Metabolomics-assisted proteomics identifies succinylation and SIRT5 as important regulators of cardiac function

Sushabhan Sadhukhan[a], Xiaojing Liu[b,c,d], Dongryeol Ryu[e], Ornella D. Nelson[a], John A. Stupinski[f], Zhi Li[a], Wei Chen[g], Sheng Zhang[g], Robert S. Weiss[f], Jason W. Locasale[b,c,d], Johan Auwerx[e,1], and Hening Lin[a,h,1]

[a]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853; [b]Duke Cancer Institute, Duke University School of Medicine, Durham, NC 27710; [c]Duke Molecular Physiology Institute, Duke University School of Medicine, Durham, NC 27710; [d]Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC 27710; [e]Laboratory of Integrative and Systems Physiology, School of Life Sciences, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland; [f]Department of Biomedical Sciences, Cornell University, Ithaca, NY 14853; [g]Proteomics & Mass Spectrometry Facility, Institute of Biotechnology, Cornell University, Ithaca, NY 14853; and [h]Howard Hughes Medical Institute, Cornell University, Ithaca, NY 14853

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Cellular metabolites, such as acyl-CoA, can modify proteins, leading to protein posttranslational modifications (PTMs). One such PTM is lysine succinylation, which is regulated by sirtuin 5 (SIRT5). Although numerous proteins are modified by lysine succinylation, the physiological significance of lysine succinylation and SIRT5 remains elusive. Here, by profiling acyl-CoA molecules in various mouse tissues, we have discovered that different tissues have different acyl-CoA profiles and that succinyl-CoA is the most abundant acyl-CoA molecule in the heart. This interesting observation has prompted us to examine protein lysine succinylation in different mouse tissues in the presence and absence of SIRT5. Protein lysine succinylation predominantly accumulates in the heart when *Sirt5* is deleted. Using proteomic studies, we have identified many cardiac proteins regulated by SIRT5. Our data suggest that ECHA, a protein involved in fatty acid oxidation, is a major enzyme that is regulated by SIRT5 and affects heart function. *Sirt5* knockout (KO) mice have lower ECHA activity, increased long-chain acyl-CoAs, and decreased ATP in the heart under fasting conditions. *Sirt5* KO mice develop hypertrophic cardiomyopathy, as evident from the increased heart weight relative to body weight, as well as reduced shortening and ejection fractions. These findings establish that regulating heart metabolism and function is a major physiological function of lysine succinylation and SIRT5.

sirtuin | lysine succinylation | fatty acid metabolism | desuccinylation | hypertrophic cardiomyopathy

Protein posttranslational modifications (PTMs) contribute toward the functional diversity of proteomes through regulating their activity, stability, and cellular localization. Many novel PTMs have been identified recently that result from enzymatic or nonenzymatic reactions with metabolites (1–5). Lysine, being the most frequently posttranslationally modified amino acid, has become the target of various PTMs such as acetylation, methylation, propionylation, butyrylation, crotonylation, succinylation, malonylation, glutarylation, long-chain fatty acylation, ubiquitination, and 2-hydroxyisobutyrylation (1, 3–9). Unlike lysine acetylation, lysine succinylation is a relatively new PTM and the succinyl donor is presumably succinyl-CoA. Acetylation on lysine neutralizes the positive charge of lysine side chain and is known to affect the structure and function of chromatin (10) as well as cellular metabolism (11). However, succinylation on lysine undergoes a complete charge reversal by changing a positively charged side chain to a negatively charged one. Regarding the change in charge, lysine succinylation is similar to phosphorylation, producing a two-unit charge shift in the modified residues. So, it can be anticipated that lysine succinylation would have a significant role in metabolic pathways, as was previously found for acetylation or phosphorylation.

Sirtuins are an evolutionarily conserved family of NAD-dependent lysine deacylases. Among the seven mammalian sirtuins (SIRT1–7), SIRT3–5 are located in mitochondria (12, 13). Unlike SIRT3, both SIRT4 and SIRT5 have very weak deacetylase activities (14). SIRT5 possesses unique enzymatic activity on hydrolyzing negatively charged lysine modifications such as lysine succinylation, malonylation, and glutarylation (1, 4, 8). The presence of two positively charged amino acids, Tyr102 and Arg105, in the active site of SIRT5 explained its preference for negatively charged acyl groups such as succinyllysine (1). Although proteomic studies (15–19) in mouse liver and skeletal muscle have identified hundreds of potential desuccinylation substrates of SIRT5 and several of these have been biochemically confirmed, the physiological significance of SIRT5 and lysine succinylation remains unclear. Deletion of *Sirt5* in mice produced only subtle phenotypes that seemed normal under basal conditions (20, 21) despite increased serum ammonium levels (22). We thus set out to obtain crucial information that would help to reveal the function of lysine succinylation and SIRT5.

Acetyl-CoA and succinyl-CoA are important intracellular metabolites involved in diverse metabolic pathways including the TCA cycle. Differences in metabolism could lead to a differential distribution of acyl-CoAs across different tissues. In many recently discovered PTMs, the lysine side chains of proteins react with acyl-CoAs through their ε-amino groups. Thus, the distribution of acyl-CoA may significantly affect the PTMs. Herein, we have conducted a metabolomics study to first profile acyl-CoAs in various murine tissues and found that different tissues have very different acyl-CoA profiles. This has led us to examine protein lysine succinylation across different tissues. Protein lysine succinylation predominantly accumulates in the heart when *Sirt5* is

Significance

Lysine succinylation is a recently discovered protein posttranslational modification and SIRT5 is an efficient desuccinylase. Although many mammalian proteins have recently been found to be regulated by lysine succinylation and SIRT5, the physiological significance of succinylation and SIRT5 remains unknown. Here we report that protein lysine succinylation predominantly accumulates in the heart when *Sirt5* is deleted. *Sirt5*-deficient mice exhibit defective fatty acid metabolism, decreased ATP production, and hypertrophic cardiomyopathy. Our data suggest that regulating heart metabolism and function is a major physiological role of lysine succinylation and SIRT5.

Author contributions: S.S. and H.L. designed research; S.S., D.R., and O.D.N. performed research; X.L., J.A.S., Z.L., R.S.W., and J.W.L. contributed new reagents/analytic tools; S.S., D.R., W.C., S.Z., and J.A. analyzed data; and S.S. and H.L. wrote the paper.

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[1]To whom correspondence may be addressed. Email: admin.auwerx@epfl.ch or hl379@cornell.edu.

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deleted. We have identified many desuccinylation substrates of SIRT5 using proteomics, among which ECHA, a protein involved in fatty acid oxidation, is a major substrate in the heart. SIRT5 activates ECHA via desuccinylation and, as a result, *Sirt5*-deficient mice exhibit defective fatty acid metabolism and decreased ATP production. *Sirt5* knockout (KO) mice exhibit both reduced shortening fraction and ejection fraction, implying a reduced cardiac function. Taken together, these findings reveal that a major physiological role of lysine succinylation and SIRT5 is to regulate heart metabolism and function.

Results

Different Mouse Tissues Have Unique Acyl-CoA Profiles. To obtain information that would help reveal the function of lysine succinylation and SIRT5, we profiled acyl-CoA concentrations, including succinyl-CoA (the presumed donor of succinyl for lysine succinylation), in major mouse organs such as liver, heart, kidney, brain, and muscle. This targeted metabolomics study conducted on acyl-CoAs from wild type (WT) mouse tissues revealed that different tissues have unique acyl-CoA profiles. For example, succinyl-CoA is the most abundant acyl-CoA in the heart. In the liver, the absolute concentration of succinyl-CoA is similar to that in the heart, but acetyl-CoA and free CoA are more abundant than succinyl-CoA (Fig. 1*A*). This interesting acyl-CoA profile suggested that different tissues might have differential patterns of protein lysine succinylation and prompted us to examine succinylation in different mouse tissues.

Protein Lysine Succinylation Predominantly Occurs in the Heart of *Sirt5* KO Mice. We next investigated the protein lysine succinylation and acetylation status in different tissues from *Sirt5* WT and KO mice. Western blot analysis for succinyllysine demonstrated that although the level of succinylation increased in all tissues when *Sirt5* was knocked out, it increased most dramatically in the heart (Fig. 1*B*). Importantly, concentrations of succinyl-CoA and succinyl-carnitine were comparable in the *Sirt5* WT and KO mice's hearts (Fig. S1 *A* and *B*), implicating that the observed hypersuccinylation was generated by the deficiency of SIRT5 and not by increased succinyl donors. Levels of most of the short-chain acyl-CoAs remained unaltered in *Sirt5* WT and KO mice tissues (Fig. S1 *C*–*F*). Western blot analysis for acetyllysine showed no significant changes in acetylation in *Sirt5* WT and KO tissues (Fig. 1*C*). The data suggested that among the mouse tissues tested, the desuccinylase activity of SIRT5 might play a very important role in the heart. Consistent with this hypothesis, among the different mouse tissues tested, the heart had the highest SIRT5 protein level (Fig. S2). Very recently SIRT5 is found to possess deglutarylation activity in addition to its known deacetylation, demalonylation, and desuccinylation activity (8). In general, glutaryl-CoA concentration is much lower compared with acetyl-CoA and succinyl-CoA. It is highest in the liver tissue among the tissues studied and hence, as expected, we found that the changes in protein lysine glutarylation level were rather small in all tissues examined when *Sirt5* was knocked out (Fig. S1*G*). These results suggested that although SIRT5 can remove several different negatively charged acyl lysine modifications, the major acyl group removed in vivo is likely succinyl.

Quantitative Proteomics on Lysine Succinylation from *Sirt5* WT and KO Heart. We next sought to identify proteins that are succinylated and regulated by SIRT5 in mouse heart. We used a proteomics approach involving reductive dimethylation (23) of the tryptic peptides followed by the enrichment of the succinylated peptides for identification by mass spectrometry (MS) (Fig. 2*A*). Tryptic peptides from equal amounts of total lysate of *Sirt5* WT and KO mice heart tissues were separately labeled with heavy and light dimethyl groups, respectively. The labeled peptides were then mixed and succinylated peptides were enriched using an anti-succinyllysine polyclonal antibody. Nano liquid chromatography (LC)-MS/MS analysis was then carried out to compare the abundance of succinylated peptides in *Sirt5* WT and KO samples. MS analysis revealed 124 succinylated proteins that are potentially regulated by SIRT5. Among all of the identified succinylated proteins, more than 75% were mitochondrial proteins (Dataset S1). More than 90% of succinylation sites showed increased abundance in *Sirt5* KO heart with an average KO/WT ratio of 8.37 and a median of 1.64 (Dataset S2). Significantly, over 25% of the sites showed over threefold greater abundance in *Sirt5* KO heart (Dataset S2).

To gain insight into how lysine succinylation and SIRT5 might affect mitochondrial metabolic networks, we performed pathway enrichment analysis using DAVID bioinformatics resources (24, 25). Consistent with earlier reports, a number of metabolic pathways including branched-chain amino acids metabolism, the TCA cycle, fatty acid metabolism, propanoate metabolism, oxidative phosphorylation, pyruvate metabolism, and ATP synthesis are significantly enriched among the SIRT5 desuccinylation targets (Fig. 2*C*) (16, 18, 19). It is possible that the regulation of all these metabolic enzymes collectively contributes to the biological function of SIRT5 and lysine succinylation in the heart. Nevertheless, to gain a better understanding of the physiological roles for SIRT5, we sought to identify the pathway that is significantly affected in the heart when *Sirt5* is knocked out and that can play important roles in regulating heart function.

One feature that caught our attention was that the number of succinylation sites per protein varied significantly (from 1 to 28) depending on the protein (Fig. 2*B*). ECHA was identified to have the most succinylation sites (at 28 Lys residues) in the *Sirt5* KO heart. Among the 66 lysine residues of ECHA, 28 were succinylated and the majority of succinylated residues (26 out of 28) were only found in *Sirt5* KO heart, indicative of ECHA being a target of SIRT5. We focused on ECHA for biochemical validations for two considerations. First, we examined several other desuccinylation targets of SIRT5 (e.g., citrate synthase and ATP synthase) and found that the activities of these targets were not

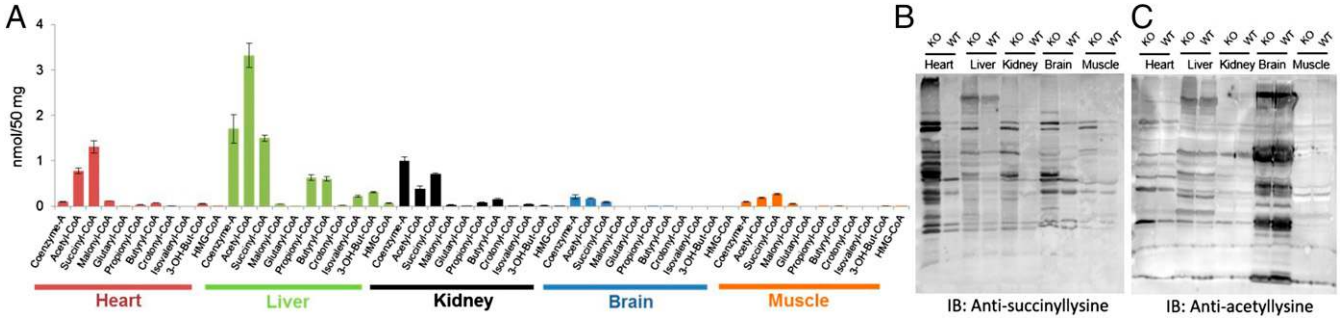

Fig. 1. Protein lysine succinylation occurs to the greatest extent in the heart. (*A*) Profiling of short-chain CoAs among different tissues from *Sirt5* WT mice using LC-MS/MS (mean ± SEM, $n = 3$ mice). (*B* and *C*) Western blot of different tissue lysates (25 μg each) against (*B*) antisuccinyllysine antibody and (*C*) against antiacetyllysine antibody. *Sirt5* KO heart has the highest succinylation level. Coomassie-stained gels (loading control) are shown in Fig. S2 *D* and *E*.

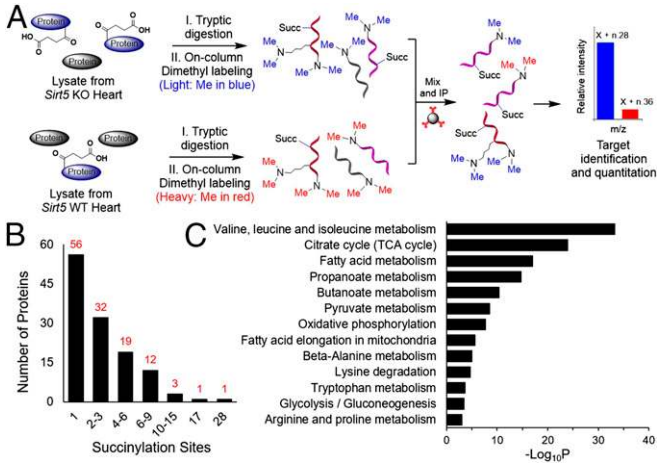

Fig. 2. Workflow of the dimethyl-labeling strategy for the succinylome analysis. (*A*) One milligram of total protein from *Sirt5* KO and WT heart was separately digested with trypsin and labeled with light and heavy dimethyl groups, respectively. The isotopically labeled peptides were mixed together and immunoprecipitated with antisuccinyllysine antibody. Succinyl-lysine peptides were then analyzed by nano LC-MS/MS. (*B*) Distribution of number of lysine succinylation sites per protein. (*C*) Metabolic pathways enriched with lysine succinylated proteins.

significantly affected by SIRT5 in the heart. Second, ECHA is most abundant in the heart compared with other tissues. ECHA is the α-subnit of mitochondrial trifunctional enzyme, which is important for fatty acid β-oxidation and has three distinct activities: enoyl-CoA hydratase (ECH), 3-hydroxyacyl-CoA dehydrogenase (HACD), and 3-ketoacyl-CoA thiolase (KCAT) (26). The α-subunit (ECHA) has the ECH and HACD activities whereas the β-subunit (ECHB) harbors the KACT activity.

SIRT5 Activates ECHA by Desuccinylation. To confirm that ECHA was indeed hypersuccinylated in SIRT5-deficient mice, we immunoprecipitated ECHA from *Sirt5* WT and KO mouse heart and analyzed the succinylation level by Western blot using anti-succinyllysine antibody. ECHA was highly succinylated in the absence of SIRT5 (Fig. 3*A*). Similarly, Flag-tagged mouse ECHA (Flag-ECHA) in *Sirt5* knockdown (KD) HEK-293T cells was hypersuccinylated compared with ECHA from control KD cells (Fig. 3*B*). When Flag-ECHA was cotransfected with an expression vector for either SIRT5, or its catalytic mutant SIRT5-H158Y, into HEK-293T cells, ECHA succinylation level was decreased when coexpressed with SIRT5, but not with SIRT5-H158Y (Fig. 3*C*). Additionally, when coexpressed, Flag-ECHA was able to immunoprecipitate V5-tagged SIRT5, suggesting that ECHA and SIRT5 interact with each other (Fig. 3*D*).

Next we aimed to determine whether the succinylation of ECHA modulates its enzymatic activity. The combined ECH and HACD activities of ECHA were measured by monitoring the formation of NADH from NAD at 340 nm (27) using 2-(*E*)-decenoyl-CoA as a substrate. ECHA from *Sirt5* KO heart showed a 32% decrease in activity compared with that from *Sirt5* WT heart, suggesting that ECHA succinylation down-regulates its activity (Fig. 4*A*). Similarly, ECHA purified from *Sirt5* KD HEK-293T cells showed a lower activity than that from the control cells (Fig. 4*B*). Coexpression of ECHA with SIRT5 decreased ECHA succinylation (Fig. 3*C*) and led to a 24% increase in enzymatic activity (Fig. 4*B*). Coexpression of ECHA with SIRT5-H158Y did not change ECHA succinylation (Fig. 3*C*) or increase its activity (Fig. 4*B*). We also purified recombinant mouse trifunctional protein complex (ECHA and ECHB) from *Escherichia coli* to test the consequence of succinylation on its activity in vitro. We first treated the recombinant ECHA and ECHB complex with succinyl-CoA for 10 min at 27 °C to prompt nonenzymatic succinylation, and then the reaction mixture was further incubated with or without SIRT5 for 10 min at 27 °C. Nonenzymatically succinylated ECHA and ECHB complex showed a 40% reduction in activity, but upon SIRT5 treatment the activity was restored (Fig. 4*C*). To evaluate whether lysine acetylation also regulates ECHA activity, we performed a chemical acetylation of ECHA and ECHB complex by incubating it with acetic anhydride and checked its activity. As shown in Fig. S3*A*, we did not observe any change in ECHA activity after it was acetylated. This result further demonstrates that ECHA activity is regulated by succinylation and SIRT5-catalyzed desuccinylation.

Lys351 Is the Major Succinylation Site of ECHA That Regulates Its Activity. To elucidate which of the 28 succinyllysine residues (Fig. S3 *B* and *C*) identified on mouse ECHA down-regulates its enzymatic activity, we examined the crystal structure of a homologous ECHA from *Mycobacterium tuberculosis* in complex with free CoA bound at the ECH active site (28). The crystal structure shows that several lysine residues (K351, K406, and K644) targeted by SIRT5 are present at the interface between ECHA and ECHB or are close to the bound CoA in the ECH site. For example, K351 is very close to the bound CoA (the distance between the ε-N of K351 and the phosphate of CoA is less than 4 Å) and hence the succinylation on K351 could disrupt the interaction between ECHA and CoA (Fig. S3*D*). To test the effect of succinylation of these lysine residues (K351, K406, and K644) on ECHA activity, we expressed Flag-tagged ECHA WT, K-to-R mutants (mimicking the desuccinylated state) or K-to-E mutants (mimicking the negatively charged succinyllysine modification) in HEK-293T and carried out enzymatic activity assays after immunoprecipitation. Whereas all of the K-to-R mutants maintained basal activity, only K351E showed a significant loss (more than 70%) in ECHA enzymatic activity compared with the WT (Fig. 4*D*). These data identify Lys351 as a critical lysine residue for the regulation of ECHA enzymatic activity.

We further checked the enzymatic activity of K351R and K351E mutants immunopurified from HEK-293T control and *Sirt5* KD cells. Whereas WT ECHA from *Sirt5* KD cells showed a decrease in activity compared with that from control KD cells, neither K351R nor K351E showed any difference in enzymatic activity in control and *Sirt5* KD cells (Fig. 4*E*). We also showed that upon chemical succinylation with succinyl-CoA, K351R and K351E ECHA did not lose additional activity (Fig. 4*F*). Thus, our mutational data suggest that SIRT5 regulates ECHA enzymatic activity mainly through desuccinylation of Lys351.

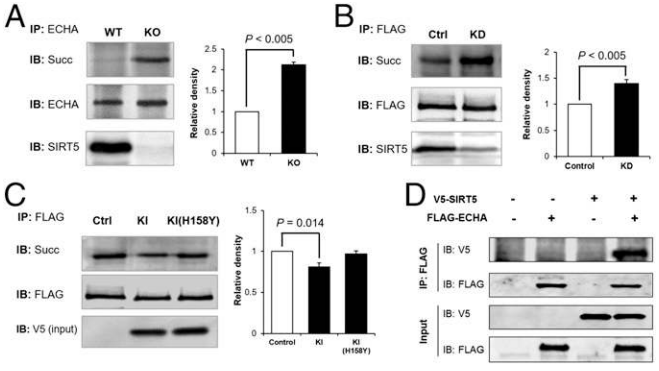

Fig. 3. Lack of SIRT5 leads to hypersuccinylation on ECHA. (*A*) ECHA was immunoprecipitated from *Sirt5* WT and KO mouse heart using ECHA-specific antibody. *Sirt5* KO mouse heart had increased succinylation on endogenous ECHA. (*B*) Flag-ECHA expressed in HEK-293T *Sirt5* KD cells showed increased succinylation compared with Flag-ECHA from control KD cells. (*C*) Overexpression of WT SIRT5, but not catalytically inactive SIRT5-H158Y, decreased the succinylation level of ECHA. Quantitative representation of relative density of succinylation (mean ± SEM, $n = 3$) is shown for *A*–*C*. (*D*) Flag-ECHA and V5-tagged SIRT5 were co-overexpressed in HEK-293T cells. Immunoprecipitation of Flag-ECHA pulled down V5-tagged SIRT5.

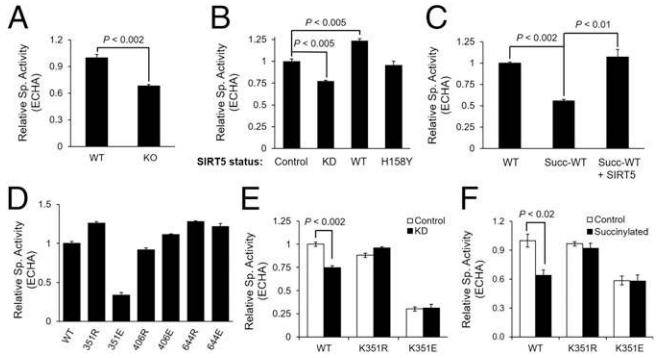

Fig. 4. SIRT5 increases ECHA activity by desuccinylation. (*A*) ECHA activity was higher in *Sirt5* WT mouse hearts than in *Sirt5* KO mice. (*B*) Flag-ECHA expressed in HEK-293T control, *Sirt5* KD, and SIRT5 (WT or H158Y) overexpressing cells showed activities consistent with the hypothesis that SIRT5 increases ECHA activity by desuccinylation. (*C*) Recombinant ECHA and ECHB (coexpressed and purified in *E. coli*) could be nonenzymatically succinylated, which decreased the ECHA activity. Incubation with SIRT5 and NAD restored ECHA activity. (*D*) K351 is the only residue that decreases ECHA activity when mutated to E. (*E*) Neither K351R nor K351E show any change in activity when purified from HEK-293T control or *Sirt5* KD cells. (*F*) Unlike WT, K351R and K351E mutant ECHA does not lose any additional activity when incubated with succinyl-CoA. Data shown as mean ± SEM, $n = 3$.

Absence of SIRT5 Resulted in Reduced Fatty Acid Oxidation and Accumulation of Long-Chain Fatty Acyl-CoAs. We wanted to determine the consequence of decreased ECHA enzymatic activity due to succinylation on the level of long-chain fatty acyl-CoAs in the heart. Defective ECHA would significantly slow down the β-oxidation of long-chain fatty acids, leading to accumulation of long-chain acyl-CoAs. Indeed, after 30 min of endurance exercise, long-chain acyl-CoA levels were elevated in the KO heart compared with the WT (Fig. 5*A*). Significant accumulation of long-chain acyl-CoAs in the heart was also observed in *Sirt5* KO mice that were fasted for 24 h (Fig. S4*A*). *Sirt5* KO heart also showed an accumulation of odd-chain fatty acyl-CoA with a chain length higher than 11 but not the shorter ones (Fig. S4*B*). The data suggest that the loss in ECHA activity in *Sirt5* KO heart also slows down the odd-chain fatty acid oxidation. Endurance exercise or fasting forces the mice to use β-oxidation to get the necessary energy and therefore requires optimal ECHA activity. Our data suggest that succinylation impairs fatty acid oxidation through down-regulation of ECHA activity. Hence, SIRT5 is important to maintain efficient fatty acid oxidation in the heart during energy-demanding situations such as fasting and exercise. In addition, fatty acid oxidation, measured in permeabilized heart tissues from *Sirt5* WT and KO mice, was clearly reduced in *Sirt5* KO mice (Fig. 5*B*). Our findings are in agreement with the previous report of reduced fatty acid oxidation and accumulation of acylcarnitines in *Sirt5* KO liver and muscles (18).

Lack of SIRT5 Results in Lower Cardiac ATP Levels. To further test the hypothesis that the regulation of ECHA by SIRT5 is important for cardiac energy production, we measured ATP levels in *Sirt5* WT and KO hearts. As would be expected in the case of defective fatty acid oxidation, we observed more than 20% reduction in ATP in *Sirt5* KO heart compared with WT (Fig. 5*C*). Other metabolic enzymes regulated by SIRT5 might also contribute to the decreased ATP production in *Sirt5* KO hearts. For example, citrate synthase controls the flow of acetyl-CoA into the TCA cycle and any loss in its activity might also contribute to the observed decrease of fatty acid oxidation. To test this possibility, we measured the enzymatic activity of citrate synthase from *Sirt5* WT and KO heart lysates and found that WT and KO heart had comparable citrate synthase activities despite citrate synthase being hypersuccinylated in KO heart (Fig. 5*D* and Fig. S4*C*). Another likely candidate for reduced ATP level in *Sirt5* KO heart is ATP synthase, which was found to be hypersuccinylated in the proteomics study. However, we did not observe any significant change in ATP synthase (complex V) activity in *Sirt5* WT and KO heart (Fig. 5*E*). These data support the conclusion that the observed succinylation-induced fatty acid oxidation deficit is mainly driven by the regulation of ECHA.

SIRT5 KO Mice Exhibit Reduced Cardiac Function and Develop Hypertrophic Cardiomyopathy with Aging. The heart has a very rapid and dynamic rate of ATP consumption (29), and hence a constant supply of ATP is necessary to keep the heart working properly. Lower cardiac ATP content might decrease the ability of *Sirt5* KO mice to effectively convert the chemical energy to contractile work (30). Fatty acid is a major energy source used to sustain contractile function in the heart, and thus a decrease in fatty acid metabolism might result in heart dysfunction. To further explore cardiac function in *Sirt5* WT and KO mice, we performed echocardiography on mice at 8 weeks of age after overnight fasting. Both the shortening fraction and ejection fraction were reduced in young adult *Sirt5* KO mice, indicating reduced cardiac function in the absence of SIRT5 (Fig. 6 *A* and *B* and Fig. S5). To see whether the cardiac phenotype becomes more prominent upon aging, we also recorded echocardiographic parameters in 39-week-old mice. The older *Sirt5* KO mice showed hallmarks of hypertrophic cardiomyopathy, such as significantly increased heart weight (normalized to body weight) and left ventricular mass (normalized to body weight) along with reduced shortening fraction and ejection fraction (Fig. 6 *C*–*G* and Fig. S6 *A*–*I*). Hematoxylin and eosin (H&E) staining and quantification of cardiomyocyte cross-sectional area shows evidence of cardiac hypertrophy in the *Sirt5* KO mice (Fig. 6 *H* and *I* and Fig. S6*J*). Furthermore, there was evidence of fibrosis (Masson's trichrome staining, Fig. 6*J*) and macrophage infiltration (F4/80 staining, Fig. S6*K*) in *Sirt5* KO hearts. In addition, well-known markers for cardiomyopathy, such as smooth muscle myosin (SMM) and atrial natriuretic peptide (ANP) levels (Fig. 6*K*), were robustly induced in *Sirt5* KO hearts. Finally, to rule out the possibility that the hypertrophic cardiomyopathy in *Sirt5* KO mice was caused by a developmental defect, we monitored the hearts of neonatal *Sirt5* WT and KO pups. In *Sirt5* KO pups, heart weight was normal

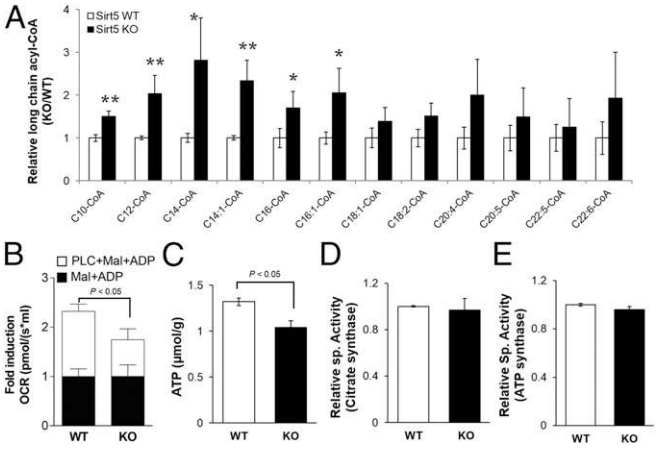

Fig. 5. SIRT5 deficiency leads to accumulation of long-chain CoAs and decreased cardiac ATP levels. (*A*) Relative levels of long-chain CoA thioesters in *Sirt5* KO hearts compared with WT (after 30 min of exercise, $^{**}P < 0.05$, $^{*}P < 0.1$). (*B*) Normalized fatty acid oxidation was significantly reduced in permeabilized *Sirt5* KO heart tissue. Mitochondrial respiration in response to palmitoyl-L-carnitine (PLC) was monitored. Malate (2 mM) and ADP (2.5 mM) were used as a pretreatment. (*C*) Cardiac ATP levels were measured in *Sirt5* WT and KO mice after 24 h of fasting. (*D*) Enzymatic activity of citrate synthase was measured in heart extracts from *Sirt5* WT and KO mice. (*E*) Complex V activities were measured from *Sirt5* WT and KO mice heart mitochondria. All data shown as mean ± SEM, $n = 3$ per genotype.

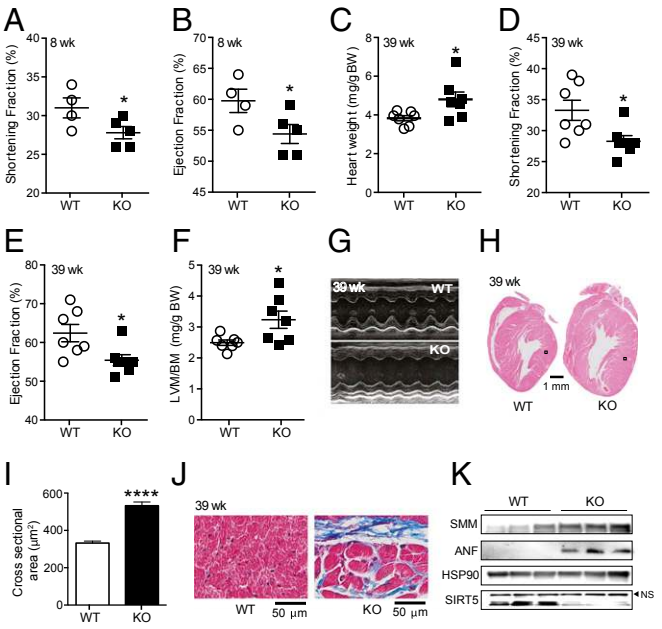

Fig. 6. SIRT5 deficiency causes hypertrophic cardiomyopathy. (*A* and *B*) Shortening fraction (*A*) and ejection fraction (*B*) were reduced in *Sirt5* KO mice (n = 4 and 5 for *Sirt5* WT and KO respectively, 8-week-old males). (*C*) Normalized heart weight of *Sirt5* WT and KO male mice (n = 7 per genotype). (*D*–*F*) The shortening fraction (*D*), and ejection fraction (*E*) were significantly reduced whereas left ventricular mass to body mass (LVM/BM, *F*) was significantly increased in hearts of *Sirt5* KO mice. (*G*) Representative M-mode images of echocardiography showing cardiac dysfunction in *Sirt5* KO mice. (*H* and *I*) H&E staining of heart cross-sections (*H*) and quantification of cardiomyocytes cross-sectional areas (n = 100 per genotype, *I*) showing cardiac hypertrophy in the *Sirt5* KO mice. The two black boxes in *H* indicate the localization of the images that are shown in larger magnification in Fig. S6*J*. (*J*) Masson's trichrome stain in cross-sections of the heart showing increased fibrosis in *Sirt5* KO hearts. (*K*) Evaluation of SMM, ANP, HSP90, and SIRT5 protein levels in *Sirt5* WT and KO mouse hearts. Experiments in *C*–*J* were performed with hearts of 39-week-old male mice. All graphs shown as mean ± SEM, $*P < 0.05$, $****P < 0.0001$.

(Fig. S6*L*). Moreover, we failed to discover an induction of the transcript levels of the cardiomyopathy markers *Smm* and *Anp* in the hearts of *Sirt5* KO pups at 2 days of age (Fig. S6*M*). Thus, the hypertrophic cardiomyopathy in *Sirt5* KO mice was not caused by a developmental defect.

Discussion

Sirtuins were originally thought to be NAD-dependent protein lysine deacetylases (31). The lack of efficient deacetylation activity for SIRT4–7 prompted studies that led to the discovery that SIRT5 is an efficient desuccinylase, demalonylase (1, 17) and deglutarylase (8). This finding also opened up directions to discover novel activities for other sirtuins (7, 9). Finding the desuccinylase and demalonylase activity of SIRT5 also led to the identification of lysine succinylation and malonylation as common PTMs (1, 4, 16–19, 32). Proteomic studies have identified about 1,000 proteins that are succinylated and regulated by SIRT5 (16, 18, 19). Despite these studies, the biological significance of lysine succinylation and SIRT5 remains unclear. *Sirt5* KO mice only display subtle changes in physiology and seem normal under basal conditions regardless of elevated ammonia levels (22). In our study, we used targeted metabolomics to profile acyl-CoA distributions in different tissues. Interestingly, the metabolomics data show that succinyl-CoA is the most abundant short-chain acyl-CoA in the mouse heart. The heart needs a constant energy supply to sustain the mechanical pumping and thus may need to optimize metabolism to favor the TCA cycle and oxidative phosphorylation, which may lead to a higher succinyl-CoA concentration. Regardless of what exactly causes this interesting acyl-CoA profile, the results we obtained suggest that different tissues have very different metabolism and that it is worthwhile to examine the function of acylation in different tissues.

The unique acyl-CoA profiling results then led us to examine succinylation in different mouse tissues with and without SIRT5. Interestingly, succinylation increases most dramatically in the heart when *Sirt5* is deleted, which suggests that SIRT5 may have important functions in the heart. Consistent with this, SIRT5 level is higher in the heart than in other mouse tissues tested. *Sirt5* KO mice exhibit a reduced cardiac function and display signs of cardiomyopathy upon aging. Thus, protein succinylation and SIRT5 exert important roles in cardiac function. To understand the molecular mechanism underlying the function of succinylation and SIRT5 in the heart, we have identified over a hundred proteins with increased lysine succinylation in *Sirt5* KO heart using semiquantitative proteomics. Among the proteins we identified, ECHA has the highest number of lysine succinylation sites. SIRT5 activates ECHA by desuccinylating it. Consistent with ECHA being inhibited by succinylation, *Sirt5* KO hearts have compromised long-chain fatty acid oxidation along with a decreased ATP levels. We believe that the inhibition of the fatty acid oxidation pathway is the major contributor to the reduced ATP production because several lines of evidence suggest that neither the TCA cycle nor ATP synthase is inhibited by *Sirt5* deletion.

Succinylation is a widespread PTM and affects major metabolic pathways including amino acid metabolism, the TCA cycle, fatty acid metabolism, oxidative phosphorylation, urea cycle, ketogenesis, and so on. SIRT5 can either activate or repress enzymatic activity via desuccinylation. SIRT5 is reported (18) to up-regulate hepatic ketogenesis through activation of 3-hydroxy-3-methylglutaryl-CoA synthase 2. There are reduced fatty acid oxidation and accumulation of medium- and long-chain acylcarnitines in *Sirt5*-deficient mouse liver and muscle, but the underlying mechanism was not investigated. In our current study, we show that SIRT5 positively modulates fatty acid oxidation in the mouse heart. In addition, we demonstrate that succinylation impairs fatty acid oxidation through down-regulation of ECHA activity. Altogether, this suggests that SIRT5 plays a critical role in regulating fatty acid metabolism in multiple tissues.

Although many of the substrate proteins that we identified here were also previously reported (16, 18, 19), the uniqueness of the current study is that for the first time to our knowledge we have connected lysine succinylation and SIRT5 to an important physiological function (i.e., the regulation of heart metabolism and function). This function likely underlies the impaired performance in an endurance run test reported earlier (21). In recent years, significant advances have been made on the role of fatty acid metabolism defects in the pathogenesis of cardiomyopathy. Fatty acid oxidation provides most of the energy required by the heart. In 1939, Herrmann and Decherd (33) proposed the energy-starvation hypothesis, which stated that deprivation of cardiac energy could lead to heart failure. Cardiomyopathy can occur in a broad range of pathological conditions. It is well documented that defects or disorders of fatty acid metabolism often lead to cardiomyopathy (34–36). Cardiomyopathy is a major symptom of inborn errors in fatty acid metabolism, such as malonyl-CoA decarboxylase deficiency, carnitine palmitoyl transferase 2 deficiency, medium-chain acyl-CoA dehydrogenase deficiency, and mitochondrial trifunctional protein (MTP) deficiency (37). A mutation of the *Hadha* gene, which encodes ECHA protein, leads to MTP deficiency with cardiac symptoms (38). In the present study, we have established that SIRT5 deficiency leads to decreased ECHA activity and deficiency in cardiac energy metabolism, and ultimately cardiomyopathy. Very recently, Boylston et al. (39) have showed that *Sirt5* KO mice are more susceptible to ischemia-reperfusion injury compared with WT.

Connecting lysine succinylation and SIRT5 to heart function was made possible by the targeted metabolomics analysis of acyl-CoA concentrations in various mouse tissues. In the last decade, many novel PTMs have been reported, including propionylation, butyrylation, crotonylation, glutarylation (8), long-chain fatty

acylation (7, 9), and 2-hydroxyisobutyrylation (6). All these PTMs likely result from reactions with cellular metabolites either via specific acyltransferases or via nonenzymatic pathways, similar to lysine succinylation (40, 41). Although our studies with ECHA suggest that chemical succinylation and SIRT5-catalyzed desuccinylation in vitro is able to recapitulate the effects of succinylation and desuccinylation on ECHA in vivo, protein-catalyzed succinylation cannot be completely ruled out. Regardless of the enzymatic or nonenzymatic nature of lysine acylation, our study here suggests that combining metabolomics of acyl-CoAs and proteomic identification of substrate proteins in different tissues is useful to understand the functions of these newly identified PTMs.

Materials and Methods

Full details are provided in *SI Materials and Methods*. In vitro chemical succinylation was achieved by incubating 50 nM recombinant ECHA and ECHB complex with 3 mM succinyl-CoA in a mixture of 100 mM Hepes, pH 7.4, 100 mM KCl, 10% (vol/vol) glycerol, 1 mM free CoA, and 1 mM NAD at 27 °C for 20 min. The activity of succinylated ECHA was measured by adding 100 μM 2-(*E*)-decenoyl-CoA into the above reaction mixture and monitoring formation of NADH at 340 nm. In a separate reaction, 50 nM ECHA and ECHB complex was chemically succinylated as described above for 10 min and then 0.5 μM SIRT5 was added to that reaction mixture and incubated for an additional 10 min. Then, 100 μM 2-(*E*)-decenoyl-CoA was added and activity was measured similarly to determine whether SIRT5 can recover the activity of ECHA. Student's *t* test was used for statistical analysis.

All animal experiments were approved by the veterinary ethics committee of the canton of Vaud, Switzerland (permit ID 2444) and Cornell Institutional Animal Care and Use Committee protocol 2011-0098.

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