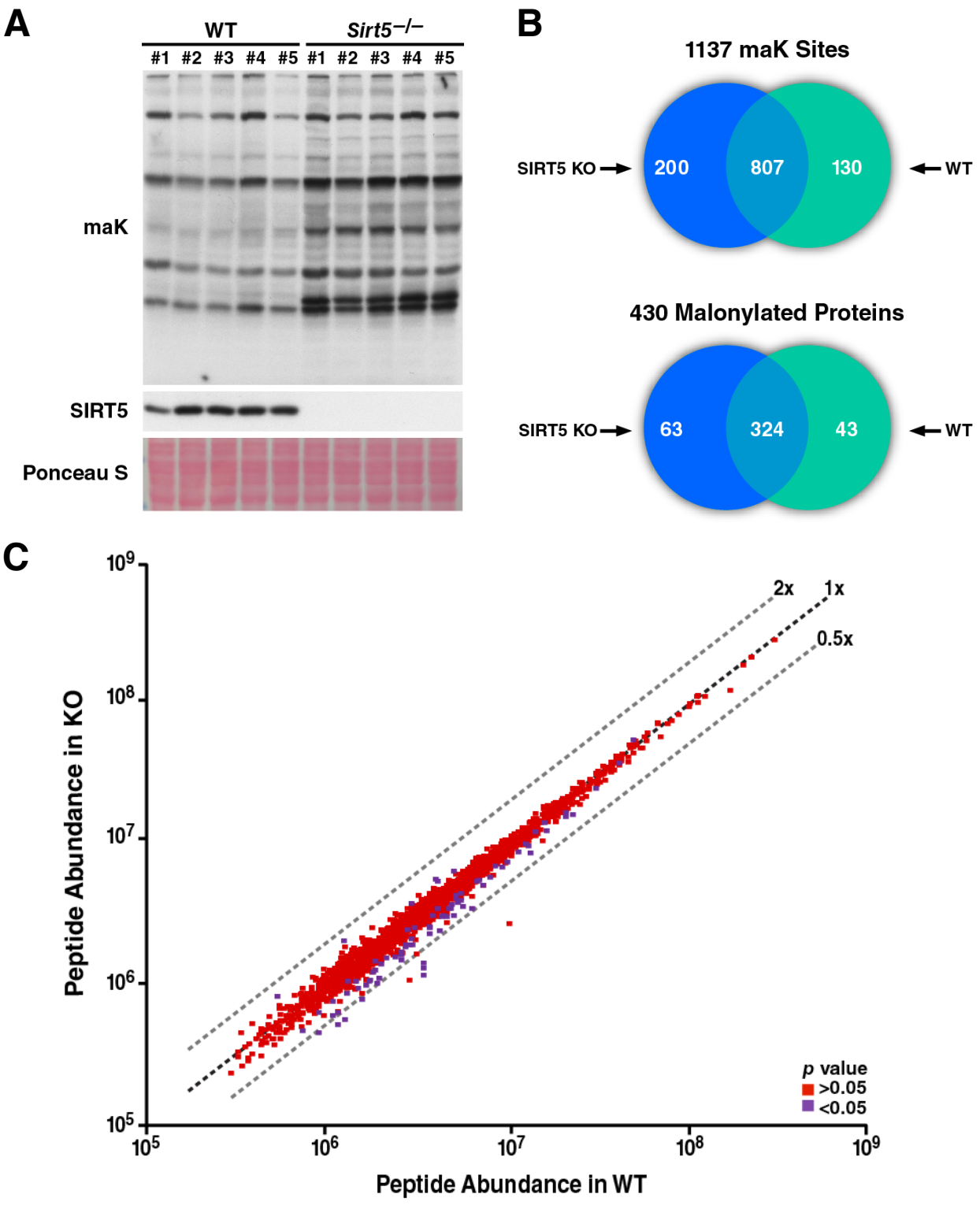

Figure S1. Identification of Whole Liver Lysine Malonylome by Label-free Quantitation. Related to Figure 2.

(A) Western blot showing malonylation of whole cell lysates from WT or *Sirt5*$^{-/-}$ mouse

livers. Western blot for SIRT5 confirmed the absence of SIRT5 protein expression in *Sirt5*$^{-/-}$ tissues. (B) Identification of malonylated lysine sites and proteins. Venn diagrams show the overlap of identified malonylated lysine sites and proteins in WT and *Sirt5*$^{-/-}$ mouse liver. (C) Scatterplot of unmodified peptides quantified by MS1 Filtering. Dashed lines represent the fold change with peptides significantly different (p-value < 0.05) in the *Sirt5*$^{-/-}$ animals in purple and all other sites in red. Peptides were normalized to a peptide from a non-malonylated protein prior to averaging of the data. All peptides for a given protein were then averaged and a protein ratio was generated. See Table S4 for individual peptide and protein ratios.

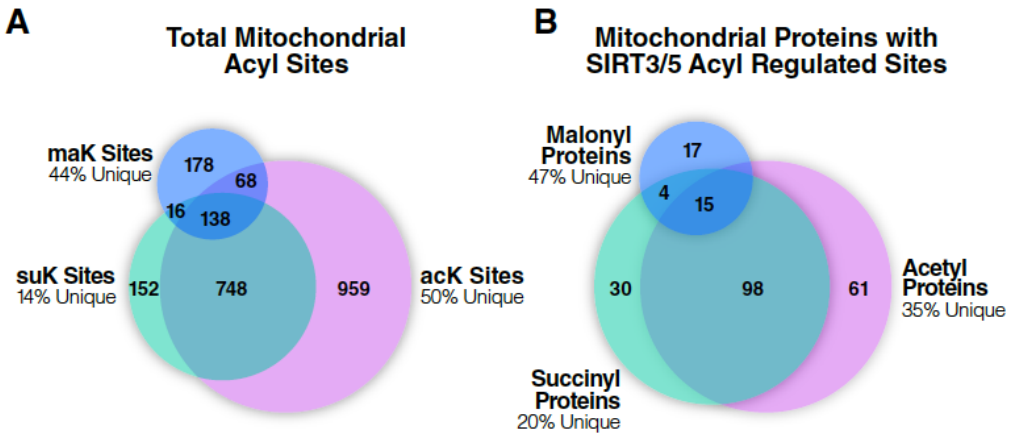

Figure S2. Overlap of malonylation sites with acetylation/ succinylation sites and their regulation by sirtuins. Related to Figure 2.

(A) Venn diagram showing the overlap of lysine acetylated, malonylated and succinylated sites identified on mitochondrial proteins from mouse liver tissue.

(B) Venn diagram showing the number of mitochondrial proteins in liver with at least one residue significantly increased (KO:WT ≥ 1.5, p-value < 0.05) by acetylation, succinylation or malonylation in either the SIRT3 or SIRT5 KO animals.

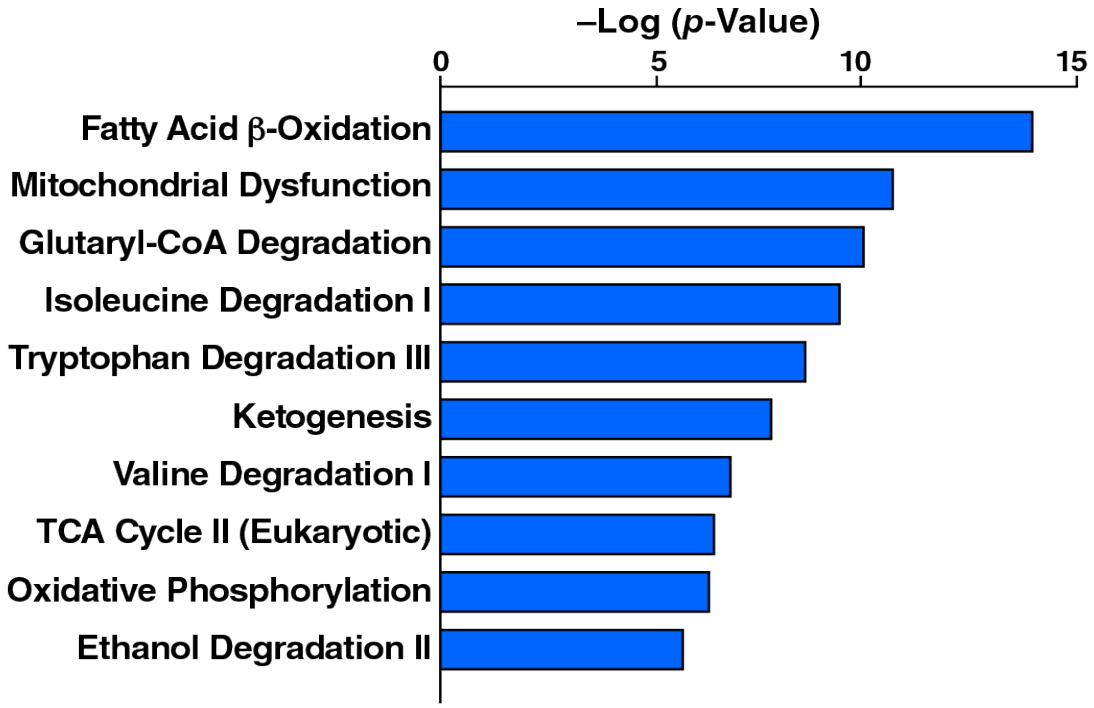

Figure S3. Enriched Pathways by Mitochondrial Proteins with Three Acylation Marks. Related to Figure 6.

53 mitochondrial proteins with three acylation marks are examined by Ingenuity CPA analysis to identify the enriched pathways.

Supplemental tables

Table S1. Mass Spectromety details for malonllysine containing peptides isolated by affinity immunoprecipitation. Related to Figure 2.

Table S2. Quantification of lysine malonylated peptides by MS1 Filtering. Related to Figure 2.

Table S3. Mass Spectromety details for peptides identified in whole liver lysates. Peptides were then used for quantifying changes in protein expression (Table S4). Related to Figure 2.

Table S4. Malonylated proteins quantified by MS1 filtering using peptides identified from whole cell lysates of WT and *Sirt5*$^{-/-}$ animals. Related to Figure 2.

Table S5. Conservation of malonylated lysine residues across species. Related to Figure 3.

Supplemental Methods

Materials.

HPLC solvents, including acetonitrile and water, were obtained from Burdick & Jackson (Muskegon, MI). Reagents for protein chemistry, including iodoacetamide, dithiothreitol (DTT), ammonium bicarbonate, formic acid, trifluoroacetic acid, trichostatin A, dodecyl-maltoside, urea, nicotinamide, and bovine serum albumin (BSA), were purchased from Sigma Aldrich (St. Louis, MO). Malonylated synthetic peptide containing a stable isotope labeled arginine residue (TVDGPSGmaK$\underline{\text{LWR}}$[^{13}C6 ^{15}N4]) was obtained from Thermo Fisher Scientific at >90% purity. Tris (2-carboxyethyl)phosphine (TCEP) and protein A/G magnetic beads were purchased from Thermo Scientific (Rockford, IL), and HLB Oasis SPE cartridges were purchased from Waters (Milford, MA). Proteomics grade trypsin was from Promega (Madison WI). Trypsin-predigested β-galactosidase (a quality control standard) was purchased from AB SCIEX (Foster City, CA).

Sample preparation of mouse liver for MS analysis.

Sample homogenization was performed with 15 passses in a tight-fitting dounce homogenizer. 10 mg of whole liver lysate was denatured with 1% dodecyl-maltoside and 8 M urea per h), alkylated with 10 mM iodoacetamide (30 min at RT in the dark), and incubated overnight at 37°C with sequencing grade trypsin added at a 1:50 enzyme:substrate ratio (wt/wt). Samples were then acidified with formic acid and desalted using HLB Oasis SPE cartridges. Samples were eluted, concentrated to near dryness by vacuum centrifugation, and resuspended in NET buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). 20 mg of the protein digest from each sample was desalted using C-18 zip-tips for total peptide analysis of samples by MS1 Filtering, while the remaining protein digest was used for affinity purification of lysine-malonylated peptides.

Generation and characterization of malonyl-lysine-specific antibodies.

Malonyl-lysine antibodies were generated at Cell Signaling Technology (Danvers, MA) by immunizing New Zealand White rabbits. We used the mixture of peptides whose sequence is CXXXXXX(maK)XXXXXX, where X is an equimolar mix of amino acids, but degenerated malonylated peptide library. The reactive rabbit serum was then purified over peptide affinity column.

Mass spectrometry (MS) and chromatographic parameters.

The autosampler was operated in full injection mode filling a 3-μl loop with 3 μl of analyte for sample delivery. Briefly, after injection, peptide mixtures were transferred onto the analytical C18-nanocapillary LC column (C18 Acclaim PepMap100, 75 μm I.D. x 15 cm, 3-μm particle size, 100Å pore size, Dionex, Sunnyvale, CA) and eluted at a flow rate of 300 nL/min with the following gradient: at 5% solvent B in A (from 0–13 min), 5–35% in A (from 63–66 min), and 80–5% solvent B in A (from 66–68 min), with a total runtime of 90 min, including mobile phase equilibration. Solvents were prepared as follows, mobile phase A: 2% acetonitrile/98% of 0.1% formic acid (v/v) in water, and mobile phase B: 98% acetonitrile/2% of 0.1% formic acid (v/v) in water. Mass spectra and tandem mass spectra were recorded in positive-ion and "high-sensitivity" mode with a resolution of ~35,000 full-width half-maximum in MS1 and 15,000 in MS2. The nanospray needle voltage was typically 2,400 V in HPLC-MS mode. After acquisition of two samples, TOF MS spectra and TOF MS/MS spectra were automatically calibrated during dynamic LC-MS & MS/MS autocalibration acquisitions injecting 50 fmol of a β-galactosidase tryptic digest. For collision-induced dissociation tandem mass spectrometry (CID-MS/MS), the mass window for precursor ion selection of the quadrupole mass analyzer was set to ± 1 *m/z*. The precursor ions were fragmented in a collision cell by nitrogen as the collision gas. Advanced information dependent acquisition (IDA) was used for MS/MS collection on the TripleTOF 5600 to obtain MS/MS spectra for the 20 most abundant parent ions after each survey MS1 scan (allowing were based on value M not *m/z* and were set to an exclusion mass width of 50 mDa and an exclusion duration of 15–20 sec. All mass spectrometry data has been uploaded to the Center for Computational Mass Spectrometry under the 'Mass spectrometry Interactive Virtual Environment',

MassIVE, and can be downloaded using the following link: ftp://MSV000079116:a@massive.ucsd.edu.

Affinity purification of lysine-malonylated peptides.

The polyclonal anti-malonyl lysine antibodies (Cell Signaling Technology, H4597 and BL13640) were immobilized on protein A/B magnetic beads (4°C, 2 h). Malonyllysine peptide standard (150 fmol of TVDGPSGmaKLWR[^{13}C6 ^{15}N4]) was added to the digested peptides from the above liver protein lysate and incubated overnight at 4°C at a 1:50 antibody:peptide ratio (wt/wt). Beads were washed three times in NET buffer, and the peptides eluted by washing three times in 1% trifluoroacetic acid/40% acetonitrile. Peptides are concentrated to near dryness by vacuum centrifugation and resuspended in equal amounts of 0.1% formic acid/1% acetonitrile. The malonyllysine peptide enrichments were subsequently desalted using C-18 zip-tips. After evaporation of organic solvents, samples were suspended in 0.1% formic acid/1% acetonitrile and auto-calibration was examined between sample acquisitions to prevent carry over that would affect downstream quantitative analysis.

Bioinformatic database searches.

Search parameters in Mascot for malonylated peptides were as follows. Trypsin digestion with four missed cleavages accounted for the inability of trypsin to cleave at malonylated lysine residues. Trypsin specificity was set to C-terminal cleavage at lysine and arginine. Variable modifications included lysine malonylation, methionine oxidation, conversion of glutamine to pyroglutamic acid, and deamidation of asparagine. Carbamidomethyl cysteine was set as a fixed modification. Precursor ion and fragment ion mass tolerances were set to 20 ppm and 0.2 Da, respectively. Peptides with an expectation value below the 1% false discovery rate (FDR) were chosen for further data processing. The following sample parameters were used in Protein Pilot: trypsin digestion, cysteine alkylation set to iodoacetamide, urea denaturation, and malonylation emphasis. Processing parameters were set to "biological modification," and a thorough discovery rate analysis tool (PSPEP) algorithm (Shilov et al., 2007). All mass spectral details for malonylated peptides are

available in Table S1. For non-malonylated peptide searches the malonylation emphasis was not used and a peptide confidence value below the local FDR of 1% was used for further peptide processing. Mass spectral details for non-enriched peptides are available in Table S4.

Skyline MS1 Filtering tool algorithm and data analysis.

Skyline is an open-source software project and can be freely installed. Additional details and tutorials for creating spectral libraries and MS1 filtering can be viewed on the Skyline website (http://proteome.gs.washington.edu/software/skyline). Spectral libraries were generated in Skyline with the BiblioSpec algorithm (Frewen and MacCoss, 2007) from database searches of the raw data files as described (Schilling et al., 2012). Raw files were directly imported into Skyline in their native file format, which Skyline achieves using the ProteoWizard data access library (Kessner et al., 2008). After data import, graphical displays of chromatographic traces for the top three isotopic peaks were performed in this study were done on the peptide level, using a peptide centric approach. Only the most abundant isotope for each peptide was used for quantification. The following data extraction peptide areas were normalized to the spiked in malonyl peptide standard (TVDGPSGmaKLWR[^{13}C6^{15}N4] with the most abundant precursor at *m/z* 727.38^{++}; where maK is N-malonyllysine and R=^{13}C6^{15}N4-Arg) and multiplied by a normalization factor 1e^{7} to ensure all values were greater than 1. The normalized peptide area was then averaged across all WT or *Sirt5*$^{-/-}$ acquisitions, and a ratio generated (KO:WT). *P*-values were calculated using a two-tailed, unpaired Student's t-test. For protein quantification peptides identified from the liver lysate (Table S3) that corresponded to malonylated proteins from our enrichment analysis were analyzed by MS1 Filtering. Protein expression was determined by averaging the individual peptide KO:WT ratios. All details for peptide quantitation by MS1 filtering are provided in Table S2 and S4.

Generation of sequence logos.

To generate sequence logos, we first determined the 11–amino acid sequence context for each modified peptide. These sequences were analyzed with IceLogo by comparison to context sequences from an appropriate set of control lysines.

Conservation index of lysine malonylation sites.

Non-redundant malonylated peptides with distinct peaks were mapped to orthologous proteins for conservation analysis (Table S5). We downloaded the full sequence from UniProt and aligned it to the nr database using blastpgp (BLAST suite 2.2.18) (Altschul et al., 1997). To ensure high-quality multiple alignment, we required (i) sequence identity of 30–94% to the query mouse protein, and (ii) >10 such hits could be found. We generated the multiple alignments with CLUSTALW (2.0.12) using default settings (Larkin et al., 2007). We computed conservation indices for each modified lysine by: (i) counting the number of conserved lysines across the seven queried species and (ii) using AL2CO on the alignment (Pei and Grishin, 2001). When calculating rates of mutation to various amino acids, we excluded sites that were absent from the multiple alignment in a given species.

Cell Culture and Plasmid Construction.

Expression vector of mouse GAPDH with C-terminal Flag, coding sequence was PCR amplified and cloned into the pcDNA3.1+-derived vector pcDNA-Flag (Invitrogen, Carlsbad, CA). For the constructs expressing shRNAs, we used the modified pSicoR lentiviral vector (Ventura et al., 2004) that expresses the mCherry reporter gene driven by an EF-1α promoter (pSicoRMS). shRNAs targeting GAPDH (target sequences of human GAPDH #1: GGATATTGTTGCCATCAAT, GAPDH #2: GGTTTACATGTTCCAATAT) were cloned into pSicoRMS.

Dot blot assay and Western blot:

Antibodies used are listed as follows: α-malonyl-lysine (Cell Signaling Technology, H4597, BL13640), α-succinyl-lysine (Cell Signaling Technology, H1007), α-acetyl-lysine (Cell Signaling Technology, #9814), anti-laminA/C (Cell Signaling Technology, #2032), anti-tubulin (Sigma-Aldrich, #T6074), anti-VDAC (Cell Signaling

Technology, #4866), anti-Flag M2 (Sigma-Aldrich), anti-GAPDH (Millipore, MAB374) and anti-SIRT5 (Cell Signaling Technology, #8782).

Tissue subcellular fractionation.

The subcellular extraction was performed based on previous reports (Cox and Emili, 2006). In short, liver tissues were homogenized in ice-cold 250-STMDPS buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF, 25 μg/ml spermine and 25 μg/ml spermidine) with a Dounce homogenizer. Then the extract was centrifuged at 800 g for 15 min to remove nuclei. The supernatant was again centrifuged at 6000 g for 15 min to spin down mitochondria (Pellet II). Residual supernatants were resuspended in hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1 mM DTT, plus the Halt inhibitor cocktail; ThermoScientific) and incubated on ice for 30 min. The suspension was sonicated to lyse mitochondria. Protein concentrations were determined for each fraction using the standard BCA assay (ThermoScientific). The successful subcellular fractionation was examined by Western blotting.

Mouse hepatocyte isolation and deuterium-glucose oxidation assay.

Mouse liver primary hepatocytes were isolated at UCSF Liver Center according to previously described protocols (Seglen, 1972). In short, livers of wild-type or *Sirt5* $^{-/-}$ mice were perfused in-situ with a calcium-chelating solution, followed by a collagenase buffer. Upon digestion, the liver was removed and minced and filtered to remove any remaining liver tissue. The cell suspension was centrifuged two times at 90 g, and the supernatant was discarded. The pellet was re-suspended and underlain with 50% Percoll and spun at 200 g. After two more washes, cell pellets were resuspended and viable cells were counted. 10^6 cells were seeded in 6 well plates and cultured in DMEM with 10% FBS, 25mM 6,6 2d-glucose. The critical feature of this assay is that over 90% of the deuterium on 6,6-2d-Glucose is transferred to water during glycolysis (Beysen et al., 2007). This deuterated water then disperses uniformly into the medium in a culture plate. The culture media was collected following the time course, and the amount of glucose that was oxidized can be calculated from the amount of the deuterated water detected by sensitive IR-MS.

Demalonylation assay of GADPH by SIRT5 in MEFs

Human SIRT5 was cloned into pMSCV-IRES-hygromycin vector (Clontech). Retrovirus was produced by transfecting the plasmid to PLAT-E packaging cells (Morita et al., 2000). Retrovirus for SIRT5 or SIRT5 H158Y were infected to SIRT5 WT or KO MEFs, following the drug selection by hygromycin 50-100μg/ml for 2 weeks. Lentivirus for overexpressing Flag tagged GADPH was infected one day prior to the cell collection. Immediately after cell collection, the immunoprecipitation by anti-Flag M2 antibody was performed against Flag-GAPDH. The level of maK in GAPDH was assessed by Western blotting.

Demalonylation assay of GADPH by SIRT5 *in vitro:*

Briefly, Flag-tagged human SIRT5 WT, SIRT5 H158Y mutant, or mouse GAPDH was cloned into pCAGGs-vector (Provided by Dr. Leonard Guarente). After overexpression in HEK293T cells, protein was immunoprecipitated by anti-Flag M2 agarose beads (Sigma-Aldrich) and subsequently dialyzed and spin concentrated by Amicon MWCO 10Da (Millipore). *In vitro* de-malonylation was assayed by adapting a SIRT3-mediated deacetylation protocol described previously(Schwer et al., 2006). β-NAD(Sigma-Aldrich N6522) was added at the final concentration of 1mM, along with 4 mM $MgCl_2$. After 60 min reaction, the samples were heat-denatured with adding Laemmli sample buffer for Western blotting.

Measurement of GAPDH activity

The concentration of the whole cell lysates was adjusted to 1 mg/ml, and 10 μL / sample was used to measure GAPDH activity with dual time points for the assay (20 and 40 min).

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