Appendix

Analysis of acetylation stoichiometry suggests that SIRT3 repairs nonenzymatic acetylation lesions

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Appendix Figure S1. Estimating the degree of partial chemical acetylation based on median reductions in CP abundance.

A Partial chemical acetylation by acetyl-phosphate (AcP) caused a significant reduction in median CP abundance in a concentration- and pH-dependent manner. The column graph shows the median CP ratios at the indicated concentration AcP and pH. The degree of chemical acetylation was predicted (predicted % acetylation) by subtracting the median CP ratio from 1. Significant differences between the CP ratio distribution and the internally-controlled comparison (peptides generated by cleavage at two arginine residues, see Materials and Methods) was determined by Wilcoxon test (N.S. = not significant).

B Table showing that the degree of acetylation predicted from the median CP ratio (predicted % acetylation, N.D. = not determined) is similar to the fraction of acetylated peptides identified in the mass spectrometer without affinity enrichment of acetylated peptides (% acetylated). This supports the idea that the median CP ratio can be used to predict the degree of acetylation, and further indicates that acetylated peptides are as readily detected as unmodified peptides.

C Number of identified acetylation sites (observed acetylation) is correlated to the degree of chemical acetylation as predicted from the median CP ratio (predicted % acetylation). The scatterplot
shows the number of acetylation sites identified by mass spectrometry without affinity enrichment of acetylated peptides compared to the degree of chemical acetylation predicted from the median CP ratio in panel A. Note that while the Spearman’s correlation is high (0.9) it is not significant (p = 0.08) due to the small number of observations.
Appendix Figure S2. Comparing estimated acetylation stoichiometry with SILAC and TMT quantification.

A  The scatterplot shows the correlation between estimated stoichiometry based on partial (5%) chemical acetylation using either SILAC or TMT quantification. Spearman’s correlation (Corr.), p-value (p) by two-tailed test, and number (n) of estimates analyzed are shown.

B  The scatterplot shows the correlation between estimated stoichiometry based on partial (5%) chemical acetylation using TMT quantification and comprehensive (100%) chemical acetylation using SILAC quantification. Spearman’s correlation (Corr.), p-value (p) by two-tailed test, and number (n) of estimates analyzed are shown.

C  The box plots show the distributions of stoichiometry estimates based on partial (5%) chemical acetylation. The median stoichiometry of acetylation is shown above each box plot and the number of sites analyzed is shown in parenthesis. Proteins were classified by association with the UniProt keywords “nuclear” (nuclear), “cytoplasmic” (cyto.), or “mitochondrion” (mito.). SIRT3-targeted sites were classified based on the degree of increased acetylation in SIRT3 KO liver tissue (Hebert et al, 2013); untargeted sites (S3 <2), and sites with >2-fold (S3 >2), >4-fold (S3 >4), or >8-fold (S3 >8) increased acetylation are shown.
Appendix Figure S3. Predicted acetylation stoichiometry in the wild-type mice used to compare acetylation under fed and fasted conditions, and to ATGL-deficient animals. The box plots show the predicted acetylation stoichiometry based on comparison to SILAC liver tissue as a common standard. Only sites for which differences in both protein and acetylation levels were quantified were used to predict acetylation stoichiometry. Proteins were classified by association with the UniProt keyword “mitochondrial” (mito.). SIRT3-targeted sites were classified based on the degree of increased acetylation in SIRT3 KO liver tissue (Hebert et al, 2013); untargeted sites (S3 <2), and sites with >2-fold (S3 >2), >4-fold (S3 >4), or >8-fold (S3 >8) are shown.
Appendix Figure S4. SIRT3 is similarly expressed in fed and fasted, wild-type and ATGL KO liver tissue. The same protein lysates used to measure acetylation changes in liver tissue were subjected to Western blot analysis using rabbit monoclonal SIRT3 antibody (Cell Signaling Technology #5490S) and mouse monoclonal β-actin antibody (Sigma-Aldrich A2228).
Appendix Figure S5. SIRT3-targeted sites are mostly unaffected by calorie restriction, fasting, and obesity in wild-type animals.

A The box plots show the distributions of acetylation site ratios comparing liver tissues of calorie restricted mice to ad libitum fed mice. The data was taken from (Hebert et al, 2013). Note that sites with >2-fold increased acetylation in SIRT3 knock out (KO) animals (SIRT3-targeted sites) are unaffected in calorie-restricted, wild-type (WT) animals.

B The box plots show the distributions of acetylation site ratios comparing liver tissues of either fasted or obese mice to re-fed or lean mice, respectively. The data was taken from (Still et al, 2013). Proteins were classified by association with the UniProt keyword “mitochondrial” (mito.). SIRT3-targeted sites were classified as follows, >2-fold increased acetylation in SIRT3 KO mice (S3>2) or >2-fold increase acetylation in calorie-restricted, SIRT3 KO mice (S3>2 CR) (Hebert et al, 2013), and >2-fold increase acetylation in fasted, SIRT3 KO mice (S3>2 fasted) (Dittenhafer-Reed et al, 2015).
Appendix Figure S6. Comprehensive acetylation affects peptide cleavage at arginine residues. The histograms show the distributions of *E. coli* peptides generated by cleavage at two arginine residues (arginine-flanked, AF) in control and 100% acetylated samples (100% Ac). The control sample had a median SILAC ratio of -0.17 (Log2 scale) and a standard deviation of 0.29, the 100% acetylated sample had a median SILAC ratio of -0.54 (Log2 scale) and a standard deviation of 0.76. These data indicate that 100% acetylation slightly reduced the efficiency of AF peptide cleavage globally, and resulted in greater variability of AF peptide cleavage. Note that *E. coli* protein was used for this experiment since we could not quantify arginine-containing peptides from SILAC liver tissue since only lysine residues were labeled.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Regulated Ac sites</th>
<th>Estimated stoichiometry</th>
<th>Number of sites analyzed and range of stoichiometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoyl–coenzyme A hydratase (EHHADH)[Zhao et al, 2010]</td>
<td>K165, K171, K346, K584</td>
<td>K163* (0.07%) K172* (0.11%) K344* (0.35%) K579* (0.30%) homologous residues</td>
<td>18 sites (0.02% to 0.84% acetylated)</td>
</tr>
<tr>
<td>Malate dehydrogenase (MDH2)[Hebert et al, 2013; Zhao et al, 2010]</td>
<td>K185, K239, K301, K307, K314</td>
<td>K185 (0.25%) K301 (0.19%) K307 (0.42%) K314 (0.36%)</td>
<td>12 sites (0.05% to 0.76% acetylated)</td>
</tr>
<tr>
<td>Argininosuccinate lyase (ASL)[Zhao et al, 2010]</td>
<td>K288</td>
<td>K288 (0.01%)</td>
<td>11 sites (0.01% to 0.39% acetylated)</td>
</tr>
<tr>
<td>3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGC2)[Shimazu et al, 2010]</td>
<td>K310, K447, K473</td>
<td>K310 (0.70%) K447 (0.04%) K473 (0.05%)</td>
<td>18 sites (0.04% to 0.70% acetylated)</td>
</tr>
<tr>
<td>Glutamate dehydrogenase (GDH/GLUD1)[Schlicker et al, 2008]</td>
<td>Regulated sites not identified</td>
<td>18 sites (0.01% - 1.6%)</td>
<td>18 sites (0.02% - 1.6% acetylated)</td>
</tr>
<tr>
<td>Ornithine transcarbamylase (OTC)[Hallows et al, 2011; Yu et al, 2009]</td>
<td>K88 and not identified</td>
<td>K88 (0.11%)</td>
<td>10 sites (0.01% - 0.40% acetylated)</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase (PPIF)[Hafner et al, 2010; Shulga et al, 2010]</td>
<td>K145, K166</td>
<td>K166 (0.06%)</td>
<td>5 sites (0.02% - 0.39% acetylated)</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (IDH2)[Schlicker et al, 2008; Someya et al, 2010; Yu et al, 2012]</td>
<td>K242/243, K413, and not identified</td>
<td>N.D.</td>
<td>19 sites (0.02% - 2.0% acetylated)</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD2)[Chen et al, 2011; Qiu et al, 2010; Tao et al, 2010]</td>
<td>K53, K68, K89, K122</td>
<td>K68 (0.86%) K89 (0.02%) K122 (0.30%)</td>
<td>8 sites (0.02% - 0.86% acetylated)</td>
</tr>
<tr>
<td>Electron transport chain complex I subunit (NDUFA9)[Ahn et al, 2008]</td>
<td>Regulated sites not identified</td>
<td>4 sites (0.01% - 0.22%)</td>
<td>4 sites (0.02% - 0.22% acetylated)</td>
</tr>
<tr>
<td>Succinate dehydrogenase (SDHA)[Cimen et al, 2010; Finley et al, 2011]</td>
<td>Regulated sites not identified</td>
<td>17 sites (0.03% - 0.34%)</td>
<td>17 sites (0.03% - 0.34% acetylated)</td>
</tr>
<tr>
<td>acetyl-coenzyme A acetyltransferase 1 (ACAT1)[Still et al, 2013]</td>
<td>K260, K265</td>
<td>K260 (0.01%)</td>
<td>14 sites (0.01% - 1.19% acetylated)</td>
</tr>
<tr>
<td>Long-chain acyl-CoA dehydrogenase (ACADL)[Bharathi et al, 2013]</td>
<td>K318, K322</td>
<td>K322 (0.23%)</td>
<td>9 sites (0.01% - 1.24% acetylated)</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase B (ALDOB)[Lundby et al, 2012]</td>
<td>K147</td>
<td>K147 (0.02%)</td>
<td>8 sites (0.02% - 0.09% acetylated)</td>
</tr>
</tbody>
</table>

**Appendix Table S1. Estimated acetylation stoichiometry at previously characterized sites.** The table shows proteins that were previously suggested to be regulated by acetylation, the acetylation sites implicated in their regulation (Regulated Ac sites), the estimated stoichiometry at these positions (SILAC quantified sites only), and the total number of sites analyzed and range of stoichiometry. Stoichiometry was estimated from the liver tissue of four month old, ad libetum fed mice.
Dataset EV1
SILAC liver tissue (Lys<sup>6</sup>) was used as an internal standard. AcP-treated liver protein from 4 month old animals was unlabeled (Lys<sup>0</sup>) and was mixed with SILAC labeled peptides in the following ratios; 0mM AcP (1:1), 1mM AcP (1:2), 10mM AcP (1:5), 100mM AcP (1:25). AcP-treated peptides were diluted in order to ensure quantitative accuracy based on preliminary experiments.

Dataset EV2
AcP-treated liver protein from 4 month old animals was labeled using Isobaric mass tags; 0mM AcP (TMT 128), 1mM AcP (TMT 129), 10mM AcP (TMT 130), 100mM AcP (TMT 131).

Dataset EV3
Native peptide intensity was compared to heavy-isotope-labeled AQUA QuantPro heavy isotope labeled peptide standards (Thermo Fisher Scientific). For further details see Materials and Methods.

Dataset EV4
SILAC liver tissue (Lys<sup>6</sup>) was 100% acetylated and mixed with unlabeled (Lys<sup>0</sup>) liver protein from 4 month old animals at 1/100 (1%), 1/1000 (0.1%), and 1/10000 (0.01%) stoichiometry.

Dataset EV5
SILAC liver tissue (Lys<sup>6</sup>) was used as an internal standard and mixed with equal parts liver peptides from WT-fed, WT-fasted, ATGL-fed, ATGL-fasted animals.

Dataset EV6
Liver, heart, brain, and brown adipose tissue was labeled using Isobaric mass tags, WT-fed (TMT 128), WT-fasted (TMT 129), ATGL-fed (TMT 130), ATGL-fasted (TMT 131)

Dataset EV7
Tissues were unlabeled (Arg<sup>0</sup>, Lys<sup>0</sup>), MEFs were SILAC labeled (Arg<sup>10</sup>, Lys<sup>6</sup>)

Appendix Table S2. Summary of quantitative mass spectrometry approaches used in different experiments. The table shows the approach used to quantify acetylation for the indicated datasets.
Appendix References


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Hafner AV, Dai J, Gomes AP, Xiao CY, Palmeira CM, Rosenzweig A, Sinclair DA (2010) Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy. Aging 2: 914-923


