SIRT5 is a lysine desuccinylase known to regulate mitochondrial fatty acid oxidation and the urea cycle. Here, SIRT5 was observed to bind to cardiolipin via an amphipathic helix on its N terminus. In vitro, succinyl-CoA was used to succinylate liver mitochondrial membrane proteins. SIRT5 largely reversed the succinyl-CoA-driven lysine succinylation. Quantitative mass spectrometry of SIRT5-treated membrane proteins pointed to the electron transport chain, particularly Complex I, as being highly targeted for desuccinylation by SIRT5. Correspondingly, SIRT5−/− HEK293 cells showed defects in both Complex I- and Complex II-driven respiration. In mouse liver, SIRT5 expression was observed to localize strictly to the periportal hepatocytes. However, homogenates prepared from whole SIRT5−/− liver did show reduced Complex II-driven respiration. The enzymatic activities of Complex II and ATP synthase were also significantly reduced. Three-dimensional modeling of Complex II suggested that several SIRT5-targeted lysine residues lie at the protein-lipid interface of succinate dehydrogenase subunit B. We postulate that succinylation at these sites may disrupt Complex II subunit-subunit interactions and electron transfer. Lastly, SIRT5−/− mice, like humans with Complex II deficiency, were found to have mild lactic acidosis. Our findings suggest that SIRT5 is targeted to protein complexes on the inner mitochondrial membrane via affinity for cardiolipin to promote respiratory chain function.

Mitochondrial oxidative energy metabolism is fundamental to human health, and changes in mitochondrial function are now recognized as playing an etiological role in diseases such as cancer, diabetes, and neurodegeneration. Of particular importance to mitochondrial function are the ~200 proteins that reside on the inner mitochondrial membrane (IMM). IMM proteins include a large family of solute transporters, several ion channels, four electron transport chain (ETC) complexes, and ATP synthase. Additionally, the IMM has many peripherally associated proteins that bind to the membrane electrostatically rather than via transmembrane domains. This group includes enzymes such as carnitine palmitoyltransferase-2, mitochondrial trifunctional protein (MTP), and very-long-chain acyl-CoA dehydrogenase (VLCAD), which together catalyze long-chain fatty acid oxidation (FAO). All three show membrane localization through electrostatic binding to cardiolipin on the IMM (1–3). Cardiolipin also appears to facilitate assembly of higher-order ETC “supercomplexes” that mediate maximal respiratory efficiency (4). In an additional layer of complexity, there is increasing evidence that metabolic pathways that directly produce reducing equivalents, such as FAO and the tricarboxylic acid cycle (TCA), can physically interact with the ETC and potentially with each other (5).

To date, the factors that regulate metabolic complex assembly and facilitate protein-protein and protein-lipid interactions on the IMM are poorly understood. Reversible post-translational protein modifications such as lysine acylation are widespread in the mitochondria and represent a potential regulatory mechanism for the formation and function of IMM protein complexes. For example, we recently showed that succinylation of three lysine residues in the VLCAD C terminus leads to a complete loss of membrane binding (1). Succinylation converts the positively charged lysine residues to negative charges, thereby disrupting the electrostatic interaction between the amphipathic helix of VLCAD and cardiolipin. Incubation with the mitochondrial desuccinylase SIRT5 restores membrane binding of VLCAD. Mass spectrometry studies have identified SIRT5-targeted lysines on other IMM proteins such as MTP, ADP/ATP translocase, isocitrate dehydrogenase, and hydroxymethylglutaryl-CoA synthase-2 (6–10), but its role in maintaining the integrity of the IMM proteome has not been directly

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3 The abbreviations used are: IMM, inner mitochondrial membrane; ETC, electron transport chain; MTP, mitochondrial trifunctional protein; VLCAD, very-long-chain acyl-CoA dehydrogenase; FAO, fatty acid oxidation; IVD, isovaleryl-CoA dehydrogenase; SDH, succinate dehydrogenase; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; BNGE, blue native gel electrophoresis; LCAD, long-chain acyl-CoA dehydrogenase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; DDM, n-dodecyl β-D-maltoside.

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SIRT5 binds to cardiolipin

Figure 1. SIRT5 binds to cardiolipin on the inner mitochondrial membrane. A, liver mitochondria from wild-type (WT) and SIRT5−/− (KO) mice were fractionated into membrane and matrix fractions and Western blotted (25 μg of protein/lane) with anti-succinyllysine (Suc-Lys) antibody. Bottom, markers of the mitochondrial inner membrane (Tim23) and the matrix (HSP60). B, liver mitochondria from three wild-type mice were fractionated and Western blotted for SIRT5 (left) or the matrix marker protein IVD (right). Densitometry was used to measure the relative intensities of the SIRT5 bands in each fraction. C, recombinant SIRT5 and SIRT3 (200 ng) were incubated with mixed-composition unilamellar liposomes and pelleted. The pellet was washed once, and the resulting three fractions (membrane (Memb), wash, and supernatant (Sup)) were Western blotted to visualize SIRT3 and SIRT5. D, increasing amounts of cardiolipin (CL), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) were spotted onto nitrocellulose, which was then incubated with 0.5 μg/ml recombinant SIRT5 diluted in blocking buffer. Bound SIRT5 was visualized by Western blotting. Avg, average.

Results

SIRT5 binds to cardiolipin on the inner mitochondrial membrane

Fractionated mitochondria from wild-type and SIRT5−/− mouse liver were subjected to Western blotting with anti-succinyllysine antibody. Proteins in both the matrix and membrane fractions were observed to be hypersuccinylated in the absence of SIRT5 (Fig. 1A). After four cycles of washing the membrane fraction, known matrix proteins like isovaleryl-CoA dehydrogenase (IVD) were absent, but SIRT5 was still detectable by Western blotting (Fig. 1B). The amount of membrane-bound SIRT5 is estimated to be about one-third of the amount observed in the matrix fraction.

To investigate whether SIRT5 interacts directly with membrane lipids, we incubated recombinant human SIRT5 with mixed-composition unilamellar liposomes designed to mimic the mitochondrial inner membrane (43% phosphatidylcholine, 37% phosphatidylethanolamine, 20% cardiolipin). The liposomes were pelleted, washed once, and pelleted again; the amount of SIRT5 was then detected in the pellet, wash, and supernatant fractions. Mixed composition liposomes efficiently pulled down recombinant SIRT5, but not recombinant SIRT3, from solution (Fig. 1C). To determine which of the components in the mixed-composition liposomes was binding SIRT5, we performed a “fat blot” by spotting increasing amounts of cardiolipin, phosphoethanolamine, and phosphatidylcholine onto nitrocellulose and incubating the membrane with recombinant SIRT5. The results showed dose-dependent binding of SIRT5 to cardiolipin but no detectable binding to phosphoethanolamine or phosphatidylcholine (Fig. 1D).

An N-terminal amphipathic helix mediates SIRT5 binding to cardiolipin

Mitochondrial targeting signals typically contain several positive charges and form an amphipathic helix that serves as an important recognition element for the mitochondrial protein import machinery (11). This amphipathic helix is then removed when the mitochondrial targeting signal is cleaved by the mitochondrial processing peptidase. Interestingly, the mitochondrial processing peptidase cleavage site of SIRT5 leaves an amphipathic helix on the mature protein at the extreme N terminus (12). This N-terminal amphipathic helix has three positively charged residues that orient into the solvent as seen in the SIRT5 crystal structure (Fig. 2A). We hypothesized that these positively charged residues might confer cardiolipin binding to SIRT5, similar to the amphipathic helix we previously identified in VLCAD (1). To test this hypothesis, we mutated the three charged residues to uncharged glutamine residues.
Figure 3. SIRT5 counteracts succinylation of mitochondrial membrane proteins. A, a freshly isolated lobe of SIRT5−/− liver was stained with X-Gal to reveal the pattern of β-galactosidase expression from the SIRT5 mutant allele (blue color indicates zones expressing SIRT5). The “honeycomb” structure of the hepatic lobules can be seen outlined by the staining for β-galactosidase, which localizes to the peribiliary (PP) zone. The central vein (CV) can be seen as a brown spot in the middle of each lobule (red arrow as example), and no blue X-Gal staining appears in the region around the central vein. B, washed mitochondrial membranes from SIRT5−/− liver were treated with 0.5 mM succinyl-CoA (Succ-CoA) from 0 to 5 h, and the effects were visualized by Western blotting with anti-succinyllysine (Suc-Lys) antibody with Ponceau S staining as a loading control. Below the Ponceau image are the -fold changes in global succinylation estimated by densitometry. C, quadruplicate samples of succinyl-CoA-treated SIRT5−/− membranes were treated with either inactive mutant recombinant SIRT5 H158Y (Control) or wild-type recombinant SIRT5 (SIRT5-Tx), and the resulting desuccinylation was visualized by Western blotting with anti-succinyllysine antibody. D, the blot from C was quantified by densitometry of the entire Western blot lanes; shown are the means of the four Control and four SIRT5-Tx lanes; error bars represent S.D. E and F, the remainder of the eight samples shown in C were subjected to quantitative mass spectrometry to identify the specific lysine residues targeted by SIRT5 (see supplemental Table 1). Shown is a summary of the unique succinylation sites identified and the number of SIRT5 target sites defined as showing a statistically significant change at p < 0.05. F is a volcano plot (log p value plotted against log -fold change) of the mass spectrometry results.

(SIRT5-3Q). Compared with wild-type SIRT5, SIRT5-3Q showed reduced affinity for cardiolipin in the fat blot assay (Fig. 2, B and C). Similarly, when constant amounts of SIRT5 and SIRT5-3Q were subjected to pulldown assays with increasing concentrations of cardiolipin liposomes, the pulldown was less efficient with SIRT5-3Q (Fig. 2, D and E). This confirms a role for the N-terminal amphipathic helix in SIRT5-cardiolipin binding.

SIRT5 counteracts succinylation of mitochondrial membrane proteins

Li et al. (13) recently reported that SIRT5 expression may be subject to hepatic zonation. To confirm their findings, we performed whole-mount liver X-Gal staining for β-galactosidase, which is expressed from the mutant allele in SIRT5−/− mice. β-Galactosidase staining was localized exclusively to the peribiliary zones that outline the hepatic lobules (Fig. 3A), thereby confirming the hepatic zonation of SIRT5. SIRT5 deficiency may only affect a subset of hepatocytes, and thus the protein succinylation levels are likely not uniform across the zones of the liver. For the purpose of identifying potential SIRT5 target proteins on the mitochondrial membrane, we created a uniformly succinylated mitochondrial membrane fraction by isolating mitochondria from whole SIRT5−/− liver, separating the membranes, and treating them with the endogenous succinylating agent succinyl-CoA (14, 15). Incubating the membrane fraction with 0.5 mM succinyl-CoA for 1 h increased global succinylation levels by 2.8-fold; further incubation did not have any additional effect (Fig. 3B). Our previous work using chemically acylated proteins as substrates for recombinant sirtuins has shown that SIRT3 and SIRT5 have highly specific target sites and will not deacylate lysine residues indiscriminately (1, 16). Therefore, we used our succinyl-CoA-treated membrane protein fraction as substrate for recombinant SIRT5 to unveil potential target proteins/pathways. Quadruplicate succinyl-CoA-treated membrane-enriched samples were treated with either human SIRT5 or inactive mutant SIRT5 H158Y as a control. SIRT5 reduced the global succinylation level by about 60% (Fig. 3, C and D). The same protein samples shown in Fig. 3C were subjected to quantitative mass spectrometry to identify the specific succinylation sites targeted by SIRT5. A total of 2238 succinylated peptides representing 1969 unique sites of succinylation were detected. Of these, 1238 peptides representing 1150 unique succinylation sites showed statistically significant changes between control and SIRT5-treated samples. The
results are visually summarized in Fig. 3E and in the volcano plot shown in Fig. 3F, and the 1238 individual peptides are listed in supplemental Table 1.

**SIRT5 targets the mitochondrial respiratory chain**

The mass spectrometry data were used for Reactome pathway analysis (17). The top five pathways targeted by SIRT5 were electron transport, Complex I biogenesis, peroxisomal lipid metabolism, ATP synthesis, and cristae formation (Fig. 4A). We selected the top pathway, electron transport, for further investigation. With a p value cutoff of 0.05, the mass spectrometry experiment identified a total of 223 SIRT5 target sites on the five complexes of the ETC. Complex I had 63 sites showing an average change in succinylation of 5.9-fold upon SIRT5 treatment (Fig. 4B). The highest observed -fold changes for any peptide in our mass spectrometry analysis were 61- and 37-fold,
Complex II and ATP synthase activities are reduced in SIRT5<sup>−/−</sup> liver

We turned to the SIRT5<sup>−/−</sup> mouse model to determine whether SIRT5 regulates hepatic ETC function in vivo with the caveat that zonation may reduce the robustness of the functional changes when assessed in the whole organ. Liver mitochondria were isolated, and the architecture of the ETC was examined using blue native gel electrophoresis (BNGE) followed by Coomassie staining to visualize Complexes I, III, IV, and V (ATP synthase) and supercomplexes (SC) to control for any potential variability in mitochondrial content or cell counting. Complex I function was probed by measuring respiration on pyruvate/malate in the presence of ADP, whereas Complex II was probed by addition of succinate. Pyruvate/malate respiration (Complex I) was significantly lower both in the basal state and in cells that had been starved by changing the medium to 0.1 mM glucose for 3 h (Fig. 4D). Succinate-driven respiration through Complex II, however, was only significantly affected when the cells had been glucose-starved (Fig. 4E).

Complex II and ATP synthase activities are reduced in SIRT5<sup>−/−</sup> liver

In our hands, pyruvate/malate-driven respiration through Complex II was significantly lower both in the basal state and in cells that had been starved by changing the medium to 0.1 mM glucose for 3 h (Fig. 4). Succinate-driven respiration through Complex II, which can readily be immunoprecipitated, was only significantly affected when the cells had been starved by changing the medium to 0.1 mM glucose for 3 h (Fig. 4B). To validate ETC protein succinylation in vivo, we selected succinate dehydrogenase subunit A (SDHA), a soluble subunit of Complex II, which can readily be immunoprecipitated. Western blotting of immunoprecipitated SDHA from wild-type and SIRT5<sup>−/−</sup> liver homogenates showed a 3.4-fold increase in succinylation in fed SIRT5<sup>−/−</sup> liver (Fig. 4C). Succinylation decreased with fasting in wild-type liver but not SIRT5<sup>−/−</sup> liver, resulting in a 9.5-fold difference in succinylation levels in the fasted state. This experiment was repeated once with similar results.

To probe the effects of SIRT5 deficiency on ETC function, we utilized a human cell line (HEK293) in which CRISPR (clustered regularly interspaced short palindromic repeats) had been used to delete SIRT5. Equal numbers of SIRT5<sup>−/−</sup> and control cells were permeabilized with digitonin and used for respirometry studies in an Oroboros Oxygraph-2K. The data were normalized to the maximal mitochondrial respiration induced by the chemical uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to control for any potential variability in mitochondrial content or cell counting. Complex I function was probed by measuring respiration on pyruvate/malate in the presence of ADP, whereas Complex II was probed by addition of succinate. Pyruvate/malate respiration (Complex I) was significantly lower both in the basal state and in cells that had been starved by changing the medium to 0.1 mM glucose for 3 h (Fig. 4D). Succinate-driven respiration through Complex II, however, was only significantly affected when the cells had been glucose-starved (Fig. 4E).

**Figure 5.** SIRT5 deficiency does not affect the abundance of the respiratory chain complexes in liver mitochondria. A, blue native gel electrophoresis of wild-type (WT) and SIRT5<sup>−/−</sup> (KO) liver mitochondria. Respiratory chain Complexes I, III, IV, and V (ATP synthase) and supercomplexes (SC) are indicated with arrows. B, Western blot of the two soluble subunits of Complex II (SDHA and SDHB) in liver mitochondrial extracts with Tim23 as a loading control. Densitometry was used to evaluate the protein levels in each lane relative to Tim23, and the results are shown in the bar graph (error bars represent S.D.).

was significantly lower in SIRT5<sup>−/−</sup> liver homogenates (Fig. 6B). In agreement with this finding, immunocapture-based enzyme activity assays demonstrated no change in Complex I NADH dehydrogenase activity in SIRT5<sup>−/−</sup> liver mitochondria, whereas Complex II succinate dehydrogenase activity was significantly reduced (Fig. 6, C and D).

To determine whether the reduction in succinate-stimulated respiration in liver homogenates was strictly due to reduced Complex II activity or also involved changes in ETC complexes downstream of Complex II, we measured the activity of Complexes III, IV, and V. Complexes III and IV were probed with in-gel activity stains following BNGE and did not differ between genotypes (Fig. 6E). Because the in-gel activity stain for ATP synthase may be affected by the presence of the Coomassie dye (19), ATP synthase was assayed in mitochondrial lysates by following its ATPase activity. SIRT5<sup>−/−</sup> liver mitochondria showed a statistically significant reduction of ATPase activity (Fig. 6F).

**SIRT5 targets lysines at the protein-lipid interface of Complex II**

Mass spectrometry identified 14 SIRT5 target sites on the SDHA subunit of Complex II and another eight on SDHB. SDHC and SDHD, which are hydrophobic and embedded in the IMM, had no identified sites as was expected from their lack of solvent exposure. The crystal structure of porcine Complex II has been solved (20), and we utilized three-dimensional molecular modeling to visualize the SIRT5 target sites. Of the 14 sites on SDHA, two were not conserved in the pig enzyme. The remaining 12 all oriented into solution and were not near the FAD cofactor or the active site, providing no obvious rationale for targeting these sites.
for how they might influence activity (modeling not shown). On SDHB, all eight target sites were conserved across species. SDHB is sandwiched between SDHA (which catalyzes the FAD-dependent oxidation of succinate to fumarate) and the membrane-embedded subunits SDHC/SDHD. It contains three iron-sulfur clusters and channels the electrons from SDHA to ubiquinone, which binds in a gap formed at the intersection of subunits SDHB, SDHC, and SDHD. There is some evidence to suggest that cardiolipin is critical for optimal formation of this gap and conductance of electrons through SDHB to ubiquinone (21). Molecular modeling revealed that six of the eight SIRT5 target sites on SDHB orient toward the predicted membrane interface where SDHB interacts with SDHC/SDHD (21). We speculate that these six positively charged lysines play a role in Complex II cardiolipin binding and therefore proper conformation for electron transfer. Indeed, another group recently substituted one of these lysine residues (Lys-53) with a negatively charged glutamate and observed significantly reduced Complex II activity (22).

In humans, genetic Complex II deficiencies are often associated with a mild, persistent lactic acidosis (23). SIRT5−/− mice were similarly observed to have mild lactic acidosis (Fig. 7C). This is unrelated to the known defect in mitochondrial FAO in SIRT5−/− mice because mice lacking the key FAO enzyme long-chain acyl-CoA dehydrogenase (LCAD) do not show the lactic acidosis phenotype.

**Discussion**

In the present work, we showed that SIRT5 can directly interact with cardiolipin. Cardiolipin is unique to the IMM. With its dimeric structure (four alkyl chains and a double negative charge), it has highly specialized roles in cristae formation, ETC supercomplex formation, and promoting the stability and function of all four individual ETC complexes as well as ATP synthase (4, 24–26). Our results suggest that SIRT5 may also localize to the cardiolipin-rich domains on the IMM that are critical for mitochondrial functionality. In addition to the aforementioned ETC complexes, which all interact with cardiolipin, other known SIRT5 substrate proteins such as MTP, VLCAD, carbamoyl-phosphate synthetase-1, and isocitrate dehydrogenase also bind to cardiolipin (2, 27). Together, these findings all point to SIRT5 as being directed to the IMM to reverse succinylation of IMM proteins.

Our data suggest that SIRT5 desuccinylates multiple subunits of all four respiratory chain complexes and ATP synthase. However, in SIRT5−/− liver in vivo, only Complex II and ATP synthase were functionally affected. It must be kept in mind that our functional assays utilized liver homogenates or mitochondria isolated from the whole organ, which could result in an underestimation of functional effects due to the strong hepatic zonation of SIRT5. We speculate that the effect of lysine succinylation on Complex II and ATP synthase in the periportal...
zone may be much stronger than what we observed in mitochondria from whole-tissue homogenates. Similarly, the use of a mixed mitochondrial population may have masked more subtle effects that could be present on Complexes I, III, and IV, which all bear many SIRT5-targeted lysine residues. In support of this, we observed significantly reduced malate/pyruvate-driven respiration in SIRT5/−/−/− HEK293 cells. Complex I may very well be affected in periportal hepatocytes in vivo or in other tissue types where Complex I activity is more prominent such as the heart and muscle.

Two previous studies explored effects of SIRT5 on Complex II SDH activity in cell culture models. First, Park et al. (6) observed higher SDH activity in cultured cells following SIRT5 knockdown and suggested that succinylation activates SDH. Li et al. (22) expressed a succinylation mimetic SDHB mutant (K53E) in cultured cells and observed lower SDH activity, indicating, in opposition to Park et al. (6), that succinylation reduces SDH function. They also overexpressed SIRT5 in cells and saw increased SDH activity, further suggesting that succinylation suppresses, whereas SIRT5 desuccinylation promotes, SDH activity. Our results are in agreement with those of Li et al. (22), pointing to SIRT5 as a positive regulator of Complex II rather than a suppressor. Our work in SIRT5/−/− liver further documents compromised Complex II and ATP synthase function in vivo, adding evidence to support the view of SIRT5 as a promoter of mitochondrial energy metabolism rather than a suppressor.

The workflow used in the present experiments to identify succinylation sites on hepatic proteins was different from those used in previously published studies. Rardin et al. (7) isolated liver mitochondria from wild-type and SIRT5/−/− mice, trypsinized the lysates, enriched for succinylated peptides, and then quantified the succinylation sites by mass spectrometry. Park et al. (6) used a similar workflow but started with whole
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liver rather than isolated mitochondria. In the present study, we isolated liver mitochondria, lysed them, collected the mem- 
brane fraction, and treated it with succinyl-CoA prior to the anti-succinyllysine enrichment step and mass spectrometry. 
This workflow allowed for characterization of membrane- 
bound proteins that may otherwise be lost as non-soluble mate-
rial while clearing mitochondrial or whole-liver lysates for mass spectrometry analysis. Furthermore, we enhanced protein 
succinylation by succinyl-CoA treatment prior to analysis. 
Although this served as a work-around for the problem pre-
presented by SIRT5 zonation, we recognize that this step altered 
the natural state of the proteins in the analysis and that further 
validation is required for any particular SIRT5 target site of 
interest.

Finally, our method and the resulting membrane succiny-
lome data set may provide valuable leads for future discov-
eries. For example, one of the top targeted pathways identified here was peroxisomal lipid metabolism. Peroxisomal proteins 
likely entered our analysis due to the common problem of per-
oxosomes co-isolating with mitochondria. Other studies have 
identified succinylation sites on peroxisomal proteins as well 
(curated on PhosphoSitePlus®). Park et al. (6), in their analysis 
of liver homogenates, identified several hundred extramito-
chondrial succinylation sites and demonstrated extramito-
chondrial localization of SIRT5. Their study and ours raise 
questions and future lines of investigation regarding the 
extramitochondrial role of SIRT5 as well as the origins of 
extramitochondrial lysine succinylation. We observed SIRT5 
targeting of several enzymes involved in peroxisomal FAO (see 
supplemental Table 1). Peroxisomal FAO can produce, in addi-
tion to acetyl-CoA from fatty acids, succinyl-CoA and glutaryl-
CoA from the chain shortening of long-chain dicarboxylic acids 
(28, 29). Peroxisomally generated acetyl-CoA, succinyl-CoA, 
and glutaryl-CoA could acylate peroxisomal proteins. These 
metabolites are ultimately exported to the mitochondria and 
could potentially acylate proteins in the cytosol, outer mito-
chondrial membrane, and intermembrane space during 
transport, thereby explaining the observed acylation of inter-
membrane space proteins such as mitofilin (Ref. 10 and supple-
mental Table 1). Future investigations are necessary to deter-
mine whether SIRT5, already known to localize to the cytosol 
and mitochondria, may also localize to peroxisomes to coordi-
nate lipid metabolism across cellular compartments or to the 
intermembrane space to influence crista formation.

Experimental procedures

Animal studies

SIRT5−/− and wild-type control mice were purchased from 
The Jackson Laboratory (Bar Harbor, ME) and bred in-house. 
LCAD−/− mice were obtained from the Mutant Mouse 
Regional Resource Center. All breeding and experimentation 
met the approval of the University of Pittsburgh Institutional 
Animal Care and Use Committee. Blood lactate was measured 
in unrestrained animals by nicking the tip of the tail and col-
lecting droplets of blood into the assay strips for a handheld 
lactate analyzer (Lactate Scout, EKF Diagnostics). Euthanasia 
was conducted using inhaled CO₂ gas according to Institutional 
Animal Care and Use Committee recommendations. In some 
experiments, the mice were fasted overnight (16 h) prior to 
euthanasia and tissue harvest.

Staining for β-galactosidase

Whole-mount staining was performed using the protocol of 
West et al. (30). A liver lobe was freshly harvested, washed in 
PBS, fixed for 30 min, washed again, and then stained with 
X-Gal at 37 °C for 4 h. The tissue was then viewed/photograph-
ed using a dissecting microscope (Leica).

Mitochondrial membrane isolation

All steps were performed at 4 °C or on ice essentially as 
described (1). Briefly, freshly isolated mouse liver was collected 
into HMS buffer (5 mM HEPES, pH 8, 220 mM mannitol, 70 mM 
sucrose), minced on a glass plate, transferred to a prechilled 
glass homogenizing vessel, and subjected to 20 passes with a 
Teflon pestle at 500–1000 rpm. Following slow-speed cen-
trifugation (2540 × g for 1 min) to remove cellular debris, mito-
chondria were pelleted by centrifugation at 12,000 × g for 15 
min. After resuspending and washing twice in cold HMS buffer, 
the mitochondrial pellet was resuspended in 50 mM KPO₄, 
ph 7.0, sonicated, and centrifuged 15 min at 40,000 × g to pellet the 
membranes. The membrane pellet was washed four times in the 
same buffer, then resuspended, and quantified spectrophoto-
metrically by measuring protein content (A₂₈₀ nm − A₃₁₀ nm).

Protein expression and purification

Recombinant human His-tagged SIRT3, SIRT5, and inactive 
mouse mutant SIRT5 H158Y were expressed and purified exactly as we 
described previously (1). Mutations in SIRT5 were introduced 
with the QuikChange II site-directed mutagenesis kit (Agilent 
Technologies) and confirmed by DNA sequencing.

Succinyl-CoA acylation of proteins

Succinylation of SIRT5−/− mouse liver mitochondrial extracts was accomplished by incubation (1–5 h as indicated in 
the figure legends) with freshly prepared succinyl-CoA (0.5 mM 
final concentration). Excess succinyl-CoA was removed by dial-
ysis prior to mass spectrometry experiments.

Membrane/cardiolipin binding

Membrane binding assays were conducted either by using a 
pulldown assay or by spotting the desired lipids onto nitrocell-
ulose membranes (fat blotting). Pulldown assays used either 
SIRT5−/− mouse liver mitochondrial membrane prepa-
rations (described above) or unilamellar liposomes prepared 
with an extruder (Avanti Polar Lipids, Alabaster, AL) as indi-
cated in the text and figure legends. Liposomes were either 
prepared from 100% cardiolipin or from a mixture of phospho-
lipids (43% phosphatidylcholine, 37% phosphoethanolamine, 
20% cardiolipin). Pulldown assays were carried out by mixing 
recombinant SIRT5 with either the washed SIRT5−/− mito-
chondrial membranes or the extruded liposomes, incubating 
for 1 h at 37 °C, and then fractionating the mixture by centrifu-
gation at 78,000 × g for 20 min. In some experiments, the 
pellet was washed by resuspension followed by centrifugation. 
For fat blotting, the desired phospholipids were dissolved
in chloroform and spotted onto nitrocellulose following the method of Munnik and Wierzchowiecka (31). After drying, the nitrocellulose membrane was incubated with recombinant SIRT5 protein, washed, and subjected to immunoblotting to visualize lipid-bound SIRT5.

**Quantitative mass spectrometry**

Quadruplicate samples of succinyl-CoA-treated (1 h) and dialyzed SIRT5+/− liver mitochondrial membranes were incubated with either active SIRT5/NAD+ or inactive mutant SIRT5/NAD+ as controls. Succinylated peptides were enriched and quantified as described previously (1, 7). Briefly, reverse-phase LC-electrospray ionization-MS/MS analysis was done with an Eksigent Ultra Plus nano-LC 2D HPLC system (Dublin, CA) connected to a quadrupole time-of-flight TripleTOF 5600 mass spectrometer (AB SCIEX). Peptides were searched against Swiss-Prot entry P49748 and chosen based on a 95% confidence level followed by manual inspection. Raw MS data files were imported into Skyline, and precursor ion chromatograms were extracted for label-free quantification using MS1 filtering (32). Peptide areas were then averaged across all sample acquisitions, and a ratio was generated. The resulting list of gene identifiers was functionally grouped using Reactome pathway analysis (17).

**Immunoblotting**

Western blotting was performed after electrophoresis on Criterion SDS-polyacrylamide gels (Bio-Rad) and transfer to nitrocellulose membranes. Antibodies used were: rabbit anti-succinyllysine (PTM Biolabs), anti-SIRT5 (Cell Signaling Technology), anti-IVD (a gift from Dr. Jerry Vockley, Children’s Hospital of Pittsburgh), anti-SDHA (Cell Signaling Technology), anti-SDHB (Santa Cruz Biotechnology), and anti-Tim23 (BD Transduction Laboratories). After incubation with HRP-conjugated secondary antibodies (1:5000), blots were visualized with chemiluminescence. In some experiments, the blots were scanned and subjected to densitometric analysis using ImageJ software.

**Oroboros high-resolution respirometry**

Mitochondrial respiration was measured in an Oroboros Oxygraph-2K. SIRT5+/− and control HEK293 cells were gifts from Dr. Matthew Hirschey (Duke University) and were grown under standard conditions. The cells were resuspended in Mir05 respiration medium (110 mM sucrose, 0.5 mM EGTA, 3 mM MgCl2, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, pH 7.2, 1 mg/ml fatty acid-free BSA), permeabilized with digitonin, and placed into the Oroboros high-resolution respirometry software.

Mitochondrial respiration was measured as described previously (1, 7). Next, 10 mM succinate was added to assess the combined activity of Complexes I + II. Next, 10 µM cytochrome c was added to assess mitochondrial integrity followed by 0.5 mM CCCP to uncouple the mitochondrial membrane and induce maximal respiration. Complex II respiration was defined as respiration after addition of 10 µM succinate (Complexes I + II) minus the rate of respiration on malate/pyruvate/ADP (Complex I). The experiments were performed on four sets of wild-type versus SIRT5−/− HEK293 cells and five sets of wild-type versus SIRT5−/− livers. To allow combination of data across runs, respiration on each substrate was expressed as a percentage of maximum respiration (CCCP response).

**Blue native gel electrophoresis and activity stains**

Mouse liver mitochondria were isolated as described above and resuspended in HB buffer (50 mM KPO4, 2.5% glycerol, 250 mM sucrose buffer, pH 7.4). Blue native gel electrophoresis was carried out as described (5). First, digitonin was added to solubilize mitochondria (8:1, w/w). Coomassie Blue (5% Coomassie Blue G-250 in 750 mM 6-aminoacaproic acid) was added (1:30, v/v), and 80 µg of each sample was electrophoresed on a 3–12% native PAGE Novex Bis-Tris gel (Invitrogen) at 80 V for 4 h at 4°C.

In-gel assays of ETC complex activities were performed as described for Complex III (decylubiquinol cytochrome c oxidoreductase) and Complex IV (cytochrome c oxidase). Gels were first incubated with Complex III assay solution overnight with gentle agitation to visualize Complex III, and then the gel was moved to fresh Complex III assay solution supplemented with 1 nM catalase, 10 mg of cytochrome c, and 750 mg of sucrose to visualize Complex IV.

**Mitochondrial solubilization for ETC spectrophotometric assay**

Spectrophotometric assays of Complexes I, II, and V were conducted using snap-frozen pellets of mouse liver mitochondria that were solubilized as follows. Mitochondrial pellets were diluted to 5.5 mg/ml (or 11 mg/ml for Complex V assay) in 50 mM Tris, pH 7.5, with Complete protease inhibitors (Roche Diagnostics). Then 1/10 volume of 10% (w/v) n-dodecyl-β-D-maltoside (DDM; Sigma-Aldrich). The final protein concentration was 5 mg/ml (or 10 mg/ml for Complex V assay), and the final DDM concentration was 1%. The sample was left on ice for 20 min and centrifuged at 25,000 × g at 4°C for 20 min. The supernatant was transferred to a clean new tube for use in spectrophotometric assays as described below.

**Complex I immunocapture activity assay**

Complex I activity was measured using an immunocapture method as described by Nadanaciva et al. (34) to reduce background artifacts. Immunocapture was performed by coating Nunc Maxisorp 8-well strips (Thermo Scientific) with 20 µg/ml anti-Complex I immunocapture antibody (Abcam ab109798) in PBS overnight at 4°C. The strip wells were then aspirated and replaced with blocking solution (5% fat-free milk in 50 mM Tris, pH 7.5) for 2 h at room temperature. The wells were washed once in 200 µl of 50 mM Tris, pH 7.5, and then incubated for 2 h at room temperature with 75 µg of solubilized mouse liver mitochondria in 100 µl of 50 mM Tris, pH 7.5, with Complete
protease inhibitors. The captured Complex I was washed gently three times (20 mM Tris, 50 mM KCl, 0.015% (w/v) DDM, pH 7.5) and then incubated for 45 min at 4 °C with 0 µl of 56 µg/ml phosphatidylcholine (Sigma-Aldrich) dissolved in 20 mM Tris, 50 mM KCl, 0.015% (w/v) DDM, pH 7.5. Finally, Complex I activity was measured by adding 200 µl of 25 mM KPO₄, pH 7.2, 5 mM MgCl₂, 0.12% BSA, 0.15 mM coenzyme Q1 (Sigma-Aldrich), 0.26 mM NADH (Sigma-Aldrich). The oxidation of NADH was monitored by the decrease at 340 nm in a FluorStar Omega plate reader (BMG Labtech) in kinetic mode for 35 min.

**Complex II immunocapture activity assay**

Complex II was immunocaptured similarly to Complex I using anti-Complex II immunocapture antibody (Abcam ab109865) at 10 µg/ml in 50 mM KPO₄, pH 7.2, overnight at 4 °C. The wells were blocked with 5% fat-free milk in 50 mM KPO₄, pH 7.2, for 2 h at room temperature; washed with 50 mM KPO₄, pH 7.2; and incubated with 50 µg/well solubilized mouse liver mitochondria for 2 h at room temperature. Captured Complex II was washed twice with 20 mM KPO₄, pH 7.2, 0.015% (w/v) DDM. Complex II activity was measured by adding an assay solution containing 25 mM KPO₄, pH 7.2, 20 mM sodium succinate, 65 µM coenzyme Q2, 50 µM dichlorophenolindophenol (Sigma-Aldrich 33125), 0.115% (w/v) DDM. The decrease in absorbance of dichlorophenolindophenol was measured at 600 nm in a FluorStar Omega plate reader in kinetic mode at room temperature for 1 h.

**Complex VATPase activity**

Fifty micrograms of solubilized mouse liver mitochondria in 50 mM Tris, pH 7.5, 1% DDM, protease inhibitors was added to a 96-well plate. ATPase activity was measured by addition of an assay solution (pH 7.5) containing 25 mM HEPES, 25 mM KCl, 2 mM MgCl₂, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.5 mM NADH, 30 units/ml pyruvate kinase (Sigma-Aldrich), 30 units/ml l-lactic dehydrogenase (Sigma-Aldrich). The decrease in absorbance of NADH was measured at 340 nm in a FluorStar Omega plate reader in kinetic mode at 30 °C for 30 min.

**Protein molecular modeling**

Three-dimensional modeling of the SIRT5 enzyme (Protein Data Bank code 5BWL) and Complex II (Protein Data Bank code 1ZOY) was performed with Yasara software (Yasara Biosciences, Vienna, Austria).

**Author contributions**—Overall study design was by E. S. G. and Y. Z. with contributions from B. W. G., M. J. R., and E. V. P. The manuscript was written by E. S. G. and Y. Z. with editing by B. W. G., M. J. R., and E. V. P. Experiments were performed by Y. Z., S. S. B., M. J. R., J. L., K. V. M., and S. S.-L. Data analysis and figure preparation were by E. S. G. and Y. Z. with assistance from M. J. R., J. L., and S. S.-L.

**References**


SIRT5 binds to cardiolipin


