INVENTORY OF SUPPLEMENTAL INFORMATION

- **SUPPLEMENTAL DATA** – Includes 2 Figures and 2 Tables

    - Figure S1, Related to Figure 1

    - Figure S2, Related to Figure 4

    - Table S1: Microsoft Excel file containing our analyzed quantitative acetylproteomic data from the high fat diet condition (for each file, the 1st tab contains a description and the subsequent tabs have increasing levels of data analysis), Related to Figure 2.

    - Table S2: Microsoft Excel file containing our analyzed qualitative acetylproteomic data from human samples (one tab only), Related to Figure 4.

- **SUPPLEMENTAL EXPERIMENTAL PROCEDURES –** Includes detailed explanations of experimental methods and the legends for Figures S1 and S2 and Tables S1 and S2. Related to the EXPERIMENTAL PROCEDURES.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mitochondrial Isolation

Quadriceps muscles were removed under nembutol anesthesia (100 mg/kg body weight) and immediately placed in ice cold KMEM buffer (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO4, 1 mM ATP, pH=7.4). Muscles were transferred to a 1.7 mL microcentrifuge tube containing 600 µL KMEM buffer on ice. Muscles were then crudely minced 200 times and pieces were transferred to a 5 ml polystyrene tube. The 1.7 mL microcentrifuge tube was washed with 600 µL KMEM buffer and transferred to the 5 ml polystyrene tube. An additional 1.2 mL of KMEM buffer was added to a final volume of 2.4 mL. Minced muscle tissue was then subjected to tissue tearing for 5 seconds. The muscle tissue was then transferred to a 10 mL round bottom glass homogenization tube. The polystyrene tube was washed with 600 µL of KMEM and transferred to the glass homogenization tube. An additional 1.8 mL of KMEM was added to the homogenization tube for a final volume of 4.8 mL. Samples were homogenized on ice using a Teflon pestle at ~800 rpm over 10 slow passes (~3 min), transferred to a 50 ml conical vial and 5 mL of KMEM was added. Homogenates were centrifuged (800 x g, 10 min, 4ºC) and the supernatant was transferred through 2 layers of gauze into a sterile 50 ml conical vial. This suspension was centrifuged (12,000 x g, 10 min, 4ºC) and the supernatant was discarded while the pellet was gently re-suspended in 1 mL KMEM and transferred to a sterile 1.7 mL microcentrifuge tube. The samples were centrifuged (7,000 x g, 10 min, 4ºC), the supernatant was decanted and the pellet was re-suspended in 1 mL KMEM buffer. The sample underwent one final centrifugation (3,500 x g, 10 min, 4 ºC), the supernatant was thoroughly aspirated and the pellet was snap frozen and prepared for subsequent testing.

Protein Expression

Mitochondrial lysates were prepared using cell lytic-M lysis buffer (Sigma-Aldrich) and protease inhibitors (Roche). Mitochondrial lysates required 3x freeze-fractures and sonication (5x-1 sec pulses). Protein concentrations were determined using the Pierce BCA protein assay (Thermo Fisher Scientific Inc.). Proteins were separated on Criterion gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Blots were blocked with 1% fish geletain and exposed to primary antibodies to detect: global acetylated lysine residues (Cell Signaling), CrAT (Abcam), and ETFα (Abcam). Proteins were visualized using FITC-conjugated secondary antibody with band intensity being quantified using LiCor imaging software.

Preparation of Crude Extracts and Tryptic Digests

Pulverized quadriceps tissue samples (100 mg tissue powder) were solubilized in 1.2 mL 8 M urea with 50 mM Ammonium Bicarbonate (AmBic), pH 8.0. Protein solubilization was performed using a three-step approach: First, a Tissue Tearor (Biospec Products, Inc) was used at high speed for approximately 10 seconds per sample. Next, the samples were taken through three freeze-thaw cycles utilizing freezing in -80ºC and thawing on ice. After the third thaw, samples were subjected to probe sonication at power level 3 for three five-second bursts, cooling on ice in between. Samples were centrifuged at 20,000 x g for 5 mins. Clear supernatant (0.85 mL) from each sample was diluted with 2.93 mL AmBic to dilute Urea to 1.8 M; each sample then was divided into three equal aliquots for digestion. Protein yield was determined using a mini-Bradford Assay (Bio-Rad), for each sample in duplicate. Prior to digestion, each aliquot was spiked with 10 ng (10 x 10^{-9} g) acetylated bovine serum albumin (Ac-BSA), a standard generated by Duke Proteomics Core by reacting BSA with NHS-acetate (Pierce), for a total of 30 ng acetylated-BSA total per sample. Cysteine residues were reduced with 10 mM dithiothreitol for 25 min at 32°C, alkylated at 20 mM iodoacetamide for 20 min at RT in the dark. TPCK trypsin (67 µg) was added to each eppendorf tube (25:1 substrate:enzyme ratio), and digestion was allowed to proceed at 32°C overnight. The final volume of each aliquot was 1,353 µL during digestion. Samples were then acidified to 0.5% v/v TFA, aliquots per sample combined, and desalted using 500 mg SEP-PAK C18 cartridges (Waters), per manufacturer's protocol. The peptide-containing eluate (2.25 mL of 50/49.9/0.1 v/v/v MeCN/H_2O/TFA) was diluted with 3 mL water, frozen at -80ºC, and lyophilized overnight.

Enrichment of Acetylpeptides

For each sample, the digested peptides (5 mg endogenous material supplemented with Ac-BSA internal standard) were resuspended in 1.4 mL IAP Buffer (Cell Signaling Technology) by vortexing and brief sonication in a water bath. Samples were transferred to an aliquot of Acetyl-K PTMScan enrichment beads (Cell Signaling Technology). IP was performed overnight at 4°C using end-over-end mixing. Ac-K enrichment beads were washed with a single aliquot of 0.25% NP40 in IAP buffer, one aliquot of IAP buffer, and two times with water. Elution was performed with two 50 µL aliquots of 0.15% TFA in water, for approximately 10 minutes. Eluates were combined and taken through a C18 STAGE tip desalting cleanup, and resulting peptides were once again dried via lyophilization. Samples were finally re-suspended in 12 µL of 1/2/97 v/v/v/TFA/MeCN/H_2O containing 10 fmol/µL ADH1_YEAST MassPrep Standard (Waters) and transferred to autosampler vials.

Quantitative LC/MS/MS Analysis

Quantitative LC/MS/MS was performed in duplicate for each sample (5 µL per injection), using a nanoAcquity UPLC system (Waters Corp) coupled to a LTQ-Orbitrap XL high resolution accurate mass tandem mass spectrometer (Thermo Fisher Scientific) *via* a nanoelectrospray ionization source. Samples were first trapped on a Symmetry C18 20 mm × 180 µm trapping column (5 µl/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.7 µm Acquity BEH130 C18 75 um × 250 mm column (Waters Corp.) using a 90-min gradient of 5 to 40% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55ºC. Data collection on the LTQ-Orbitrap XL was performed with MS1 (precursor) analysis in the Orbitrap mass analyzer at 60,000 resolution and AGC target of $1x10^6$ ions. Tandem mass spectra (MS/MS) were collected in a data-dependent manner on the top 5 most abundant precursor ions per scan, in the LTQ using 35% normalized collision energy and an AGC target of $5x10^4$ ions. Dynamic exclusion was enabled with a repeat count of 2 and 120 sec exclusion window.

Spectral Processing and Peptide Quantitation

Sample order of data collection was interwoven between conditions in order to minimize temporal bias (see **Supplemental Table 1**). Duplicate analyses for each sample were run together. Following analyses, data was imported into Rosetta Elucidator v3.3 (Rosetta Biosoftware, Inc), and all LC-MS/MS runs were aligned based on the accurate mass and retention time of detected ions ("features") using PeakTeller algorithm (Elucidator). The relative peptide abundance was calculated based on area-under-the-curve (AUC) of aligned features across all runs. The overall dataset had 77,124 quantified isotope (peptide) groups, and 95,050 MS/MS spectra were acquired for peptide sequencing by database searching. This MS/MS data was searched against a custom NCBI RefSeq database with *mus musculus* taxonomy (26682 forward entries created on 11/8/2012, www.ncbi.org), which also contained a reversed-sequence "decoy" database for false positive rate determination as well as several proteins used as surrogate standards (ADH1_YEAST, ALBU_BOVIN, PYGM_RABIT, CASA1_BOVIN, and ENO1_YEAST). Database searching was performed in an automated fashion from the Elucidator software package using Mascot search engine (v2.2), assigning precursor ion tolerance of 5 ppm and product ion tolerance of 0.8 Da. Searching allowed variable modification of N and Q (deamidation, +1 Da), M (oxidation, +16 Da), and K (acetylation, +42 Da). After aggregating all search results, annotation (assignment of peptide sequence to the quantitative

MS signal) was performed for peptides whose ion score was greater than 30. For the high fat diet cohorts, this resulted in a total of 4204 peptides mapping to 932 proteins, including 1111 peptides containing at least one acetylated K residue, at 1% peptide false discovery rate (45 decoy/reverse hits out of 4204 total). For quantitative processing, the data was first curated to contain only high quality peptides with appropriate chromatographic peak shape (1,087 acetylpeptide features in the high fat fed mice). Analyzed data for the high fat diet cohorts is included as **Supplementary Table 1**. Analyzed data for the human pilot study is included as **Supplementary Table 2**. All raw mass spectrometry files for the acetylproteomics work described herein are available online through Chorus (https://chorusproject.org, Public Project ID989) *via* the following links:

LF and HF diet mouse acetylproteomics (Public Experiment ID2047):

https://chorusproject.org/anonymous/download/experiment/22e926ba037a45d79abb3dd51e24d3df

Human pilot study acetylproteomics (Public Experiment ID2048):

https://chorusproject.org/anonymous/download/experiment/50216a62d2284df1a991eb2cc96fa1c3

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Mitochondrial protein immunoblot analysis in $CrAT^{fl/f}$ and $CrAT^{skm-/-}$ mice. Immunublot and quantification of (**a**) citrate synthase, (**b**) indicated oxidative phosphorylation complex subunits**,** and (**c**) SIRT3 proteins in skeletal muscle (quadriceps) from $CrAT^{fl/f}$ and $CrAT^{skm-/-}$ mice. In panels **a** and **b**, muscle lysates from MCK-Pgc1α transgenic mice (TG) and non-transgenic littermates (NT) were used as a positive control, along with lysates from isolated mitochondria. In panel **c**, lysates from 293 cells treated with SiRNA against Sirt3 (KD) and corresponding control (C) cells were used for a negative control and purified Sirt3 protein (P) was used as a positive control.

Figure S2. Submitochondrial localization of lysine acetylation. Venn Diagrams illustrating suborganelle distribution considering the mitochondrial proteome (**a,c**) and the acetylpeptides identified in the current study (**b,d**), for both mouse (**a-b**) and human (**c-d**) samples. Overlap in proteins (**a,c**) and acetylpeptides (**b,d**) localizing to the mitochondrial matrix (Matrix) and mitochondrial inner membrane space is illustrated. The white circle indicates the entire mitochondrion, black represents the matrix, and grey represents the IMS, with compartmental overlap depicted.

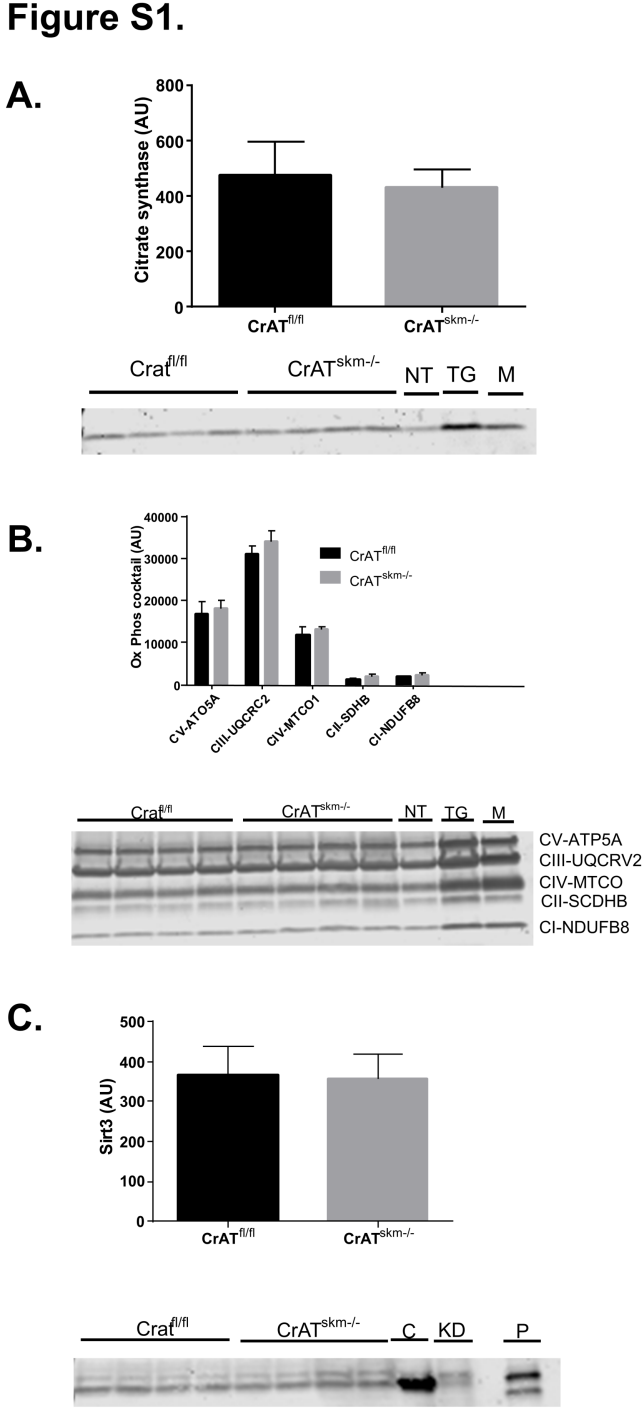
SUPPLEMENTAL TABLE LEGENDS

Table S1. Analyzed quantitative acetyl proteomic data comparing $CrAT^{fl/f}$ and $CrAT^{skm-/-}$ mice, all fed a high fat diet. Supplementary Excel file with label-free quantitative data on abundance levels for 1,087 acetyl peptides (*i.e.*, aligned peptide features containing at least one acetylated lysine residue) identified at 1% FDR, with internal standard peptides indicated. The first tab (Explanation of Table S1) defines abbreviations and indicates the order that the raw files were acquired for each sample. On the subsequent tabs the quantitative data is shown

with increasing levels of analysis, from raw quantitative values of peptides in each LC-MS/MS run (Raw Quantitation) to condition-centric statistics for each acetyl peptide along with the mitochondrial localization status of its target protein (Stats with Mito Localization). On the Stats with Mito Localization tab, a “1” in a black shaded cell indicates that a given acetyl peptide is on a protein that is localized to the mitochondrion (column G), the mitochondrial matrix (column H), or the mitochondrial intermembrane space (column I). Cell containing quantitative values (column J) for statistically significant changes ($P<0.05$, as indicated in column K) in acetyl peptide abundance with CrAT deficiency are highlighted in red (increasing) or blue (decreasing).

Table S2. Qualitative acetyl proteomic data in humans. Supplementary Excel file with pooled qualitative acetyl proteomic data from quadriceps biopsies from human subjects, all having a BMI over 29. The data shows 515 acetylpeptides at a 1% FDR, with internal standard peptides indicated. A “1” in a black shaded cell indicates that a given acetyl peptide is on a protein that is localized to the mitochondrion (column D), the mitochondrial matrix (column E), or the mitochondrial intermembrane space (column F).

Figure S1.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CIII-UQCRV2
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CI-NDUFB8
C.
Sirt3 (AU)
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CrAT skm-/-
Crat fl/fl
CrAT skm-/-
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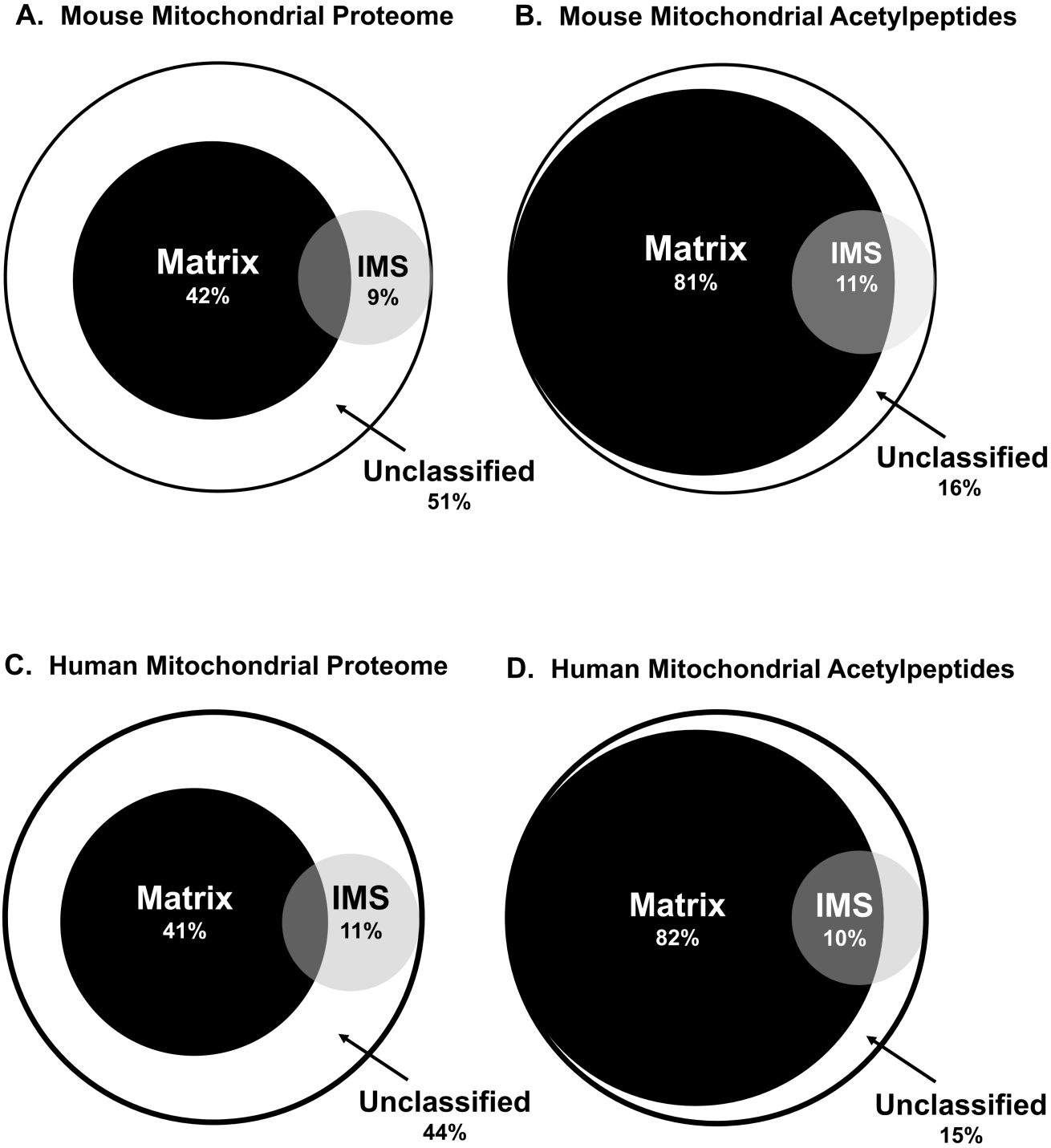
KD
P

Figure S2.


A. Mouse Mitochondrial Proteome
Matrix
42%
IMS
9%
Unclassified
51%
B. Mouse Mitochondrial Acetylpeptides
Matrix
81%
IMS
11%
Unclassified
16%
C. Human Mitochondrial Proteome
Matrix
41%
IMS
11%
Unclassified
44%
D. Human Mitochondrial Acetylpeptides
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