Moran AE, Barber R, Nguyen G, Feigin VL, Naghavi M, Mensah GA, Murray CJ. Demographic and epidemiologic drivers of global cardiovascular mortality. *N Engl J Med*. 2015;372:1333–1341. doi: 10.1056/NEJMoa1406656.
- Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB, American Heart Association statistics C and stroke statistics S. Heart disease and stroke statistics 2016 update: a report from the American Heart Association. *Circulation*. 2016;133:e38–e360.
- Bayeva M, Gheorghide M, Ardehali H. Mitochondria as a therapeutic target in heart failure. *J Am Coll Cardiol*. 2013;61:599–610. doi: 10.1016/j.jacc.2012.08.1021.
- Rosca MG, Tandler B, Hoppel CL. Mitochondria in cardiac hypertrophy and heart failure. *J Mol Cell Cardiol*. 2013;55:31–41. doi: 10.1016/j.yjmcc.2012.09.002.
- Wang W, Karamanlidis G, Tian R. Novel targets for mitochondrial medicine. *Sci Transl Med*. 2016;8:326rv3. doi: 10.1126/scitranslmed.aac7410.
- Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M. The growing landscape of lysine acetylation links metabolism and cell signalling. *Nat Rev Mol Cell Biol*. 2014;15:536–550. doi: 10.1038/nrm3841.
- Cantó C, Menzies KJ, Auwerx J. NAD(+) metabolism and the control of energy homeostasis: a balancing act between Mitochondria and the nucleus. *Cell Metab*. 2015;22:31–53. doi: 10.1016/j.cmet.2015.05.023.
- Lee CF, Tian R. Mitochondrion as a target for heart failure therapy—role of protein lysine acetylation. *Circ J*. 2015;79:1863–1870. doi: 10.1253/circj.CJ-15-0742.
- Sauve AA, Wolberger C, Schramm VL, Boeke JD. The biochemistry of sirtuins. *Annu Rev Biochem*. 2006;75:435–465. doi: 10.1146/annurev.biochem.74.082803.133500.
- Karamanlidis G, Lee CF, Garcia-Menendez L, Kolwicz SC Jr, Suthamarak W, Gong G, Sedensky MM, Morgan PG, Wang W, Tian R. Mitochondrial complex I deficiency increases protein acetylation and accelerates heart failure. *Cell Metab*. 2013;18:239–250. doi: 10.1016/j.cmet.2013.07.002.
- Kolwicz SC Jr, Olson DP, Marney LC, Garcia-Menendez L, Synovec RE, Tian R. Cardiac-specific deletion of acetyl CoA carboxylase 2 prevents metabolic remodeling during pressure-overload hypertrophy. *Circ Res*. 2012;111:728–738. doi: 10.1161/CIRCRESA.112.268128.
- Boehm EA, Jones BE, Radda GK, Veech RL, Clarke K. Increased uncoupling proteins and decreased efficiency in palmitate-perfused hyperthyroid rat heart. *Am J Physiol Heart Circ Physiol*. 2001;280:H977–H983.
- Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem*. 2002;74:5383–5392.
- Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem*. 2003;75:4646–4658.
- Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods*. 2007;4:207–214. doi: 10.1038/nmeth1019.
- Ma K, Vitek O, Nesvizhskii AI. A statistical model-building perspective to identification of MS/MS spectra with PeptideProphet. *BMC Bioinformatics*. 2012;13 Suppl 16:S1. doi: 10.1186/1471-2105-13-S16-S1.
- Liu H, Sadygov RG, Yates JR 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem*. 2004;76:4193–4201. doi: 10.1021/ac0498563.
- Eng JK, Jahan TA, Hoopmann MR. Comet: an open-source MS/MS sequence database search tool. *Proteomics*. 2013;13:22–24. doi: 10.1002/pmic.201200439.
- Siegel S and Castellan Jr. NJ. *Nonparametric Statistics for the Behavioral Sciences*. Second Edition ed: McGraw Hill; 2000.
- Hsu CP, Oka S, Shao D, Hariharan N, Sadoshima J. Nicotinamide phosphoribosyltransferase regulates cell survival through NAD⁺ synthesis in cardiac myocytes. *Circ Res*. 2009;105:481–491. doi: 10.1161/CIRCRESA.109.203703.
- Errami M, Galindo CL, Tassa AT, Dimaio JM, Hill JA, Garner HR. Doxycycline attenuates isoproterenol- and transverse aortic band-

- ing-induced cardiac hypertrophy in mice. *J Pharmacol Exp Ther*. 2008;324:1196–1203. doi: 10.1124/jpet.107.133975.
22. Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD⁺: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev*. 2010;31:194–223. doi: 10.1210/er.2009-0026.
 23. LaNoue KF, Williamson JR. Interrelationships between malate-aspartate shuttle and citric acid cycle in rat heart mitochondria. *Metabolism*. 1971;20:119–140.
 24. Allard MF, Schönekeess BO, Henning SL, English DR, Lopaschuk GD. Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am J Physiol*. 1994;267(2 Pt 2):H742–H750.
 25. Nascimben L, Ingwall JS, Lorell BH, Pinz I, Schultz V, Tornheim K, Tian R. Mechanisms for increased glycolysis in the hypertrophied rat heart. *Hypertension*. 2004;44:662–667. doi: 10.1161/01.HYP.0000144292.69599.0c.
 26. Wambolt RB, Lopaschuk GD, Brownsey RW, Allard MF. Dichloroacetate improves postischemic function of hypertrophied rat hearts. *J Am Coll Cardiol*. 2000;36:1378–1385.
 27. Rupert BE, Segar JL, Schutte BC, Scholz TD. Metabolic adaptation of the hypertrophied heart: role of the malate/aspartate and alpha-glycerophosphate shuttles. *J Mol Cell Cardiol*. 2000;32:2287–2297. doi: 10.1006/jmcc.2000.1257.
 28. Lewandowski ED, O'donnell JM, Scholz TD, Sorokina N, Buttrick PM. Recruitment of NADH shuttling in pressure-overloaded and hypertrophic rat hearts. *Am J Physiol Cell Physiol*. 2007;292:C1880–C1886. doi: 10.1152/ajpcell.00576.2006.
 29. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature*. 2005;434:658–662. doi: 10.1038/nature03434.
 30. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature*. 2005;434:652–658. doi: 10.1038/nature03317.
 31. Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabó I, Lippe G, Bernardi P. Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc Natl Acad Sci U S A*. 2013;110:5887–5892. doi: 10.1073/pnas.1217823110.
 32. Chinopoulos C, Adam-Vizi V. Modulation of the mitochondrial permeability transition by cyclophilin D: moving closer to F(0)F(1) ATP synthase? *Mitochondrion*. 2012;12:41–45. doi: 10.1016/j.mito.2011.04.007.
 33. Schlatter D, Thoma R, Küng E, Stihle M, Müller F, Borroni E, Cesura A, Hennig M. Crystal engineering yields crystals of cyclophilin D diffracting to 1.7 Å resolution. *Acta Crystallogr D Biol Crystallogr*. 2005;61(Pt 5):513–519. doi: 10.1107/S0907444905003070.
 34. Rees DM, Leslie AG, Walker JE. The structure of the membrane extrinsic region of bovine ATP synthase. *Proc Natl Acad Sci U S A*. 2009;106:21597–21601. doi: 10.1073/pnas.0910365106.
 35. Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res*. 2005;33(Web Server issue):W363–W367. doi: 10.1093/nar/gki481.
 36. Hafner AV, Dai J, Gomes AP, Xiao CY, Palmeira CM, Rosenzweig A, Sinclair DA. Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy. *Aging (Albany NY)*. 2010;2:914–923. doi: 10.18632/aging.100252.
 37. Bochaton T, Crola-Da-Silva C, Pillot B, Villedieu C, Ferreras L, Alam MR, Thibault H, Strina M, Gharib A, Ovize M, Baetz D. Inhibition of myocardial reperfusion injury by ischemic postconditioning requires sirtuin 3-mediated deacetylation of cyclophilin D. *J Mol Cell Cardiol*. 2015;84:61–69. doi: 10.1016/j.yjmcc.2015.03.017.
 38. Yoshino J, Mills KF, Yoon MJ, Imai S. Nicotinamide mononucleotide, a key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metab*. 2011;14:528–536. doi: 10.1016/j.cmet.2011.08.014.
 39. Cantó C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, Fernandez-Marcos PJ, Yamamoto H, Andreux PA, Cettour-Rose P, Gademann K, Rinsch C, Schoonjans K, Sauve AA, Auwerx J. The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell Metab*. 2012;15:838–847. doi: 10.1016/j.cmet.2012.04.022.
 40. Gomes AP, Price NL, Ling AJ, Moslehi JJ, Montgomery MK, Rajman L, White JP, Teodoro JS, Wrann CD, Hubbard BP, Mercken EM, Palmeira CM, de Cabo R, Rolo AP, Turner N, Bell EL, Sinclair DA. Declining NAD(+) induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell*. 2013;155:1624–1638. doi: 10.1016/j.cell.2013.11.037.
 41. Yang L, Vaitheeswaran B, Hartil K, Robinson AJ, Hoopmann MR, Eng JK, Kurland IJ, Bruce JE. The fasted/fed mouse metabolic acetylome: N6-acetylation differences suggest acetylation coordinates organ-specific fuel switching. *J Proteome Res*. 2011;10:4134–4149. doi: 10.1021/pr200313x.
 42. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, Grueter CA, Harris C, Biddinger S, Ilkayeva OR, Stevens RD, Li Y, Saha AK, Ruderman NB, Bain JR, Newgard CB, Farese RV Jr, Alt FW, Kahn CR, Verdin E. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature*. 2010;464:121–125. doi: 10.1038/nature08778.
 43. Qiu X, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell Metab*. 2010;12:662–667. doi: 10.1016/j.cmet.2010.11.015.
 44. Shimazu T, Hirschey MD, Hua L, Dittenhafer-Reed KE, Schwer B, Lombard DB, Li Y, Bunkenborg J, Alt FW, Denu JM, Jacobson MP, Verdin E. SIRT3 deacetylates mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 and regulates ketone body production. *Cell Metab*. 2010;12:654–661. doi: 10.1016/j.cmet.2010.11.003.
 45. Horton JL, Martin OJ, Lai L, Riley NM, Richards AL, Vega RB, Leone TC, Pagliarini DJ, Muoio DM, Bedi KC, Jr., Margulies KB, Coon JJ and Kelly DP. Mitochondrial protein hyperacetylation in the failing heart. *JCI Insight*. 2016;2:e84897.
 46. Chung HS, Wang SB, Venkatraman V, Murray CI, Van Eyk JE. Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system. *Circ Res*. 2013;112:382–392. doi: 10.1161/CIRCRESAHA.112.268680.
 47. Murphy E, Kohr M, Menazza S, Nguyen T, Evangelista A, Sun J, Steenbergen C. Signaling by S-nitrosylation in the heart. *J Mol Cell Cardiol*. 2014;73:18–25. doi: 10.1016/j.yjmcc.2014.01.003.
 48. Wells TN, Saxty BA. Redox control of catalysis in ATP-citrate lyase from rat liver. *Eur J Biochem*. 1992;204:249–255.
 49. Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznarski P, Li H, Nabili P, Hockensmith K, Graham M, Porter GA Jr, Jonas EA. An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. *Proc Natl Acad Sci U S A*. 2014;111:10580–10585. doi: 10.1073/pnas.1401591111.
 50. Bonora M, Bononi A, De Marchi E, Giorgi C, Lebedzinska M, Marchi S, Patergnani S, Rimessi A, Suski JM, Wojtala A, Wieckowski MR, Kroemer G, Galluzzi L, Pinton P. Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. *Cell Cycle*. 2013;12:674–683. doi: 10.4161/cc.23599.