

Supplemental Methods and Figure Legends:

SIRT3-dependent deacetylation exacerbates susceptibility to acetaminophen hepatotoxicity

Zhongping Lu, Mohammed Bourdi, Jian H. Li, Angel M. Aponte, Yong Chen, David B. Lombard, Marjan Gucek, Lance R. Pohl, and Michael N. Sack

Methods

Animal experiments. The inbred C57BL/6 SIRT3^{+/-} mice were generated by the laboratory of Dr. Fred Alt at the Howard Hughes Medical Institute, Children's Hospital, Harvard Medical School. The colony was bred using heterozygous breeders and littermate offspring used for experiments. Eight week-old wildtype and SIRT3 knockout male mice were fasted overnight prior to extraction of liver mitochondrial to identify differentially acetylated mitochondrial proteins. Mice were either fed *ad-libitum* or fasted overnight prior to intraperitoneal injection with APAP at a dose of 350 mg/kg. Temporal retro-orbital blood samples were collected at 8 and 24 hours after APAP injection for ALT measurement (Teco Diagnostics, Anaheim, CA). At 24 hours the mice were sacrificed and livers extracted for histology. Liver sections were stained with H&E, and necrosis graded in a blinded-manner using a previously defined scale¹. In brief the section was labeled as 0, no injury; 1, minimal injury; 2, mild injury; 3, moderate injury; 4, marked injury and 5, severe injury. To measure 4-HNE levels, ALDH2 levels and activity and mitochondrial respiration, mice were sacrificed at baseline and at 1 hr (4-HNE and ALDH2 assays) and 4 hours (ALDH2 assays and respiratory studies) following APAP for extraction of liver mitochondria. The animal protocols were approved by the NHLBI Animal Care and Use Committee.

Lentiviral shRNA Preparation and Mice Injection. For knockdown ALDH2, lentivirus (sigma) were produced encoding a non-targeting control sequence or a construct encoding mouse the ALDH2 shRNA sequence: CCAGTGATGCAAATCCTCAAA. Lentivirus were amplified in HEK293T cells, purified with ultracentrifugation and suspended in HBSS. 8-12 week-old male mice were subjected to tail vein injections with control or shRNA lentivirus at the dose of 1.2×10^{11} lentiviral particles. ALDH2 steady-state protein levels were quantified on day 5, in a pilot study to confirm ALDH2 knockdown. On the fifth day post virus injection, subsequent mice were fasted overnight and acetaminophen (350 mg/kg ip) was administered. Blood samples were drawn at 8 and 24 hours after acetaminophen administration for ALT measurement. At the 24 hour time point mice were euthanized and the livers removed for histologic examination.

Proteomic Studies.

2-D IEF/SDS gel electrophoresis and proteomic analysis. For 2-D IEF/SDS polyacrylamide gel electrophoresis, isolated mitochondria pellets were dissolved in buffer consisting of 7M urea,

2M thiourea and 4% CHAPS in the presence of protease inhibitor. Samples were subjected to a first dimensional separation using IPG pH 3-10 strips. SDS gel electrophoresis, Western blotting and immunodetection were carried out using standard methods. Acetylated-lysine antibody was used at a 1:1000 dilution. Spots of interest were excised from coomassie blue-stained gels and subjected to trypsin digestion and mass spectrometry analysis.

LC-MS/MS Analysis on LTQ-Orbitrap XL. The dried tryptic digest was analyzed on an LTQ-Orbitrap XL (Thermo-Fisher Scientific LLC) interfaced with an Eksigent nano-LC 1D plus system (Eksigent Technologies LLC, Dublin, CA) using CID fragmentation. Briefly, sample was loaded onto an Agilent Zorbax 300SB-C18 trap column at a flow rate of 5 μ l/min for 10 min, and then separated on a reversed-phase PicoFrit analytical column (New Objective, Woburn, MA) using a 40-min linear gradient of 2-40% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min. LTQ-Orbitrap XL settings were as follows: spray voltage 1.5 kV; full MS mass range: m/z 200 to 2000. The LTQ-Orbitrap XL was operated in a data-dependent mode; *i.e.*, one MS1 high resolution (30,000) scan for precursor ions followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 2000 with collision energy of 35%. The raw file generated from the LTQ-Orbitrap XL was analyzed using Proteome Discoverer v1.1 software (Thermo Fisher Scientific, LLC) using our six-processor Mascot cluster at NIH (<http://biospec.nih.gov>, version 2.2) search engine. The following search criteria was set to: database, Sprout (Swiss Institute of Bioinformatics); taxonomy, *Mus musculus* (mouse); enzyme, trypsin; miscleavages, 2; fixed modification, carbamidomethylation (+57 Da), variable modification, methionine oxidation (+16 Da); MS peptide tolerance as 25 ppm; MS/MS tolerance as 0.8 Da.

Immunoprecipitation. Cells or isolated mitochondria were lysed in non-denature cell lysis buffer (Cell Signaling) containing protease inhibitors. Target proteins were immunoprecipitated and washed 4 times in cell lysis buffer. For deacetylation assays, immunoprecipitated proteins were washed 3 times in cell lysis buffer and twice in sirtuin deacetylase buffer (SDAC, 50 mM Tris-HCl, pH 9.0, 4 mM MgCl₂, 0.2 mM DTT).

Determination of ALDH2 activity. ALDH2 activity was measured by monitoring the reductive reaction of NAD⁺ to NADH at λ 340 nm. The reaction mixture contained: 60 mM Na-phosphate buffer (pH 8.5), 1 mM NAD⁺, 1 mM EDTA and mitochondrial proteins (0.5 mg/assay). The enzyme reaction was initiated by adding substrate (50 μ M propionyl aldehyde). The absorbance change was monitored over 3 minutes to calculate the rate of NADH production².

In-vivo deacetylation assay. Hepa-1C1C7 cells (were cotransfected with 1 μ g of the deacetylase construct or vector DNA plus 1 μ g of the ALDH2 plasmid using FugeneHD transfection reagent (Roche) according to the instruction. Two days after transfection, cells were washed with 1x DPBS twice and lysed in 200 μ l of RIPA buffer containing Protease inhibitor cocktail (Pierce), 1 μ M of TSA and 10 mM of NAM. Cell lysates were collected, sonicated, and centrifuged at 13,000 rpm for 10 minutes. Supernatants were extracted and target proteins immunoprecipitated

with 20 ul of ANTI-FLAG M2 antibody (Sigma) at 4°C for 4 hours. Precipitated samples were subjected to western blotting analysis with anti-acetylated-lysine mouse monoclonal antibody (Cell signaling) and anti-Flag mouse monoclonal antibody (Sigma).

In-vitro Deacetylation Assays. SIRT3 and ALDH2 proteins were immunoprecipitated from wildtype liver mitochondrial protein using either anti-SIRT3 or anti-ALDH2 antibodies. Immunoprecipitated proteins were incubated in SDAC buffer with NAD⁺ (1 mM), in the presence or absence of NAM (10 mM) for 3 hr at 32°C with vortex. The reactions were stopped by adding LDS sample buffer for subsequent Western blot analysis.

Mitochondrial isolation and respiration. Liver mitochondria were isolated in STE buffer (250 mM sucrose, 5 mM Tris/HCl pH 7.4 and 2 mM EGTA) using a Teflon/glass Dounce homogenizer and separated by differential centrifugation. Protein concentration was determined using BCA protein assay. Mitochondrial respiration was measured in a fiber optic oxygen monitor (Inotech, PA, USA) at 37°C and calibrated with air-saturated assay medium (120 mM KCl, 5 mM KH₂PO₄, 3 mM Hepes, 1 mM EGTA, 2 mM MgCl₂ and 0.3% defatted BSA, pH 7.2). Mitochondria were resuspended to 0.5 mg of protein/ml in assay medium supplemented with the complex 1 inhibitor rotenone (5 uM) and succinate (5 mM) to determine state 2 respiration. ADP (0.2 mM) was added to measure state 3 respiration.

GSH/GSSG Measurements. Mice were fasted overnight and then exposed to either saline or APAP administration. Four hours later the livers were extracted and 200 mg of liver tissue was homogenized in 800 ul STE buffer (250 mM sucrose, 10 mM Tris-HCL, 1 mM EDTA). 50 ul of homogenate was used for GSH measurement, and 100 ul for GSSG using the Bioxytech GSH/GSSG-412 kit (OxisResearch).

NAPQI binding and ALDH2 overexpression in Hepa-1c1c7 cells. Hepa-1c1c7 cells were grown in 10% FBS MEM alpha media. Transfection of the pCMV6-ALDH2-FLAG plasmid was performed using FuGene 6 (Roche). To evaluate the role of SIRT3 deacetylase the wildtype (hSIRT3-Flag) and the deacetylase catalytically inactive construct (hSIRT3-H248Y-HA, both from Addgene) were additionally overexpressed. 48 hours after the transfections, cells were treated with NAPQI (200 uM) and NAM (5 mM) or vehicle control for 1 hour. Immunoprecipitation using an anti-FLAG antibody to extract the exogenous ALDH2 was performed and run on SDS-PAGE gels. Acetaminophen metabolite binding to ALDH2 was detected by Western blot using an antibody to NAPQI protein adducts³.

LC-MSMS determination of acetylated lysine residues. Five milligrams of mouse liver mitochondria were sequentially reduced with dithiothreitol, alkylated with iodoacetamide, and then digested with 100 µg trypsin (Promega, Madison, WI) at 37 °C overnight. The tryptic digest was desalted and lyophilized. Acetyl-lysine peptides were enriched with agarose-immobilized anti-acetyl lysine antibody (ImmunoChem, Burnaby, BC Canada) following the protocol described by Kim et al 2006⁴. The eluted peptides were analyzed on an LTQ Orbitrap Velos

(Thermo Fisher Scientific, San Jose, CA) that was coupled with an Eksigent nanoLC-Ultra 1D plus system (Dublin, CA). LCMS data were searched against the mouse subset of Swiss Prot (Swiss Institute of Bioinformatics) database using Mascot algorithm (Matrix Science, London, UK; version 2.3). The search result was filtered with Scaffold Q+ software (Proteome Software Inc., Portland, OR), and peptide identifications were accepted at > 80% probability or < 5% false discovery rate (FDR). Relative quantitative changes of acetyl-lysine peptides of interest were calculated with QUOIL, a label-free quantitation package previously described ⁵.

ALDH2 mutagenesis studies. The Quikchange II Site-Directed Mutagenesis kit (Agilent Technologies) was employed to substitute amino acids as per the kit instructions. The template cDNA employed was the pCMV6-ALDH2-flag and the following amino acid substitutions were performed: K370-Q (AAG – CAA) 5'-caggtggatgaaactcagtttcaaagatcctcggctacatcaa-3'; K370-R (AAG – AGG) 5'-gcctcaggtggatgaaactcagtttaggaagatcctcggcta-3'; K377-Q (AAA – CAG) 5'-aagatcctcggctacatccagtcgggacaacaagaaggg-3' and K377-R (AAA – CGA) 5'-gaagatcctcggctacatccgatcgggacaacaagaagggg-3'.

Materials. Hepa-1c1c7 cell line was purchased from ATCC. Lentivirus constructs containing ALDH2 shRNA or non-targeting sequences were purchased from Sigma. pCMV6-ALDH2-flag construct was purchased from OriGene company. Anti-SIRT3 polyclonal antibody was custom made by Covance Inc (CA, USA). 4-HNE antibody was purchased from Oxis Inc (CA, USA). Acetylated-lysine antibody was purchased from Cell Signaling. The anti-acetaminophen metabolite antibody was generated as previously described ⁶. Anti-ALDH2 was obtained from Santa Cruz. NAPQI and most other chemicals were purchased from Sigma (St. Louis, MO).

Statistical Considerations. Differences between data groups were evaluated for significance using the two-tailed Student *t*-test. Multiple comparison analysis was performed using ANOVA. Statistical analysis was performed using Graphpad Prism and data are expressed as mean ± SEM. *p* < 0.05 is considered statistically significant.

References.

- 1 Nakagawa, H. *et al.* Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation. *Gastroenterology* **135**, 1311-1321, doi:S0016-5085(08)01313-9 (2008).
- 2 Moon, K. H., Kim, B. J. & Song, B. J. Inhibition of mitochondrial aldehyde dehydrogenase by nitric oxide-mediated S-nitrosylation. *FEBS Lett.* **579**, 6115-6120 (2005).
- 3 Matthews, A. M., Roberts, D. W., Hinson, J. A. & Pumford, N. R. Acetaminophen-induced hepatotoxicity. Analysis of total covalent binding vs. specific binding to cysteine. *Drug Metab Dispos* **24**, 1192-1196 (1996).
- 4 Kim, S. C. *et al.* Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol. Cell* **23**, 607-618 (2006).
- 5 Wang, G., Wu, W. W., Zeng, W., Chou, C. L. & Shen, R. F. Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: Reproducibility, linearity, and application with complex proteomes. *J Proteome Res* **5**, 1214-1223, doi:10.1021/pr050406g (2006).

- 6 Matthews, A. M., Hinson, J. A., Roberts, D. W. & Pumford, N. R. Comparison of covalent binding of acetaminophen and the regioisomer 3'-hydroxyacetanilide to mouse liver protein. *Toxicol Lett* **90**, 77-82, doi:S0378427496038313 [pii] (1997).

Supplementary Figure Legends:

Supplementary Figure 1 Representative two-dimensional gels to demonstrate differential acetylation in liver mitochondria protein in SIRT3^{+/+} and SIRT3^{-/-} mice. Representative 2-D gel electrophoresis membranes following immunoblot analysis with antibody directed against Ac-K. The numerated arrows show differentially acetylated spots in the SIRT3 knockout mice representing 14 of the 17 proteins that fulfilled the criteria for inclusion as reproducible and high probability targets of SIRT3-mediated deacetylation. The inclusion criteria include: i) High ion score (>450); ii) Validation by correct PI and MW of picked spot and iii) Reproducibility of the prior two criteria on at least three separate experiments. In these representative blots the enumerated spots contain peptides fulfilling criteria described above. Spot 1 - Glutamate dehydrogenase 1; Methylmalonate-semialdehyde dehydrogenase. Spot 2 - Glutamate dehydrogenase 1; Aldehyde dehydrogenase, mitochondrial; ATP synthase subunit alpha. Spot 3 - Acyl-coenzyme A synthetase; Acyl-CoA synthetase family member 2; Catalase; Carboxylesterase. Spot 4 - Acyl-coenzyme A synthetase, mitochondrial; Succinate dehydrogenase [ubiquinone] flavoprotein subunit. Spot 5 - Acyl-CoA dehydrogenase 9, Acyl-CoA synthetase 2. Spot 6 - Short-chain specific acyl-CoA dehydrogenase; Ornithine carbamoyltransferase. Spot 7 - Aldehyde dehydrogenase, mitochondrial; Carboxylesterase 3. Spot 8 - Voltage-dependent anion-selective channel protein 1. Spot 9 - Haloacid dehalogenase-like hydrolase domain-containing protein 3. Spot 10 - 2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial; Elongation factor Tu; Non-specific lipid-transfer protein. Spot 11 - Carboxylesterase 3; Probable carboxypeptidase. Spot 12 - Non-specific lipid-transfer protein; Proliferation-associated protein 2G4. Spot 13 - Mitochondrial import inner membrane translocase subunit TIM44; Bile acid-CoA:amino acid N-acyltransferase; 2-oxoisovalerate dehydrogenase subunit alpha, Spot 14 - 39S ribosomal protein L39, mitochondrial; 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial.

Supplementary Figure 2 Basal 4-HNE levels in SIRT3^{+/+} and SIRT3^{-/-} mouse livers.

Immunoblot analysis to quantify baseline levels of 4-HNE adduct binding to liver mitochondrial comparing wildtype to SIRT3 knockout mice. The number of proteins showing adduct binding is greater here than those shown in Fig. 3c as the film exposure time is increased in these baseline samples to enable visualization of adduct binding.

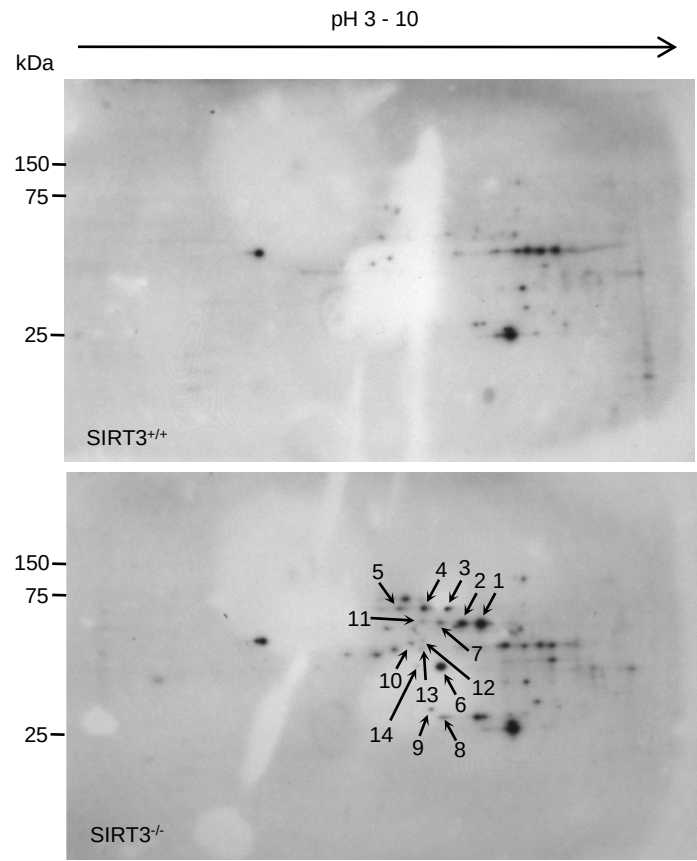
Supplementary Figure 3 Liver mitochondrial respiration in SIRT3^{+/+} and SIRT3^{-/-} mice following APAP toxicity. Oxymetry was performed on liver mitochondria isolated from wildtype and knockout mice at baseline and four hours after the administration of acetaminophen. Mitochondrial respiration was induced with succinate as substrate in the presence of rotenone and ADP. (II: state 2 respiration, III: state 3 respiration. APAP - paracetamol). Both the wildtype and knockout mice liver mitochondrial state II and III respiration is significantly depressed by acetaminophen. For oxymetry studies, the asterisk represents $p < 0.05$ between the genotypes at four hours after acetaminophen. All results shown represent the mean $\pm$ SEM. All other asterisks represent $p < 0.05$ versus the corresponding control group.

Supplementary Figure 4 Consequences of knockdown of ALDH2 in hepatocytes and in the liver (A) Immunoblot analysis of protein extracted from Hepa-1c1c7 murine liver cells transfected with distinct shRNA constructs directed against ALDH2. The relative levels of steady-state ALDH2 protein levels identified sh4 and sh5 as the two optimal shRNA's to use for the generation of lentiviral shRNA. Protein loading is confirmed by β -actin expression. **(B)** Representative H&E stained liver sections from SIRT3^{-/-} mice infected with the non-targeting shRNA (n=4) and the shRNA against ALDH2 (n=5) to confirm relative levels of hepatic injury.

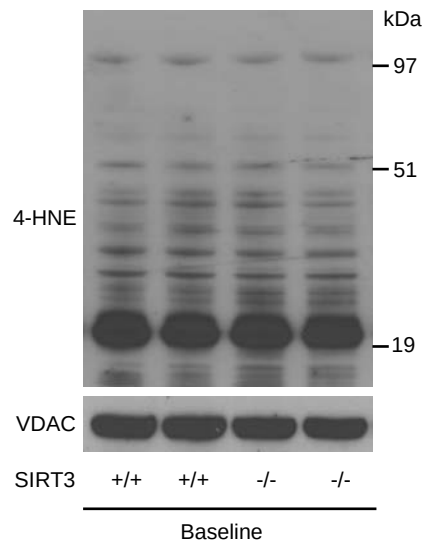
Supplementary Table 1. Increased acetylation of liver mitochondrial proteins in SIRT3^{-/-} mice.

1. 3-hydroxyisobutyryl-CoA hydrolase
2. 3-ketoacyl-CoA thiolase
3. Aldehyde dehydrogenase 2 (ALDH2)
4. Apoptosis-inducing factor 1
5. Carbamoyl-phosphate synthase
6. Dimethylglycine dehydrogenase
7. Electron transfer flavoprotein-ubiquinone oxidoreductase
8. Elongation factor Tu
9. Glutaminase liver isoform
10. Import inner membrane translocase subunit TIM44
11. Import inner membrane translocase subunit TIM50
12. Isovaleryl-CoA dehydrogenase
13. Long-chain-fatty-acid--CoA ligase 1
14. Ornithine carbamoyltransferase
15. Methylmalonate-semialdehyde dehydrogenase
16. Sarcosine dehydrogenase
17. Short-chain specific acyl-CoA dehydrogenase

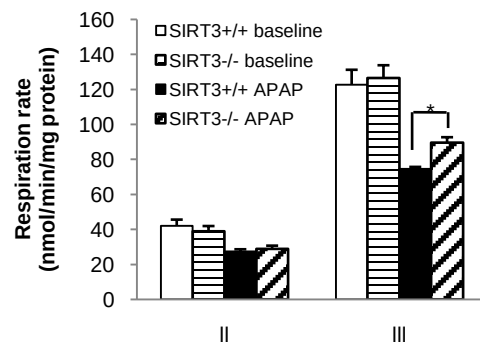
Supplemental Fig 1



Lu et al Supplemental Fig. 2



Lu et al Supplemental Figure 3



Lu et al Supplementary Figure 4

