wt SIRT3, SIRT3-H248Y (catalytically-inactive SIRT3 mutant), SIRT4, or SIRT5 were co-transfected with expression vectors for FLAG-tagged LCAD and the level of LCAD acetylation was assessed; **c.** Recombinant LCAD expressed in *E. coli* was incubated *in vitro* with recombinant SIRT3 or SIRT3-H248Y, and LCAD acetylation status was assessed; **d.** Expression vectors for wt SIRT3, SIRT3-H248Y, SIRT4 and SIRT5 (HA-tagged) were co-transfected with expression vectors for FLAG-tagged LCAD and assessed for interaction by co-immunoprecipitation; **e.** LCAD was expressed and purified with SIRT3 or SIRT3-H248Y and its enzymatic activity measured *in vitro* using 2, 6 dimethylheptanoyl-CoA as a substrate (n=4 independent assays); **f.** Recombinant LCAD was expressed in *E. coli* in the absence (Control) or presence of nicotinamide (NAM, 50 mM), purified and its enzymatic activity measured *in vitro* using 2, 6 dimethylheptanoyl-CoA as a substrate (n=4 independent assays); **g.** Expression vectors for wt LCAD, LCAD single acetylation point mutant (LCAD-K42R), or LCAD eight acetylation point mutant (LCAD-8KR) were co-transfected with expression vectors for wt SIRT3 or SIRT3-H248Y, and the level of acetylation was assessed; **h.** Wild-type LCAD, LCAD-K42R, or LCAD-8KR were expressed, and measured for enzymatic activity *in vitro* using 2, 6 dimethylheptanoyl-CoA as a substrate (n=5 measurement/sample, error bars represent data two independent protein purifications); $^{*}p<0.05$, $^{**}p<0.01$.

Figure 5. Mice lacking SIRT3 show reduced ATP production, cold intolerance and hypoglycemia.

a. Hepatic ATP levels were measured in fed and fasted wt and SIRT3-/- mice (*n*=5/genotype/condition) **b, c.** Core temperature (**b**) and blood glucose (**c**) were measured in fed and fasted wt and SIRT3-/- mice exposed to cold (4°C) for 6 h (*n*=5/genotype/condition); $^{*}p<0.05$, $^{**}p<0.01$

ONLINE ONLY METHODS

Animal Studies

All animal studies were performed according to IACUC-approved protocols. Studies used wt and SIRT3-/- 129Sv (as described[3]) male 12-week old mice, maintained on a standard chow diet (5053 PicoLab diet; Purina), unless otherwise indicated. Mice were sacrificed at 7:00 am for fed mouse studies, or transferred to a new cage without food for 24 h from 7:00 am to 7:00 am, and

then sacrificed for fasted mouse studies, unless otherwise indicated. Cold exposure studies were performed at 4°C for 6 h from 7:00 am to 1:00 pm with continuous monitoring. Core body temperature (rectal) was measured hourly with a digital thermometer (model 4600; Yellow Springs Instruments). These studies used male 4-week-old mice, either fed and given food during the cold exposure, or pre-fasted 18 h from 1:00 pm to 7:00 am and withheld from food during the cold exposure. Glucose measurements were obtained using a handheld glucometer (FreeStyle). Concentration of ketone bodies was determined using a ketone body detection kit (Stanbio Laboratory).

Biochemical assays

Citrate synthase activity was measured by monitoring the conversion of acetyl-CoA and oxaloacetate to citrate, via the CoA-thiol reaction with DNTB[29].

Metabolomics and Lipid Analysis from Tissue and Plasma

After hepatic protein precipitation with methanol, supernatants were dried, esterified with hot, acidic methanol (acylcarnitines) or *n*-butanol (amino acids), and then analyzed by tandem mass spectrometry (Quattro Micro, Waters Corporation). Acylcarnitines were assayed by adapting described methods for analysis of amino acids in dried blood spots[30]. Organic acids were extracted in ethyl acetate, dried, and then converted to their trimethyl silyl esters using N,O-bis (trimethylsilyl) trifluoroacetamide, with protection of alpha-keto groups by oximation with ethoxyamine hydrochloride, followed by gas chromatography-MS (Trace DSQ, Thermo Fisher Scientific)[31]. For lipid analysis, total lipids were extracted from tissue, cells, or plasma by the method of Folch-Lees[32] or Bligh-Dyer[33]. Individual lipid classes were separated by thin layer chromatography (TLC) on Silica Gel 60 A plates and visualized with rhodamine 6G. Lipid ester bands were scraped from the TLC plates and methylated using BF3/methanol as described by Morrison and Smith[34]. Concentration of ATP was determined spectrophotometrically in neutralized TCA filtrates by standard glucose-hexokinase assays as described previously[35,36].

Lipid Uptake, Synthesis, and Export Methods

Fatty acid transport was performed as described previously[37]. Briefly 1-^{14}C-oleic acid (58mCi/mmole) in toluene was dried under nitrogen and resolubilized in 250 μM oleic

acid/0.33% BSA in DMEM. The radiolabeled medium incubated with wild-type or SIRT3-/- primary mouse hepatocytes for 3 minutes at 37°C at which time the cells were washed extensively with PBS, lysed in 1% SDS/PBS and radioactive counts were determined by scintillation counting on a Bioscan AR-2000. Triglyceride synthesis assays were performed by incubating wild-type or SIRT3-/- primary mouse hepatocytes with 1-^{14}C-oleic acid for 2 hours. Cells were washed two times with PBS and lipids were extracted, lysates were separated by thin layer chromatography and triglyceride was quantified by radioactive counts with a Bioscan AR-2000. VLDL export assays were performed by pre-fasting wild-type or SIRT3-/- mice for 5 hours and tail vein-injecting 5mg tyloxapol. Blood samples were collected every hour for 3 hours, processed for plasma and analyzed for triglyceride content (Roche Trig/GB kit).

Electron Microscopy

Tissues from three wt and three SIRT3-/- mice were fixed by cardiac perfusion with 1.5% glutaraldehyde, 4% polyvinylpyrrolidone, 0.05% calcium chloride, and 0.1 M sodium cacodylate, pH 7.4. Livers were removed, put directly into fixative, then were embedded in Epon 812 and photographed with an electron microscope (Siemens Elmiskop 101, Siemens/CTI).

Mass Spectrometry

MS analyses were as described[38,39]. Briefly, peptides containing acetyllysines were isolated directly from protease-digested (trypsin) mitochondrial extracts from wt and SIRT3-/- mouse livers with an anti-actyllysine specific antibody and were identified by tandem MS.

Immunoprecipitation

Murine liver mitochondria were prepared and purified as described[40-42]. Mitochondria were lysed by sonication and resuspended in a low-stringency IP buffer [0.05% NP-40, 50 mM NaCl, 0.5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 10 mM nicotinamide, 1 μM trichostatin A, protease inhibitor cocktail (Roche)].

Cell Culture, Plasmid Construction and Transfection

HEK293T cells and HeLa cells were cultured in DMEM supplemented with 10% FCS. Acadl (GenBank Accession BC027412) constructs were cloned into pcDNA3.1 expression vectors

generated by standard PCR-based cloning strategies and were verified by DNA sequencing. Between 5 x10^6 and 1 x10^7 cells (corresponding to a 10 cm culture dish) were transfected according to standard procedures either by the $CaPO_4$ co-precipitation method or by lipofection (Invitrogen). Cells were maintained in growth medium for 24-48 h before harvesting. To monitor *in vivo* SIRT3 deacetylation or co-immunoprecipitation, LCAD-FLAG expression vectors were co-transfected with an empty pcDNA3.1 vector, SIRT3-HA, catalytically inactive SIRT3H248Y-HA, SIRT4-HA, or SIRT5-HA and then immunoprecipitated with anti-FLAG or anti-HA antiserum and probed for acetylation or co-immunoprecipitation, as described previously[19].

Adenovirus preparation and murine injection

Murine SIRT3 cDNA was cloned into pShuttle-IRES-GFP-1 vector (Strategene), or an empty vector as a negative control. Adenoviruses were recombined and produced using pAdeasy Adenoviral System (Stratagene). After amplification with Ad-293 as packaging cell line, virus was purified using cesium chloride gradient ultra-centrifugation and dialyzed into PBS plus 10% glycerol as described[43]. For injection, twelve-week old male mice were injected via tail vein with adenovirus over-expression either GFP (control) or SIRT3 at dose of 5×10^9 PFU/g body weight, as described[44]. The mice were monitored for signs of distress, and recovered under observation. On the sixth day after virus injection, the mice were sacrificed and livers were removed and measured for fatty acid oxidation assays, as described above.

Statistical Analyses

Results are given as the mean ± standard error. Statistical analyses represent a non-parametric Students *t-test*, and null hypotheses were rejected at 0.05.

SUPPLEMENTARY METHODS REFERENCES

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