**SUPPLEMENTAL MATERIAL**

# MITOCHONDRIAL SIRT3 PREVENTS DOXORUBICIN-INDUCED DILATED CARDIOMYOPATHY BY MODULATING PROTEIN ACETYLATION AND OXIDATIVE STRESS

Mateusz M. Tomczyk[1,2,4], Kyle G. Cheung[1,2,4], Bo Xiang[1,2,4], Nahid Tamanna[5], Ana L.F. Teixeira[5], Prasoon Agarwal[1,2,4,8,9], Stephanie M. Kereliuk[1,2,4], Victor Spicer[3,4,6], Ligen Lin[7,10], Jason Treberg[5], Qiang Tong*[7], Vernon W. Dolinsky*[1,2,4].

[1]Diabetes Research Envisioned and Accomplished in Manitoba (DREAM) Theme of the Children's Hospital Research Institute of Manitoba
[2]Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Canada
[3]Department of Internal Medicine, University of Manitoba, Winnipeg, Canada
[4]Rady Faculty of Health Science, College of Medicine, University of Manitoba, Winnipeg, Canada
[5]Department of Biological Sciences, University of Manitoba, Winnipeg, Canada
[6]Manitoba Center for Proteomics and Systems Biology, Winnipeg, Canada
[7]Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas, USA
[8]KTH Royal Institute of Technology, School of Electrical Engineering and Computer Science, Stockholm, Sweden
[9]Science for Life Laboratory, Solna, Sweden
[10] Institute of Chinese Medical Sciences, University of Macau, Macau, China.

*** Co-Corresponding Authors:** vdolinsky@chrim.ca, Children's Health Research Institute of Manitoba, 715 McDermot Avenue, Winnipeg, MB R3E 3P4 and qtong@bcm.edu, Children's Nutrition Research Center, Houston, TX 77251-1892

## Contents

## I. Supplementary Methods

### *I. 1 Reagents and Antibodies*

DOX (doxorubicin hydrochloride, D4000) was purchased from LC Laboratories and most other reagents and chemicals were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). SIRT3 (5490S), acetylated lysine (9441S) and α-tubulin (2144S) antibodies for western immunoblotting were purchased from Cell Signaling Technologies (Beverly, MA). The SOD2 (sc18504) antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

### *I. 2 Generation of M3-SIRT3 and M1-SIRT3 Transgenic Mice*

For the generation of M3-SIRT3 transgenic mice, 0.8 kb murine M3-SIRT3-FLAG fragment was released from the pCR-Blunt II-TOPO-SIRT3M3-FLAG construct [11] with XhoI and HindIII, and ligated into SalI and HindIII sites of pBS2-□MHC, which contains 5.5kb murine cardiac alpha-myosin heavy chain (□MHC) gene promoter and human growth hormone poly A signal [12], (a gift from Dr. Jeffrey Robbins through Dr. Chi-Wing Chow). The transgene fragment was released from the vector by SacII digestion. For the generation of M1-SIRT3 transgenic mice, the 1.2 kb mouse M1-SIRT3 coding sequence with FLAG was excised from the pCR-Blunt II-TOPO vector with XhoI and HindIII, and then inserted into pBluescript II vector containing human Growth Hormone (hGH) polyadenylation site. The 6.5 kb promoter of muscle creatine kinase (MCK) was cut with XhoI from the pMCK6.5-pUC118 plasmid (kindly provided by S. D. Hauschka) [13] and inserted into 5' of SIRT3. The resulting pBS-MCK-SIRT3M1-FLAG-hGH plasmid was digested with BssHII to release the 8.3 kb transgene construct. Both transgene fragments were injected into fertilized C57BL/6 mouse oocytes by the Genetically Engineered Mouse Core at Baylor College of Medicine. Gene expression studies revealed that M3-SIRT3 and M1-SIRT3 transgenic mice exhibit increased SIRT3

expression within the heart (Fig. IIA in data supplement). Hearts from both M3-SIRT3 and M1-SIRT3 transgenic animals displayed increased SIRT3 protein expression which was resistant to decreases by DOX treatment (Fig. IIB, IIC in data supplement).

*I. 3 Animal Housing and DOX Treatment*

Ten-week-old mice were treated intraperitoneally (i.p.) with 8.0mg/kg body weight of DOX 4 weeks and compared to non-transgenic saline (0.9%) treated littermates (Fig I in data supplement). DOX (doxorubicin hydrochloride, D4000) was purchased from LC Laboratories. Mice were housed under controlled temperature and lighting (20C; 12h light-dark cycle) and received *ad libitum* consumption of 5P00 Prolab RMH 3000 standard chow diet (LabDiet, St. Louis, MO). Mice were euthanized by a single i.p. dose of sodium pentobarbital (90mg/kg). All animal procedures were conducted in accordance with the University of Manitoba Animal Welfare Committee, which adheres to the principles for biomedical research involving animals developed by the Canadian Council on Animal Care and the Council for International Organizations of Medical Sciences and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

*I. 4 Mitochondrial protein isolation and mass spectrometry peptide preparation*

Mitochondrial protein was isolated from freshly harvested heart tissue using the Sigma mitochondrial isolation kit (MITOISO1) according to manufacturer's instructions. Mitochondrial integrity validated by JC-1 assay. Mitochondrial protein concentration was determined by Pierce Detergent Compatible Bradford Assay Reagent (Thermo, 1863028). Mitochondrial samples from littermates pooled to obtain 1 mg of mitochondrial protein. DTT (DL-Dithiothreitol) at a final concentration of 10mM used to reduce mitochondrial protein at

57˚C for 30mins. Alkylation was performed by adding IAA (Iodoacetamide) to a final concentration of 50mM and incubating at room temperature for 45mins in the dark. The sample was quenched with DTT. Protein clean-up was performed using Sera-Mag Carboxylate-Modified Magnetic SpeedBeads (GE Life Sciences, 45152105050350, 65152105050250) according to manufactures protocol. Proteins were trypsinzed to peptides using mass spec grade Trypsin Lys-C Mix (Promega, V5071) in 50mM HEPES (pH=8), enzyme to protein ratio was 1:50(wt/wt). C18 Sep-Pak Vac 1cc (50mg) cartridges (Waters, WAT054955) were used to clean up the peptides.

Immunoprecipitation of acetylated peptides was performed using acetylated-lysine antibody prebound to agarose beads (ImmuneChem, ICP0388). Beads were prepared according to manufactures protocol. 200 fmol of acetyl-lysine peptide standard (LVSSVSDLPacKR) was added to the resuspended peptides. Peptides were immunoprecipitated overnight at 4˚C and eluted in 0.15% TFA (Trifluoroacetic acid**)**. Acetylated peptides were dried by SpeedVac for 2 hours. Peptide clean up was performed using C18 Sep-Pak Vac 1cc (50mg) cartridges and acetylated peptides were eluted in 65% ACN in 0.1% TFA, then samples were dried by SpeedVac and eluted in 0.1% formic acid. Peptide concentrations were determined by the protein function in Nanodrop. All solutions made with LC MS grade water and filtered with 0.22µm sized filter. All reagents were made fresh the day of experiment.

*I. 5 Mass Spectrometry of Acetylated Cardiac Mitochondrial Peptides*

Analysis of peptide digests were carried out on an Orbitrap Q Exactive HF-X instrument (Thermo Fisher Scientific, Bremen, Germany). The sample was introduced using an Easy-nLC 1000 system (Thermo Fisher Scientific) at 1 µg per injection. Mobile phase A was 0.1% (v/v) formic acid and mobile phase B was 0.1% (v/v) formic acid in 80% acetonitrile (LC-MS grade).

Gradient separation of peptides was performed on a C18 (Luna C18(2), 3 μm particle size (Phenomenex, Torrance, CA)) column packed in-house in Pico-Frit (100 μm X 30 cm) capillaries (New Objective, Woburn, MA). Peptide separation was using the following gradient: 2 – 10 % increase of phase B over 5 minutes, 10 – 35 % over 35 minutes, 35 – 45 % over 5 minutes, 45 – 90% over 5 minutes, with final elution of 90% B for 10 minutes at a flow rate of 300 nL/min.

Data acquisition on the Orbitrap Q Exactive HF-X instrument was configured for data-dependent method using the full MS/DD−MS/MS setup in a positive mode. Spray voltage was set to 2.1 kV, funnel RF level at 45, and heated capillary at 275°C. Survey scans covering the mass range of 350–1500 m/z were acquired at a resolution of 60,000 (at m/z 200), with a maximum ion injection time of 60 milliseconds, and an automatic gain control (AGC) target value of 3e6. For MS2 scan triggering, up to 15 most abundant ions were selected for fragmentation at 28% normalized collision energy, with intensity threshold kept at 1.1e4. AGC target value for fragment spectra was set at 1e5, which were acquired at a resolution of 30,000, with a maximum ion injection time of 90 milliseconds and an isolation width set at 1.5 m/z. Dynamic exclusion of previously selected masses was enabled for 10 seconds, charge state filtering was limited to 2–6, peptide match was set to preferred, and isotope exclusion was on.

*I. 6 Bioinformatic Analysis of Acetylated Peptides*

A database of protein sequences for mouse were extracted from Uniprot (June 2016) and used for peptide/protein identification. Raw spectra for each of the 11 1D LC-MS runs in experiment (n=6 Non-TG-CON, n=5 Non-Tg-DOX) was converted into an MGF file using the Proteome Discoverer bundled tool; these MFG files were searched against our database using X!tandem (cyclone 2012.10.01.1).

Peptide identification settings were standard for the instrument: tryptic peptides were permitted, with a parent and fragment mass tolerance of 10 PPM. A fixed post-translational modification of C+57.021 was applied, and standard variable post translational modifications including N-terminal acetylation, deamidation, phosphorylation and oxidation were permitted. Lysine acetylation specific to this experiment design was also included, and the number of missed cleavages was increased to four. Peptide assignment into source proteins was managed by X!tandem. A maximum peptide score of log(e)<=-1 was applied; protein expectation values were computed as an aggregate of their member peptide scores under Bayes theorem.

Label-free quantitation following the peptide identification was done using in-house tools developed for this project. For all runs in the experiment, we extracted peptides containing acetylated lysine, with intensity being assigned as the sum of the spectrum's MS2 fragments, and accumulated across multiple identifications in a given run, then converted into a log2 scale.

Detection and quantitation of the spike peptide LVSSVSDLP[K+42.010][R+10.008] in each run was done using an in-house tool that scanned each run's MGF file for a parent fingerprint match (10 ppm tolerance; Z=2; 626.861 Da or Z=3; 418.243 Da) followed by a MS2 fragment evaluation (minimum 9 fragments matched at 10ppm tolerance; singly charged b and y ions). Intensity was assigned as the sum of all MS2 fragment intensities in all sequence-matching spectra, converted into a log2 scale.

All peptides in each run had their log2 expression values scaled down by subtracting out its spike peptide log2 value; these values were then normalized on a run-by-run level for differential analysis. The difference was computed as the average of the DOX values minus the average of the CON values; the two-tailed Student's T-test function built into Microsoft Excel was used to compute statistical significance. The raw mass spectrometry data (MGF format)

and the overall peptide expression matrix have been deposited to the University of California, San Diego's MassIVE data repository (massive.ucsd.edu) under accession MSV000086892.

### *I. 7 Transthoracic Echocardiography*

Mice were imaged under mild anesthesia (induced with 3% isoflurane and 1.0L/min oxygen and maintained at 1 – 1.5% isoflurane and 1.0 L/min oxygen) during echocardiography. Each mouse was placed on heated ECG platform to maintain body temperature at 37° C. Echocardiography (Vevo 2100, Fujifilm-Visual Sonics, ON Canada) was used to assess cardiac morphology and function using B-mode, M-mode, and doppler imaging[4]. Parameters were assessed at baseline and post treatment (Fig. I in data supplement) by a trained and blinded small animal echocardiographer and data were analyzed using Vevo LAB cardiovascular software and measurements were averaged over four cardiac cycles.

### *I. 8 Cell Culture*

One-to-two-day old Sprague-Dawley neonatal rats were euthanized by decapitation in accordance with study design. Primary rat neonatal cardiomyocytes (PRNCs) were isolated by enzymatic dissociation as previously described [15]. PRNC were plated at a density of $5.0x10^5$ cells per well in 24-well plates in DMEM/F-12 (HyClone, SH30126) plating media supplemented with 10% FBS (Gibco, 12483-020), 5% horse serum (Gibco, 16050122) 50 μg/ml gentamicin (Gibco, 15750-078) and 1% penicillin-streptomycin solution (HyClone, SV30010) and transduced with adenoviruses for 24h and then treated with DOX at concentrations indicated in figure legends. Human induced pluripotent stem cell (hiPSC) derived cardiomyocyte icells (Fujifilm Cellular Dynamics, CMC-100-010-000.5) were thawed at 37˚C, resuspended with plating medium (CMM-100-010-001) and seeded in gelatin-coated 96 well

plates at a density of 20 x$10^3$ cells/well. Cells were incubated at 37˚C at 7% $CO_2$. After 48 hours of plating, cells were switched to Maintenance Medium (CMM-100-120-001). Seven days post-plating cells were transduced. Full-length SIRT3 (Ad.M1-SIRT3) and truncated (Ad.M3-SIRT3) as well as deacetylase-deficient SIRT3 mutant adenoviruses (Ad.M1-SIRT3-N164A and Ad.M3-SIRT3-N87A) were prepared as previously described [6].

*I. 9 Mitochondrial $H_2O_2$ Efflux*

Mitochondrial superoxide production was also measured indirectly as $H_2O_2$ efflux. Mitochondrial $H_2O_2$ efflux was measured using an extramitochondrial fluorescence detection system based on [16] but using 10 µM Amplex Ultrared to avoid the inhibition higher concentrations can have on mitochondrial function in mouse as described [17]. The assay was modified for use in a microplate reader with a final volume of 200 µl and the other assay constituents including the respiration medium were the same final concentrations as found elsewhere [16,17] with the exception of increasing the linking enzymes to 10 and 50 IU ml-1 for horseradish peroxidase and superoxide dismutase respectively and decreasing the bovine serum albumin to 0.1% (w/v). Fluorescence was monitored at 37°C using a BMG FluoStar reader with filters set at 535nm and 590nm for excitation and emission respectively. The plate was mixed on maximal intensity for 2 seconds every 15 seconds, between readings, to minimize settling of mitochondria and to limit oxygen depletion because rates of superoxide/$H_2O_2$ formation by mitochondria are sensitive to oxygen availability under similar assay conditions [23]. Net rates of fluorescence product formation were determined by subtracting the substrate-free rate of fluorescence change from the rate in the presence of respiratory substrate. These net rates were converted from fluorescence to nmol $H_2O_2$ based on calibration curves produced in the presence of all assay components, including

mitochondria, except respiratory substrate (succinate). Rates of $H_2O_2$ formation were confirmed to be linear with mitochondrial protein added to the well and the typical experiment contained 0.025 mg ml-1 of mitochondrial protein. The two respiratory conditions tested (5 mM succinate and 5 mM succinate in the presence of 4 μM rotenone) were chosen because these will induce electron leak from primarily superoxide producing complexes in the mitochondrion but at markedly different rates due to rotenone's inhibition of the high production from complex I during reverse electron transport (reviewed in [18]).

### *I. 10 HPLC Detection of Mitochondrial Superoxide Production Ex-Vivo*

Mitochondrial superoxide production was measured directly with MitoSOXTM by a fluorescence-based-HPLC method based on [19] with modification. MitoSOXTM consists of triphenylphosphonium-linked hydroethidium that reacts with superoxide to form a superoxide-specific-product hydroxyethidium, which can be separated and detected by HPLC [20]. Previous studies reported that MitoSOXTM binds with plastics [21]. To minimize loss due to binding, glass vials and glass syringes were used for handling MitoSOXTM in all experiments. An experimental assay began with glass HPLC inserts (0.350 mL capacity) placed within HPLC vials with the following add in order with a final volume of 0.2 mL: respiration medium (120 mM KCl, 20 mM Hepes, 5mM KH2PO4, 2.5mM MgCl2, 1mMEGTA and 0.1% BSA, pH 7.2 at 37°C), succinate (5 mM), rotenone (4 μM), 0.5 μM MitoSOXTM and then mitochondria (0.05 mg mL-1 of protein) were added to initiate the reaction. Vials were sealed, vortexed, and placed in 37°C water bath. After 20 mins of incubation, 0.1 mL of acetonitrile (CH3CN) was added to destroy the mitochondrial membrane and to extract hydroxyethidium [19]. For analysis, samples were used without further filtration or processing, with 0.1 mL injected using an Ultimate-300 UHPLC to separate and detect hydroxyethidium formation with a C18 column

(AcclaimTM , 100 x 4.6 mm, 3 μm) equilibrated with 0.1% of Trifluroacetic acid (TFA) and CH3CN (HPLC grade, ≥99%) with a ratio of 80:20 (TFA: CH3CN) followed by fluorescence detection. The settings for HPLC were as follows: TFA: CH3CN was 80:20 for 11 mins, 55:45 at 12 mins, and from 12-21 mins the percentage of CH3CN was increased to 50%, then 100 % of CH3CN was run from 23-30 mins to flush the column, with a flow rate of 0.7 mL min-1 for 0-21 mins and 1 mL min-1 for 23-30 mins. The fluorescence detector used 500 nm and 595 nm for the excitation and emission wavelengths respectively. Hydroxyethidium peaks eluted around 17 mins.

For the current experiments, we used 0.5 μM of MitoSOX$^{TM}$, a concentration that is 10 fold lower than the manufacturer recommendation. Because previous studies recommended to optimize MitoSOX$^{TM}$ for each cell type given that it causes the disruption of membrane potential or increase mitochondrial permeability[22], we tested the effect of MitoSOX$^{TM}$ on mitochondrial respiration and membrane potential simultaneously in an Oroboros O2K equipped with a fluorescence module using tetramethylrhodamine methlyester as described and illustrated elsewhere [23]. We found that 5 μM MitoSOX$^{TM}$ led to near complete depolarization of respiring mitochondria, which was accompanied by a marked decrease in respiration rate (data not shown); however, decreasing to 0.5 μM of MitoSOX$^{TM}$ caused only a minor influence on membrane potential and respiration while still also providing adequate peak size for detection by HPLC.

### *I. 11 Western Immunoblotting*

Western blotting was performed as previously described[6]. Total cardiac tissue was homogenized in lysis buffer containing a mixture of protease and phosphatase inhibitors (Thermo Scientific), DTT, trichostatin-A. Protein concentration was determined using the

Bradford reagent (Bio-Rad). 4X laemmli sample buffer (Bio-Rad) and 5% 2-mercaptoethanol was added to the protein lysates. Samples were heated at 100˚C for 10mins. 15-20ug of protein was used for SDS-PAGE, transferred to nitrocellulose and probed using antibodies specified. For chemiluminescence, secondary antibodies (Santa Cruz Biotechnology) were applied, and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for detection.

## II. Supplementary Figures and Figure Legends

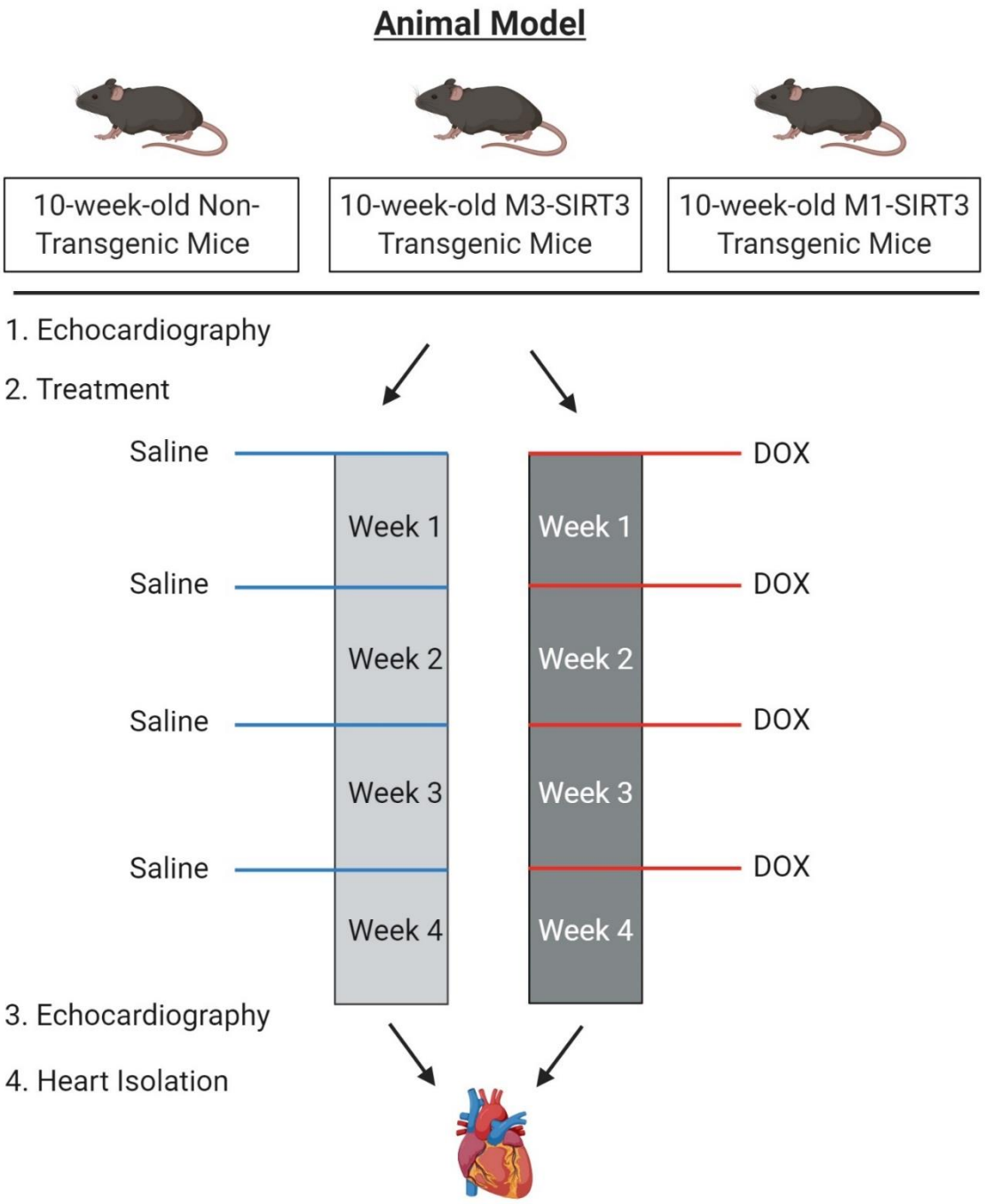


Figure S1. Non-Tg, M3-SIRT3 and M1-SIRT3 DOX Animal Model of Dilated Cardiomyopathy. M3-SIRT3, M1-SIRT3 and non-transgenic controls treated with 8.0mg/kg of DOX or saline once a week for four weeks. Echocardiography performed prior and post treatment.

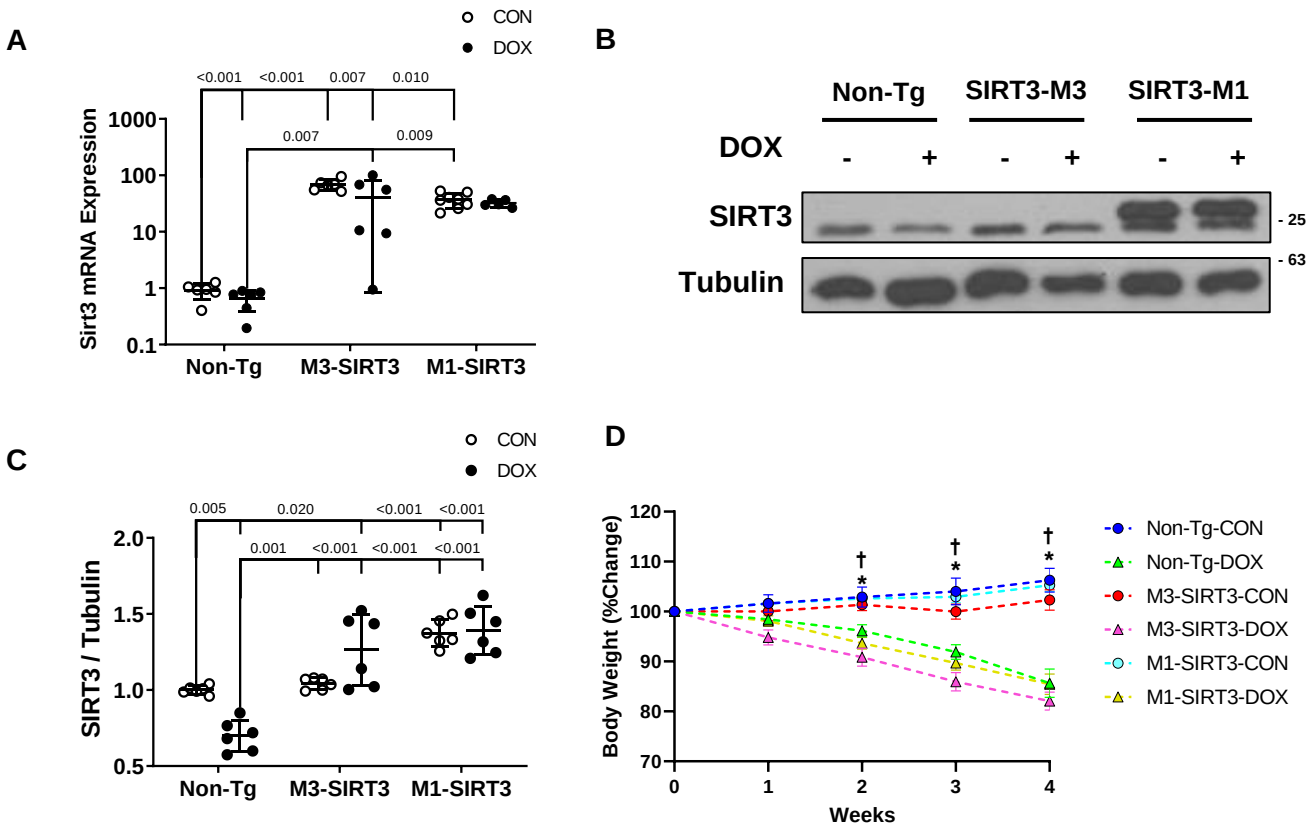


Figure S2. Cardiac and Muscle Restricted Expression of SIRT3 attenuates DOX Induced Decreases in SIRT3 Protein and Gene expression. **(A)** *Sirt3* mRNA expression in Non-Tg, M3-SIRT3 and M1-SIRT3 hearts. **(B)** Representative image of SIRT3 western blot using total cardiac protein lysates **(C)** Densitometry quantification of SIRT3 protein levels relative to tubulin. **(D)** Body weight change of animals over 4 weeks of DOX treatment represented as % change. *p<0.05 vs Non-Tg-CON,†p<0.05 vs Control Group.

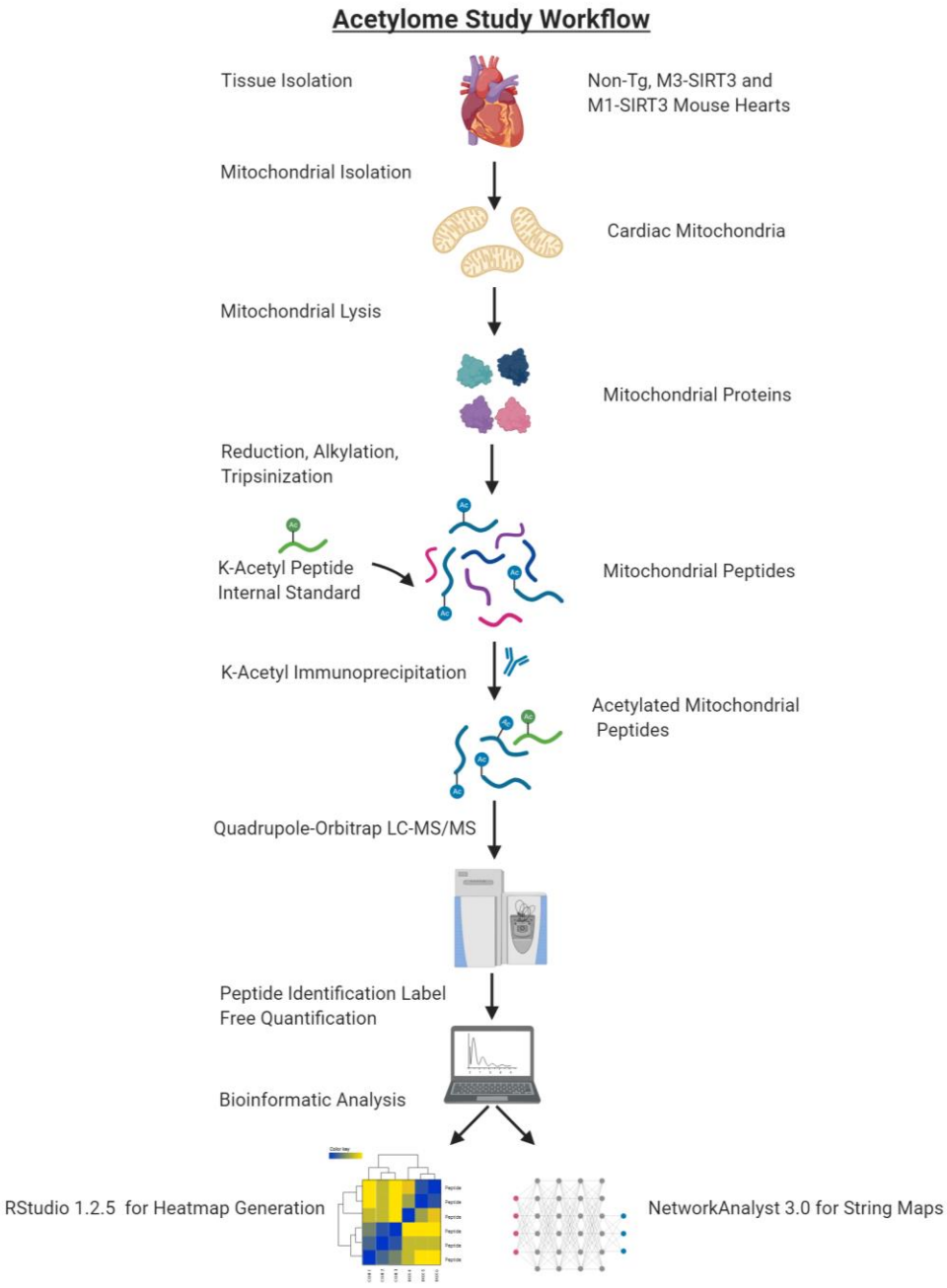


Figure S3. Cardiac Mitochondrial Peptide Mass Spectrometry Workflow. Workflow of *in vivo* mass spectrometry study. Tissue isolated from Non-Tg mice treated with saline or DOX. Mitochondrial isolation performed. Mitochondrial peptides underwent reduction, alkylation and trypsinization. Internal K-Acetyl peptide standard added to mitochondrial peptides. Immunoprecipitation performed using acetylated mitochondrial peptides. Quadrupole-Orbitrap High Performance Liquid Chromatography Mass Spectrometry (HPLC MS/MS) performed in collaboration with Manitoba Center of Proteomics and Systems Biology. Peptide identification and label free quantification. Downstream data analysis.

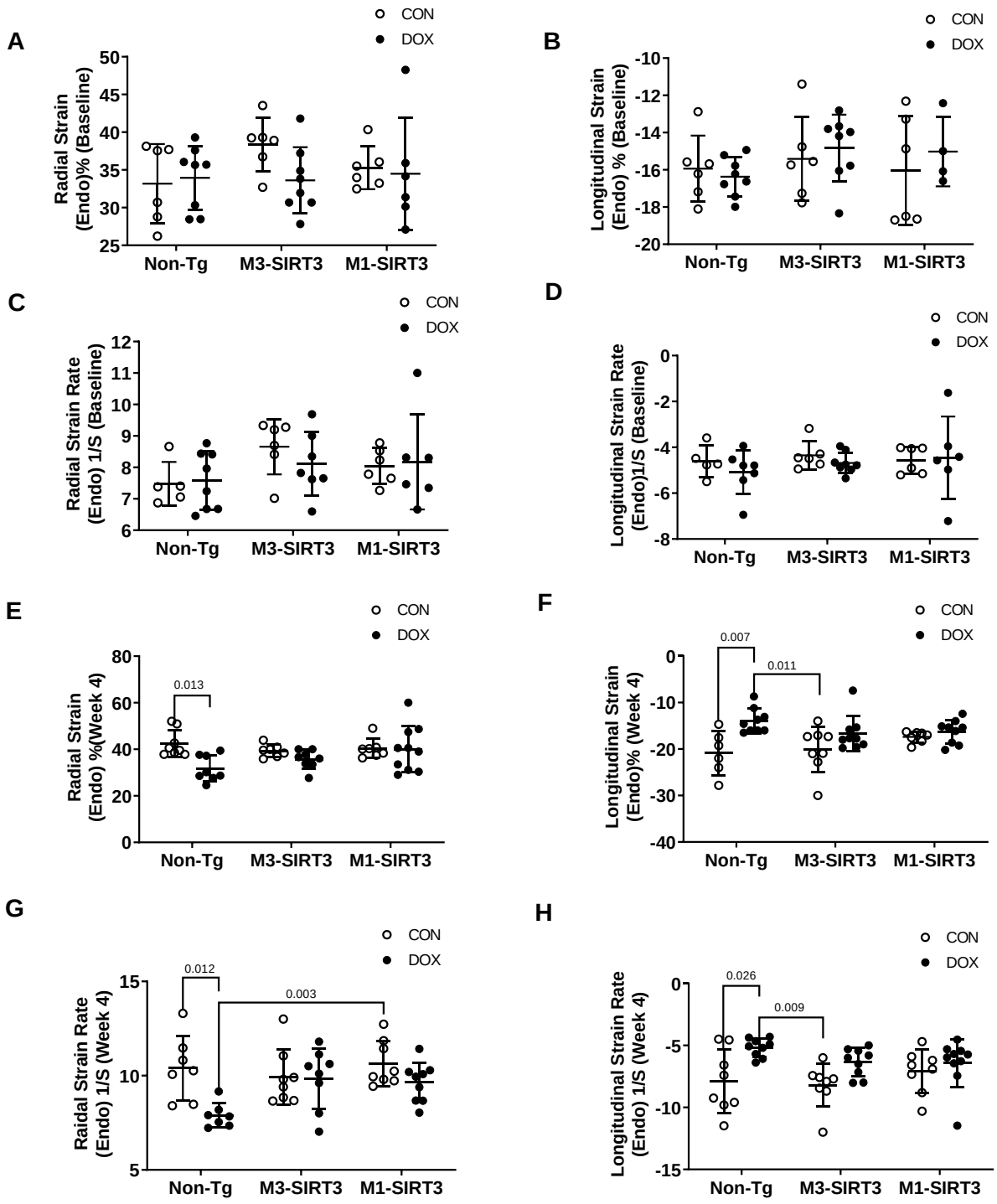


Figure S4. Baseline and Post-Treatment Measurements of Radial and Longitudinal Stain and Strain Rate. Baseline measurements of **(A)** radial strain **(B)** longitudinal strain. **(C)** radial strain rate **(D)** longitudinal strain rate. Post-treatment measurements of **(E)** radial strain **(F)** longitudinal strain. **(G)** radial strain rate **(H)** longitudinal strain rate.

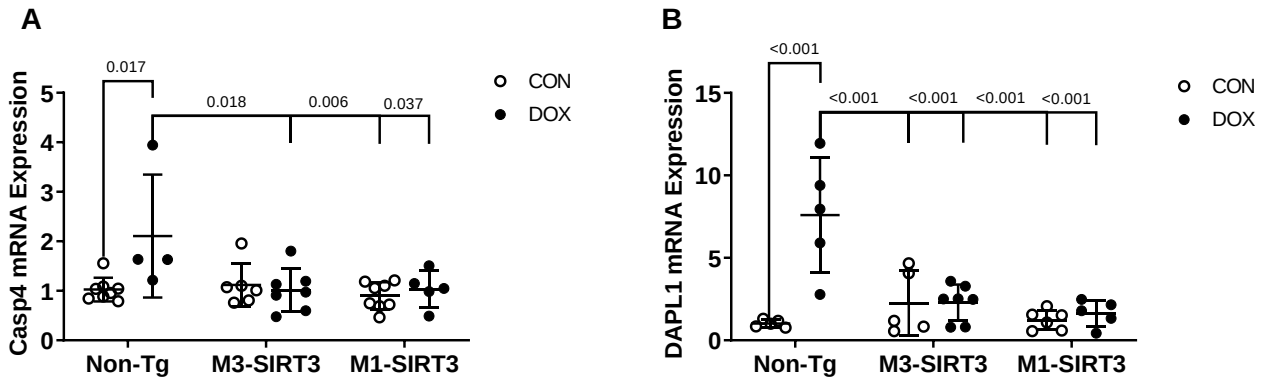


Figure S5. SIRT3 Expression Prevents Increases in the Expression of Genes Involved in Apoptosis**. (A)** *Casp4* mRNA expression in Non-Tg, M3-SIRT3 and M1-SIRT3 hearts. **(B)** *Dapl1* mRNA expression in Non-Tg, M3-SIRT3 and M1-SIRT3 hearts.

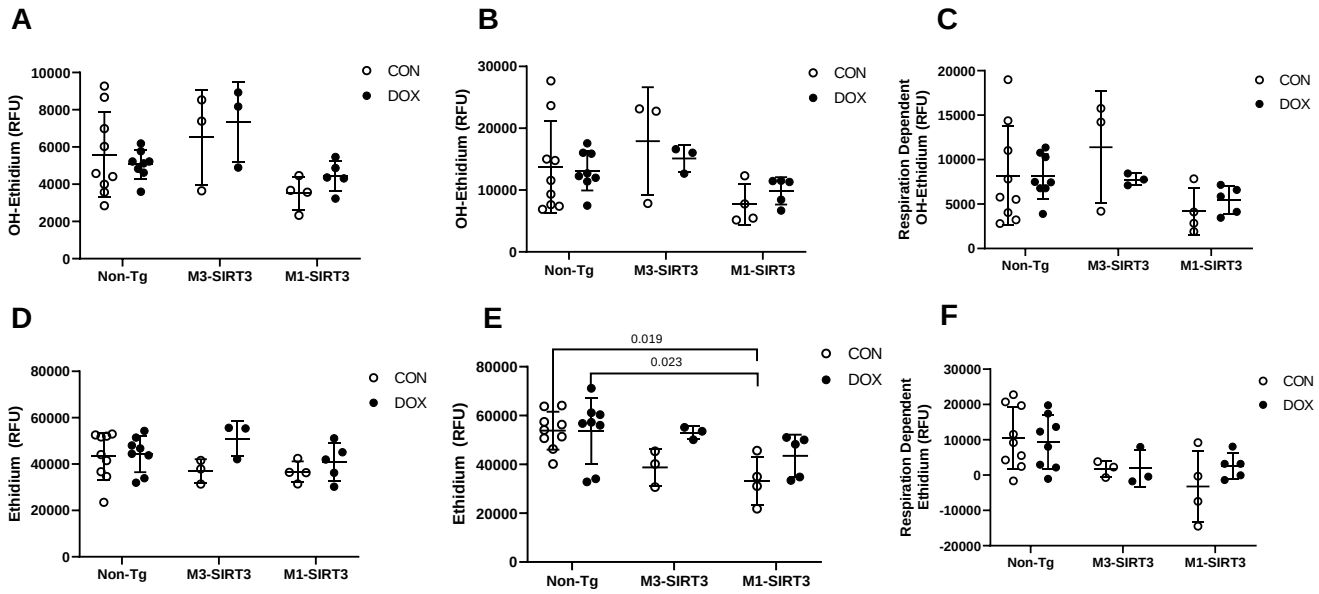


Figure S6. High performance liquid chromatography (HPLC) determination of hydroxyethidium (OH-Ethidium) and ethidium MitoSOXTM products in mitochondria isolated from Non-Tg, M3-SIRT3 and M1-SIRT3 mice. **(A)** OH-Ethidium peak fluorescence measured with no substrates. **(B)** OH-Ethidium peak fluorescence in the presence of succinate (5mM) and rotenone (4µM). **(C)** Calculated Respiration Dependent OH-Ethidium product. **(D)** Ethidium peak fluorescence measure with no substrates. **(E)** Ethidium peak fluorescence in the presence of succinate (5mM) and rotenone (4µM). **(F)** Calculated respiration dependent Ethidium product.

## III. Supplemental Tables

Table S1**.** Primer Sequences used for Quantitative Real-Time PCR

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| *Sirt3* | CCCAATGTCGCTCACTACTT | AGGGATACCAGATGCTCTCT |
| *Sod2* | GCGACCTACGTGAACAATCT | CTGAAGAGCAACCTGAGTTGTA |
| *Casp4* | TGGAAATGGAGGAACCAGAAG | TGGCCTCCTTTATTGGGTAAAT |
| *Dapl1* | CAGTGAAAGCTGGAGGGATG | CGACATTTGTGATGGCACTTG |
| *CypA* | TCCAAAGACAGCAGAAAACTTTCG | TCTTCTTGCTGGTCTTGCCATTCC |
| *Gapdh* | AACAGCAACTCCCACTCTTC | CCTGTTGCTGTAGCCGTATT |
| *Eif2a* | CCTGAAGTGTGATCCTGTGTTT | CCAAATCCAGCCAGCACTAATA |

Table S2. Pathway Analysis of Acetylated Mitochondrial Protein Interaction Network

| KEGG Pathway | P Value | FDR |
|---|---|---|
| Parkinson's disease | 1.27E-118 | 3.98E-116 |
| Oxidative phosphorylation | 2.99E-115 | 4.70E-113 |
| Metabolic pathways | 8.36E-111 | 8.75E-109 |
| Huntington's disease | 4.10E-97 | 3.22E-95 |
| Alzheimer's disease | 2.82E-87 | 1.77E-85 |
| Non-alcoholic fatty liver disease (NAFLD) | 1.18E-67 | 6.15E-66 |
| Carbon metabolism | 1.29E-53 | 5.78E-52 |
| Citrate cycle (TCA cycle) | 3.06E-42 | 1.20E-40 |
| Valine, leucine and isoleucine degradation | 9.31E-39 | 3.25E-37 |
| Pyruvate metabolism | 9.35E-36 | 2.94E-34 |
| Fatty acid degradation | 2.63E-32 | 7.50E-31 |
| Propanoate metabolism | 1.16E-31 | 3.03E-30 |
| Retrograde endocannabinoid signaling | 1.62E-31 | 3.92E-30 |
| Glycolysis / Gluconeogenesis | 6.76E-29 | 1.52E-27 |
| Cardiac muscle contraction | 1.03E-18 | 2.16E-17 |
| beta-Alanine metabolism | 1.92E-18 | 3.77E-17 |
| Fatty acid metabolism | 4.68E-17 | 8.65E-16 |
| Butanoate metabolism | 8.42E-17 | 1.47E-15 |
| Biosynthesis of amino acids | 2.44E-16 | 4.03E-15 |
| 2-Oxocarboxylic acid metabolism | 6.75E-13 | 1.06E-11 |
| Glyoxylate and dicarboxylate metabolism | 1.11E-12 | 1.66E-11 |
| Tryptophan metabolism | 3.06E-12 | 4.36E-11 |
| Lysine degradation | 8.36E-11 | 1.14E-09 |
| Histidine metabolism | 5.33E-10 | 6.97E-09 |
| Synthesis and degradation of ketone bodies | 5.09E-09 | 6.40E-08 |
| Arginine and proline metabolism | 1.39E-08 | 1.68E-07 |
| PPAR signaling pathway | 1.91E-08 | 2.22E-07 |
| Peroxisome | 1.22E-07 | 1.36E-06 |
| HIF-1 signaling pathway | 2.03E-06 | 2.20E-05 |
| Phenylalanine metabolism | 2.80E-06 | 2.93E-05 |
| Alanine, aspartate and glutamate metabolism | 1.11E-05 | 0.000112 |
| Amyotrophic lateral sclerosis (ALS) | 1.68E-05 | 0.000165 |
| Apoptosis - multiple species | 3.06E-05 | 0.000292 |
| Tyrosine metabolism | 0.000139 | 0.00128 |
| Glucagon signaling pathway | 0.000185 | 0.00166 |
| Biosynthesis of unsaturated fatty acids | 0.000285 | 0.00248 |
| Influenza A | 0.000314 | 0.00266 |
| Platinum drug resistance | 0.00039 | 0.00323 |
| Central carbon metabolism in cancer | 0.00052 | 0.00419 |
| Cysteine and methionine metabolism | 0.00058 | 0.00455 |
| Ascorbate and aldarate metabolism | 0.000987 | 0.00756 |

| | | |
|---|---|---|
| Fatty acid elongation | 0.00138 | 0.0103 |
| Legionellosis | 0.00143 | 0.0103 |
| Small cell lung cancer | 0.00144 | 0.0103 |
| Longevity regulating pathway - multiple species | 0.00212 | 0.0148 |
| Measles | 0.0033 | 0.0226 |
| Terpenoid backbone biosynthesis | 0.00411 | 0.0274 |
| p53 signaling pathway | 0.00459 | 0.03 |
| alpha-Linolenic acid metabolism | 0.0056 | 0.0359 |
| Apoptosis | 0.00653 | 0.041 |
| Endometrial cancer | 0.00674 | 0.0415 |
| Protein processing in endoplasmic reticulum | 0.00834 | 0.0503 |
| Primary bile acid biosynthesis | 0.0105 | 0.0622 |
| Non-small cell lung cancer | 0.0125 | 0.0726 |
| Toxoplasmosis | 0.0138 | 0.0786 |
| Colorectal cancer | 0.0144 | 0.081 |
| Necroptosis | 0.0149 | 0.0819 |
| Tuberculosis | 0.0154 | 0.0836 |
| Fatty acid biosynthesis | 0.017 | 0.0891 |
| Arginine biosynthesis | 0.017 | 0.0891 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 0.0213 | 0.11 |
| Pancreatic cancer | 0.0223 | 0.113 |
| Glycerolipid metabolism | 0.0326 | 0.163 |
| RNA degradation | 0.0345 | 0.169 |
| Metabolism of xenobiotics by cytochrome P450 | 0.0436 | 0.211 |
| Cellular senescence | 0.0467 | 0.222 |
| Longevity regulating pathway | 0.0482 | 0.224 |
| Drug metabolism - cytochrome P450 | 0.0486 | 0.224 |
| Hepatitis B | 0.05 | 0.228 |

Table S3. Acetylated Peptides in Cardiac Tissue Involved in Oxidative Stress Resistance

| Protein | Peptide | K-Acetyl Site | Fold Change | P Value |
|---|---|---|---|---|
| SOD2 | GELLEAI**k**R | K122 | -0.92 | 0.008* |
| SOD2 | DFGSFE**k**FK | K130 | 1.31 | 0.096 |
| SOD2 | HSLPDLPYDYGALEPHINAQIMQLHHS**k**HHAAYVNNLNATEEK | K53 | -0.75 | 0.151 |
| SOD2 | GGGEP**k**GELLEAIK | K114 | -0.42 | 0.310 |
| SOD2 | HHAAYVNNLNATEE**k**YHEALAK | K68 | -0.20 | 0.620 |
| SOD2 | RDFGSFE**k**FK | K89 | -0.05 | 0.944 |
| IDH2 | TDFD**k**NK | K280 | 4.11 | 0.034* |
| IDH2 | G**k**LDGNQDLIR | K384 | -0.82 | 0.039* |
| IDH2 | NTIL**k**AYDGR | K256 | -1.54 | 0.003* |
| IDH2 | VE**k**PVVEMDGDEMTR | K48 | -2.01 | 0.027* |
| IDH2 | FAQTLE**k**VCVQTVESGAMTK | K400 | -2.09 | 0.019* |
| IDH2 | HAHGDQY**k**ATDFVVDR | K180 | -0.62 | 0.121 |
| IDH2 | VCVQTVESGAMT**k**DLAGCIHGLSNVK | K413 | -1.17 | 0.219 |
| IDH2 | N**k**IWYEHR | K282 | -0.39 | 0.275 |
| IDH2 | LVFTP**k**DGSSAK | K199 | -0.40 | 0.277 |
| IDH2 | LILPHVDVQL**k**YFDLGLPNR | K80 | -2.35 | 0.285 |
| IDH2 | AGTF**k**LVFTPK | K193 | -0.41 | 0.308 |
| IDH2 | NILGGTVFREPIIC**k**NIPR | K155 | 0.99 | 0.321 |
| IDH2 | HY**k**TDFDKNK | K275 | 0.44 | 0.388 |
| IDH2 | HY**k**TDFDK | K275 | 0.38 | 0.469 |
| IDH2 | **k**WPLYLSTK | K243 | -0.30 | 0.584 |
| IDH2 | DQTNDQVTIDSALATQ**k**YSVAVK | K106 | 0.49 | 0.656 |
| IDH2 | EPIIC**k**NIPR | K155 | 0.01 | 0.977 |
| IDH2 | MW**k**SPNGTIR | K133 | 0.00 | 0.993 |
| IDH2 | LVPGWT**k**PITIGR | K166 | 0.00 | 0.999 |
| IDH3A | EVAENC**k**DIK | K223 | 0.87 | 0.094 |
| IDH3G | IAEYAF**k**LAQESGR | K206 | -0.43 | 0.580 |

K-Acetyl: Lysine Acetylation. Bolded lowercase **k** represents position of acetylation in peptide sequence. Location refers to amino acid position within total protein peptide sequence. Location identified using UniProt. Fold change calculated DOX-CON using normalized and scaled down log2 values. Statistics are unpaired student t-test. * P<0.05, Non-Tg-CON n=6 (males), Non-Tg-DOX n=5 (males).

## IV. Video Legends

Video 1. Echocardiography Video of Non-Tg-DOX Strain.

Video 2. Echocardiography Video of M3-SIRT3-DOX Strain.