Acetyl-CoA flux regulates the proteome and acetyl-proteome to maintain intracellular metabolic crosstalk

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AT-1/SLC33A1 is a key member of the endoplasmic reticulum (ER) acetylation machinery, transporting acetyl-CoA from the cytosol into the ER lumen where acetyl-CoA serves as the acetyl-group donor for Nε-lysine acetylation. Dysfunctional ER acetylation, as caused by heterozygous or homozygous mutations as well as gene duplication events of AT-1/SLC33A1, has been linked to both developmental and degenerative diseases. Here, we investigate two models of AT-1 dysregulation and altered acetyl-CoA flux: AT-1S¹¹³R/+ mice, a model of AT-1 haploinsufficiency, and AT-1 sTg mice, a model of AT-1 overexpression. The animals display distinct metabolic adaptation across intracellular compartments, including reprogramming of lipid metabolism and mitochondria bioenergetics. Mechanistically, the perturbations to AT-1-dependent acetyl-CoA flux result in global and specific changes in both the proteome and the acetyl-proteome (protein acetylation). Collectively, our results suggest that AT-1 acts as an important metabolic regulator that maintains acetyl-CoA homeostasis by promoting functional crosstalk between different intracellular organelles.
The acetyl-CoA transporter, AT-1 (also referred to as SLC33A1), is a key member of the endoplasmic reticulum (ER) acetylation machinery, transporting acetyl-CoA from the cytosol into the ER lumen where acetyl-CoA serves as the component of an intracellular communication network that promotes functional crosstalk between different cellular compartments and organelles to maintain acetyl-CoA homeostasis.

Results

Aberrant AT-1 activity alters lipid metabolism. AT-1S113R/+ mice are haploinsufficient for AT-1 activity (Fig. 1a), resulting in a ~50% decrease in acetyl-CoA transport from the cytosol into the ER lumen8. Histological assessment revealed that AT-1S113R/+ mice have increased propensity to liver steatosis, which could be documented by both hematoxylin and eosin (H&E) (Fig. 1b) and electron microscopy (Fig. 1c). Although observed with both regular (40.2% carbohydrate, 4.7% fat) and breeder (44.7% carbohydrate, 9% fat) diets, the steatosis was much more common in animals fed the breeder diet. Importantly, the animals displayed no differences in food intake or body weight (Fig. 1d, e). Biochemical analysis revealed a ~60% increase in free acetyl-CoA available within the cytosol and a marked accumulation of fatty acids and triglycerides in the liver (Table 1). Finally, the serum lipid profiles were not different between wild-type (WT) and AT-1S113R/+ mice (Table 2), indicating that the steatosis was not associated with dyslipidemia.

Contrary to AT-1S113R/+ AT-1 sTg mice that systemically overexpress AT-1 had increased transport of acetyl-CoA from the cytosol to the ER lumen (Fig. 1f)10. If indeed the propensity of AT-1S113R/+ to develop steatosis is caused by increased acetyl-CoA availability in the cytosol, then AT-1 sTg mice should be resistant, even when challenged with a lipogenic high-carbohydrate diet (HCD). To test this, both WT and AT-1 sTg animals were fed a HCD (70% carbohydrates, 5.2% fat) for 4 weeks15,16. As expected, WT mice on the HCD showed diffuse steatosis and lipid accumulation in the liver sections (Fig. 1g, h); however, AT-1 sTg on the HCD showed normal parenchyma and no histological evidence of lipid accumulation (Fig. 1g, h). We previously reported that AT-1 sTg eat more than their WT littermates10, and although similar behavior was observed over the 4 weeks of HCD feeding (Fig. 1i), there was no increase in body weight (Fig. 1j), suggesting that the changes in lipid metabolism were not due to altered food intake. When on a regular diet, AT-1 sTg mice showed reduced levels of cytosolic acetyl-CoA, fatty acids, and triglycerides as compared with WT littermates (Table 3). On the HCD, WT mice displayed a marked increase in fatty acids and triglycerides, whereas AT-1 sTg remained overall resistant to the lipogenic challenge (Table 3). Although there was a modest increase in triglycerides, the levels remained well below those of their WT littermates (Table 3). Furthermore, WT mice responded to the HCD by increasing serum triglyceride levels, while AT-1 sTg mice did not (Table 4).

An increase in oxidation of fatty acids is one possible explanation for the hepatic lipid-storage-resistance phenotype of AT-1 sTg mice. Consistent with this, fatty acid oxidative capacity was greater in primary hepatocytes from AT-1 sTg mice compared with WT controls (Fig. 1k). When taken together, the results obtained with AT-1S113R/+ and AT-1 sTg mice indicate that changes in AT-1 activity and acetyl-CoA flux from the cytosol to the ER lumen can cause important changes in the availability of acetyl-CoA within the cytosol, impacting lipid metabolism and propensity to hepatic steatosis.

Aberrant AT-1 activity affects the proteome. To dissect the mechanism(s) underlying the above metabolic adaptation, we used quantitative mass spectrometry coupled with liquid chromatography and investigated possible proteome changes within the liver of AT-1 sTg and AT-1S113R/+ animals17,18. We found 2056 and 373 expression levels of proteins that were significantly (P < 0.05, Fisher’s method) altered in AT-1 sTg and AT-1S113R/+ mice, respectively (Fig. 2a, see also Supplementary Data 1). The cumulative difference in the distribution indicated that there are more downregulated proteins in AT-1S113R/+ (mean FC = −0.122) than in AT-1 sTg (mean FC = −0.060) (Fig. 2b). Collectively, 61 proteins were affected in both models of AT-1 dysregulation; of them, 22 are associated with the secretory pathway, 8 with the mitochondria, 7 with the nucleus, and 24 with the cytosol (Fig. 2c, d). Comparing the proteomic response in the two mouse models, opposing changes in abundance were detected for 14 of these proteins, while the remaining 47 changed in the same direction in both models (Fig. 2d).

To assess the functional consequences of these protein expression changes, we conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the Over-Representation Analysis method. Enriched pathways passed a Benjamini–Hochberg test with a FDR < 0.05. Twenty-nine pathways were enriched in the AT-1 sTg, and 14 pathways were enriched in AT-1S113R/+ of which 6 were overlapping (Fig. 2e). These KEGG pathways were then categorized into broad key pathways (Fig. 2f). In addition to secretory pathway and mitochondrial processes anticipated based on prior work in the neuron-specific mouse model of AT-1 overexpression9, here, other pathways were also identified. Pathways responsive to changes in AT-1 included lipid metabolism, glucose metabolism, and amino acid metabolism. KEGG pathways enriched in both models of AT-1 dysregulation included carbon metabolism, fatty acid degradation, fatty acid metabolism, butanoate metabolism, PPAR signaling, and BCAA degradation. Despite both models perturbing the activity of AT-1, we observed unique protein expression consequences in each model. Specifically, AT-1S113R/+ displayed overall more downregulated proteins, and distinct enrichment in many mitochondrial pathways and lipid metabolism pathways. In contrast, AT-1 sTg displayed a wider distribution of protein expression and a wider array of enriched pathways: transcription, translation, secretory pathway, lipid...
metabolism, glucose metabolism, mitochondria adaptation, and amino acid metabolism, indicating that the overexpression of AT-1 causes highly global protein expression changes. These data show that AT-1 responsive proteins and pathways extend to metabolic pathways not previously known to be affected by changes in the cytosol-to-ER acetyl-CoA flux.

**Table 1** Hepatic lipid content in WT and AT-1S113R/+

|                  | WT                          | AT-1S113R/+                |
|------------------|-----------------------------|----------------------------|
| Cytosolic acetyl-CoA (nmol/g tissue) | 2.42 ± 0.97                 | 3.93 ± 1.65                |
| Fatty acids (µmol/g tissue)          | 18.80 ± 7.69                | 36.70 ± 5.21*              |
| Triglycerides (µmol/g tissue)        | 17.65 ± 4.60                | 67.01 ± 22.00*             |
| Total cholesterol (µmol/g tissue)    | 4.21 ± 0.87                 | 5.44 ± 1.42                |

Hepatic lipid content of WT (n = 5) and AT-1S113R/+ (n = 5) mice fed a standard diet. One-tailed Student’s t test. *P < 0.05; **P < 0.005; †P < 0.0005. All data are represented as mean ± s.d. Circles indicate individual animals.
Aberrant AT-1 activity affects the acetyl-proteome. Acetyl-CoA-dependent processes not only include fatty acid metabolism and the citric acid cycle but also Nε-lysine acetylation, which can occur in the nucleus and cytoplasm, mitochondria, and the peroxisome. To assess the ability of AT-1 and acetyl-CoA availability to modulate protein function through lysine acetylation, we used a mass spectrometry method that elucidates the stoichiometry of site-specific lysine acetylation within proteomes.

We quantified the steady-state acetylation stoichiometry on lysine sites at the proteome level, which ranged from < 1 to 99%. In the AT-1 sTg and AT-1S113R/+ liver, 3341 and 3003 lysine sites were identified, respectively. When compared with WT, 375 were significantly changing in the AT-1 sTg, and 415 were significantly changing in the AT-1S113R/+ (Fig. 3a; see also Supplementary Data 2). The distribution of stoichiometry changes (Fig. 3b) as well as the average number of lysine sites per protein identified (Fig. 3c) were significantly different between the two models (see also later). These differences can possibly be explained by changes in the availability of cytosolic acetyl-CoA as a result of less (in the AT-1S113R/+ mice) or more (in the AT-1 sTg mice) AT-1-mediated transport into the ER lumen.

A protein with multiple lysine sites can display varying stoichiometry on each site, leading to multiple acetylated protein forms capable of influencing protein stability and activity. Comparison of the AT-1 models revealed a 12% (88 sites) overlap in significantly changing acetylation sites, and 21% (119 proteins) overlap in modified proteins (Fig. 3d). To assess the functional implications of these posttranslational modifications, we conducted the KEGG pathway analysis of the acetyl-proteome.

We found a compelling 57% overlap in enriched pathways in both models of AT-1 dysregulation, which include lipid metabolism, amino acid metabolism, mitochondria, and secretory pathway and proteostasis (Fig. 3e).

The 88 acetylation sites that were detected in both models are localized as follows: 26 in the secretory pathway, 18 in the mitochondria, 33 in the cytosol, and 11 in the nucleus. Strikingly, 85 of the acetylation sites had increased acetylation stoichiometry in one mouse model and decreased acetylation stoichiometry in the other model, whereas only 3 acetylation sites had stoichiometry changes in the same direction in both models compared with WT (Fig. 3f). With the exception of eight acetylated sites, all of the lysine sites in the AT-1S113R/+ showed hyperacetylation, whereas the lysine site in the AT-1 sTg showed hypoacetylation. Lastly, we evaluated the subcellular location of all acetylated proteins significantly different from WT; the general trends again indicate hypoacetylation in AT-1 sTg and hyperacetylation in AT-1S113R/+ (Fig. 3g). When broken down by compartment, this distribution was again maintained across the secretory pathway, mitochondria, nucleus and cytosol (Fig. 3h). This differential response likely reflects changes in substrate availability. Indeed, the increased transport of cytosolic acetyl-CoA into the ER lumen in AT-1 sTg mice reduced acetyl-CoA availability in the cytosol, whereas the reduced transport to the ER in AT-1S113R/+ mice had the opposite effect. These results strongly suggest that these coordinated posttranslational acetyl modifications are intimately linked to the levels of cytosolic acetyl-CoA, and that AT-1 activity is a major driver of protein acetylation in these mouse models.

AT-1 controls cell metabolism and mitochondria bioenergetics. Comparison of the proteomic and acetyl-stoichiometric data indicate that the metabolic reprogramming induced by changes in AT-1 activity and acetyl-CoA availability is quite complex with proteins being regulated either at the level of the proteome or acetylyome, or at both levels (Fig. 4a). Comparison of the two data sets demonstrated that there are 105 and 35 acetylation sites in the AT-1 sTg and AT-1S113R/+ models, respectively, that are significantly changed at both the protein and posttranslational (acytelylation) level as compared with WT.

To further understand how these different modes of regulation can integrate into specific metabolic responses, we...
focused on the two metabolic clusters which are immediately relevant to the liver phenotype (see Fig. 1) and were heavily highlighted in the pathway analysis—lipid and mitochondrial metabolism. Proteins that were identified in the enriched KEGG pathways from either model at the level of the proteome and acetyl-proteome were included in the cluster analysis. Factors regulated at the level of the proteome and/or acetylome were represented within each cluster (Fig. 4b, c; see also Supplementary Tables 1, 2). Again, these results reveal the far-reaching metabolic impact of changes in AT-1 activity and consequent acetyl-CoA availability.

An important and unexpected finding of this study is the fact that the mitochondria dynamically adapt to changes in acetyl-CoA transport from the cytosol to the ER lumen imparted by the activity of the ER membrane acetyl-CoA transporter, AT-1. Also unexpected is the fact that the mitochondria adaptation can involve changes in stoichiometry of lysine acetylation that was expected to be buffered from acetyl-CoA levels in the cytosol. In line with this finding, we investigated the functional significance of the mitochondria adaptive response.

Direct visualization of the mitochondria by structure-illumination microscopy (SIM) in primary cultured hepatocytes
revealed significant expansion of the mitochondria network in AT-1 sTg mice (Fig. 4d), with significant increases detected in both mitochondrial area and volume (Fig. 4e). Mitochondrial remodeling was associated with a significant upregulation of the mRNA levels of the transcriptional co-activator peroxisome proliferator-activated receptor-y (PPAR-y) co-activator 1a (PGC-1a) (Fig. 4f), which is an important regulator of mitochondrial biogenesis and mitochondrial function.23,24 These results are in line with the expansion of PPAR signaling observed at the level of the proteome (see Fig. 2) and discussed later. Next, we used U-13C glutamine isotope tracing to measure metabolites in primary hepatocytes and determine the functional significance of the TCA/citric acid cycle adaptation identified in the proteome and acetyl stoichiometry data. The results revealed increased levels of 13C-labeled alpha-ketoglutarate and fumarate, increased total levels of alpha-ketoglutarate, and a trend (P = 0.055, Student’s t test) toward increased malate levels in AT-1S113R/− mice (Fig. 4g; see also Supplementary Data 3 and Supplementary Table 3). No significant differences were observed in TCA/citric acid metabolites in AT-1 sTg animals.

Together, data shown here suggest that AT-1 sTg and AT-1S113R/− mice are adapting in a different capacity to AT-1 activity and acetyl-CoA availability. In AT-1 sTg mice, we detected proteomic enrichment in PPAR signaling and acetylated protein enrichment in the TCA cycle. These changes were reflected functionally in an overall expansion in the mitochondria, but without a change in overall levels of TCA metabolites. In contrast, in AT-1S113R/− mice, we detected an increase in engagement of the TCA/citric acid cycle, which was reflected in the proteomic pathway enrichment of PPAR signaling, TCA cycle, and oxidative phosphorylation and acetyl proteoform enrichment in PPAR signaling and the TCA cycle.

The increased fatty acid oxidation capacity observed in AT-1 sTg mice (see Fig. 1k) is also reflected in the expansion of the mitochondria network (Fig. 4d) and the adaptive response of the carnitine-acylcarbina translocase machinery (Slc25a20, Cpt1a and Cpt2) and the hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase trifunctional enzyme (Hadhb, Hadha, and Hadhb). In fact, we found Hadhb, Cpt2, and Cpt1a to be modified at the proteome level, Hadha and Hadhb at the acetylome level, and Slc25a20 both at the proteome and acetylome level (Fig. 4b, c; see also Supplementary Tables 1, 2). Together, these adaptive changes at the proteome and acetylome level integrate to promote the oxidation of fatty acids within the mitochondria.

Discussion

Cell-based and mouse-based studies support the conclusion that AT-1 activity regulates ER proteostasis by maintaining the balance between quality control and the induction of reticulophagy. However, changes in intracellular acetyl-CoA flux caused by hypoactive or hyperactive AT-1 could conceivably influence cellular events beyond those already established; in particular the metabolic effects of these genetic manipulations have not been defined.

In this study, we used two models of AT-1 dysregulation and altered acetyl-CoA flux: AT-1S113R/− mice, a model of AT-1 haploinsufficiency, and AT-1 sTg mice, a model of AT-1 overexpression. We discovered that AT-1 activity has pleiotropic effects that go beyond quality control and the induction of reticulophagy. Specifically, the animals display distinct metabolic adaptation across intracellular compartments, including reprogramming of lipid metabolism and mitochondria bioenergetics. Phenotypically, this adaptive response in the AT-1S113R/− model leads to spontaneous steatosis and increased engagement of the TCA cycle. In contrast, in the AT-1 sTg model, it leads to resistance to diet-induced steatosis and increased engagement of the mitochondria network. Mechanistically, this functional adaptation is achieved by global reprogramming of several biological pathways caused by specific changes in both the proteome and the acetyl-proteome (protein acetylation). Importantly, the proteome and acetyl-proteome of AT-1 mouse models support these cellular phenotypes—also showing dramatic changes across lipid metabolism and mitochondria-related pathways. Therefore, AT-1 has emerged as a key regulator in intracellular acetyl-CoA homeostasis, which has far reaching consequences within the cell’s metabolism.

To maintain homeostasis, the cell must ensure crosstalk between different organelles and compartments. It seems likely that key cellular metabolites reflect the immediate activity of metabolic enzymes as well as the functional metabolic state of intracellular organelles. In this way, signaling molecules and/or availability respond to extracellular and/or intracellular changes and allow for implementation of the appropriate adaptive response. Our results indicate that changes in acetyl-CoA flux from the cytosol to the ER lumen, as caused by reduced or increased AT-1 activity, cause significant metabolic adaptation. These results suggest that the cytosol-to-ER flux of acetyl-CoA is a branch of the more general nutrient-signaling-pathway that enables rapid modulation and reprogramming of different intracellular activities upon fluctuation of metabolites/nutrients.

Mechanistically, AT-1 responsive proteins are involved in the secretory pathway, mitochondria, lipid, glucose, and amino acid metabolism. The AT-1 response includes changes in acetyl proteoforms in a manner that appears to be highly coordinated and occurs within different cellular organelles and compartments. These data suggest that acetyl-CoA not only serves as a cytosolic sensor but that the status of cytosol-to-ER acetyl-CoA flux is an essential component of an intracellular communication network.
Fig. 3 AT-1 sTg and AT-1S113R/+ mice show changes in stoichiometry of lysine acetylation across many metabolic pathways. a Volcano plot of percent change in lysine acetylation sites detected in AT-1 sTg (n = 4; left) and AT-1S113R/+ (n = 4; right) mice, compared with age-matched WT littermates. Statistically significant sites (375 in AT-1 sTg; 415 in AT-1S113R/) are highlighted in yellow, and all other detected sites are designated in blue. One-way ANOVA, P < 0.05. b Percent change of lysine site stoichiometry of all detected lysine sites in both models of AT-1 dysregulation. The data represented as box and whisker plots: box represents 25th to 75th inner quartile range; middle line denoting the median, and the inner square denoting the mean; whiskers represent the interquartile distance with a coefficient of 1.5; the most extreme values are the minimum and maximum, and the other dots are the 99th percentile and 1st percentile. Kolmogorov-Smirnov test. d The number of acetylation sites affected per protein. e Venn diagram showing the overlap between significant lysine sites (upper) and the overlap between acetylated proteins (lower) in AT-1 sTg and AT-1S113R/+ mice. f Heatmap showing the percent change in stoichiometry of the 88 overlapping sites found in both models. Subcellular localization was determined according to the Uniprot database annotation. The secretory pathway includes the endoplasmic reticulum, Golgi apparatus, proteasome, lysosome, extracellular, and secreted proteins. Cytoplasm includes the cytoskeleton, cytosol, ribosomes, and all other organelles. g, h The distribution of significant lysine acetylation sites according to their Uniprot subcellular annotation, as a global view in AT-1 sTg (upper) and AT-1S113R/+ (lower) mice (g), and arranged by localization: the secretory pathway (purple circles), mitochondria (yellow circles), nucleus (blue circles), and cytosol (orange circles) (h). The original data set is shown in Supplementary Data 2. All sites analyzed in d–h were significantly different (P < 0.05, one-way ANOVA) from age-matched WT littermates. Bars are mean ± s.d. Circles indicate the average value of each acetylation site.
Fig. 4 AT-1 sTg and AT-1S113R/+ mice show significant metabolic and mitochondria adaptation. a Venn diagram of overlapping significant proteins found to be modified in their expression levels (proteome) and in their stoichiometry of lysine acetylation (acylome) within AT-1 sTg (upper) and AT-1S113R/+ (lower) livers. b, c Proteins found to be enriched in lipid metabolism (b) and mitochondria-related (c) KEGG pathways are shown in clusters according to their level of regulation. Changes in the stoichiometry of acetylation are indicated by a purple (AT-1 sTg) or orange (AT-1S113R/+) acetyl group; changes in the proteome are indicated by a purple (AT-1 sTg) or orange (AT-1S113R/) circle; no change in proteome level is indicated by a gray circle. d, e Mitochondria morphology in primary-cultured hepatocytes was examined with CellLight mitochondria stain (scale bar, 1μm) (d) and quantified using Imaris reconstruction of area and volume (e) (hepatocytes from biologically independent animals; WT, n = 6; AT-1 sTg, n = 7; AT-1S113R/, n = 7). One-tailed Student’s t test. **P < 0.005; *P < 0.0005. f mRNA levels of PGC1α (pan) in liver samples (livers from biologically independent animals; WT, n = 5; AT-1 sTg, n = 5; AT-1S113R/, n = 5). One-tailed Student’s t test. **P < 0.005. g Primary cultured hepatocytes were labeled with 13C-glutamine for 30 min before collection. The total TCA intermediate metabolite levels and 13C-labeled metabolite levels are shown (hepatocytes from biologically independent animals; WT, n = 5; AT-1 sTg, n = 6; AT-1S113R/, n = 5). Two-tailed Student’s t test. *P < 0.05. All data are represented as mean ± s.d.

Methods
Animals. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and received ethical approval by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. Generation of AT-1S113R/+ animals was achieved by crossing mice carrying the Slc33a1-S113R mutation to WT animals, AT-1S113R/+ mice were studied.

in place to maintain acetyl-CoA homeostasis. Overall, the results support the conclusion that the flux of acetyl-CoA from the cytosol to the ER lumen can act as a metabolic regulator, and directly impacts crosstalk between different intracellular organelles and compartments.
between 5 and 7 months of age. Generation of AT-1 sTg mouse was achieved by injecting AT-1 (AT-1 sTg mice) 

**Hepatocyte isolation.** Primary mouse hepatocytes were prepared by anesthetizing the animals by intraperitoneal injection of 0.4 mg/kg freshly prepared 2, 2′-bipyridyldithromethanol (Avertin). The abdominal cavity was opened and a cannula was inserted into the inferior vena cava. The flow of perfusion buffer (HBSS, 59 mM HEPES, 0.6 mM EGTA; pH 7.5) began, and the portal vein was immediately cut. The liver was then perfused with collagenase buffer (HBSS, 0.25 mg/ml of collagenase type IV (Sigma-Aldrich CS136), 7 mM CaCl2, 53 mM HEPES, pH 7.5). Next, the liver was excised, and cells were harvested for tissue culture experiments by cutting into the liver capsule. Hepatocytes were passed through a 70-μm cell strainer and centrifuged (900 × g, 5 min) and re-suspended in washing media (M199, 1× glutamin), centrifuged again (100 × g, 5 min), re-suspended in plating media (10% FBS, 1× glutamin, 1× penicillin/streptomycin/glutamine, 1× BSA) and plated. For immunocytochemistry, hepatocytes were plated at 100,000 cells per collagen-coated coverslip (GC-12, Neuvitro), and transfected with CellLight Mitochondria-GFP BacMam 2.0 (ThermoFisher Scientific) overnight. Transfected cells were fixed with paraformaldehyde (4%, 15710, Electron Microscopy Sciences) for 10 min, followed by permeabilization for 10 min with 0.1% Triton-X100 (Roche Applied Science) for 5 min and incubation with F4/80 (10 μg/ml) (Abcam) and collagenase type IV (Sigma-Aldrich CS136) 100 μg/ml (in PBS) for one hour. Nuclei were stained with DAPI (62248, ThermoFisher Scientific) during the blocking step. Cells were washed three times in PBS and prolonged diamond antifade mountant (P36965, ThermoFisher Scientific) was used to mount the coverslips. Images were acquired using Streuced Illumination Microscopy (Nikon SIM), and analyzed in Imaris imaging software (Bitplane, Oxford Instruments) using the Surfaces module.

**Lipid determinations.** Whole liver samples were homogenized in PBS and the following assays were performed: Coenzyme A was measured using the Coa assay kit (K367, BioVision); free fatty acid was measured using the free fatty acid assay kit (ab65341, Abcam); triglycerides were measured using the triglyceride assay kit (ab65336, Abcam); the total cholesterol was measured using the cholesterol assay kit (ab65336, Abcam); Cytosolic acetyl-CoA was isolated from the total liver, and was measured using the acetyl-Coa assay kit (ab87546, Abcam). To isolate the cytosol, the liver was homogenized (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease and phosphatase inhibitors), then centrifuged (600 × g, 10 min). The supernatant was collected and centrifuged again (89,300 × g, 90 min) for analysis. For lipid serum analyses, blood was collected transcardially from mice with an insulin syringe. Blood was collected on ice for 15 min, then centrifuged at 200g for 10 min at 4 °C and the supernatant was collected. The lipid serum profile was performed by the University of Wisconsin-Madison Clinical laboratory.

**Real-time PCR.** Real-time PCR was performed using the Roche 480 lightcycler and Sybr Green Real Time PCR Master Mix (Life Technologies). Gene expression levels were normalized against GAPDH levels and expressed as fold of change from control. The cycling parameters for PGC1-α isoforms were selected and order of fragmentation at a normalized collision energy of 30% followed by tandem MS acquisition at a resolving power of 15 K and an AGC target of 5 × 106 with a maximum injection time of 100 ms. The top 20 intense precursor ions were selected and subjected to the HCD fragmentation at a normalized collision energy of 30%, and subjected to the HCD fragmentation at a normalized collision energy of 30%.
limit of m/z 110. Precursors were subjected to a dynamic exclusion of 45 s with a 10 ppm mass tolerance. Raw files were processed with Proteome Discoverer 2.1 engine (ThermoFisher Scientific, San Jose, CA, USA) with Byonic search engine (Protein Metrics Inc, San Carlos, CA, USA). Spectra were searched against the Uniprot Mus musculus reviewed database with trypsin as the enzyme and maximum two missed cleavages. The parent mass error tolerance was set to be 50 ppm, and fragment mass tolerance was 0.02 Da. Fixed modifications included DiLeu labels on peptide N-termini and lysine residues (+145.12601 Da) and carbamidomethylation on cysteine residues (+57.02146 Da). Dynamic modifications included oxidation of methionine residues (+15.99492 Da). Identifications were filtered to 1% peptide and protein FDR. Quantitation was performed in Proteome Discoverer with a reporter ion integration tolerance of 20 ppm for the most intense centroid. Only the PSMs that contained all reporter ion channels were considered, and protein quantitative ratios were determined using a minimum of one unique quantified peptide. Reporter ion ratio values for protein groups were exported to Microsoft Excel, and all fractions were combined for downstream analysis (see statistics section for processing). Proteins that had P < 0.05 (Fisher’s method) were filtered as significant changes. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD013736.

Stoichiometry of acetylation. Analysis was conducted using 200 μg of protein from the liver mitochondrial and cytosolic subcellular fractions, which were denatured in urea buffer (8 M urea (deionized), 500 mM ammonium bicarbonate pH 8.5 = 1). The samples were incubated at 20 min at 60 °C using the Eppendorf ThermoMixer C. Cysteines were alkylated with 50 mM iodoacetamide and incubated for 20 min Chemical acetylation using two rounds of ~20 μmol heavy isotope D_{2}-acetic anhydride (Cambridge Isotope Laboratories). The pH of the samples was spot checked and raised back to ~8.5 after each chemical modification. All samples were then premixed with 100 mM ammonium bicarbonate pH = 8.0 to 2 M urea and digested with 1:100 trypsin at 37 °C for 4 h. Samples were then diluted to 1 M urea prior to a second digestion by gluc (1:100). Chemically acetylated peptides were resuspended into ~2 mL of HPRP buffer A (100 mM Ammonium Formate pH = 10) and injected onto a prequenched Phenomenex Gemini® 250 × 10 μm (5 μm, 150 A, 200 μm) with 2% Buffer B (10% Buffer A, 90% acetonitrile). Peptides were separated with a Shimadzu LC-20AT HPLC system using a 2%–40% Buffer B linear gradient over 60 min at 0.4 mL/min flow rate, collecting 24 fractions throughout the length of the gradient. Fractions were dried down using a speedvac and pooled by concatenation in silica plates for metabolomics library generation. The samples were analyzed by a Thermo Q-Exactive Orbitrap, coupled to a Dionex Ultimate 3000 RSLC nano UPLC with a Waters Atlantic reverse phase column (100 μm × 150 mm). For data-independent acquisition (DIA) analysis by a Thermo Q-Exactive Orbitrap coupled to a Dionex Ultimate 3000 RSLC nano UPLC with a Waters Atlantic reverse phase column (100 μm × 150 mm). For data-independent acquisition (DIA), the MS survey scan was performed in profile mode with a resolution of 70,000, AGC of 1e6, maximum fill time of 100 ms in the scan range between 400 and 1000 m/z. The survey scan was followed 30 DIA scans in profile mode with a resolution of 35,000, AGC 1e6, 20 m/z window, and NCE of 30. The source voltage was set at 2000 V and capillary temperature at 250 °C. To deconvolute and analyze the DIA spectra, a spectral library containing all light and heavy acetyl-lysine feature pairs was generated. Spectra-sequence search was performed on the spectral library generated from the experiment, except they were treated with C\textsuperscript{2+}-acetylated lysine (Sgamma) and analyzed using data-dependent acquisition (DDA) mass spectrometry analysis. Using the openly available MaxQuant (v1.6.1.0) software package, we performed a database search to find peptides present in the DIA samples analyzed. Carbamidomethylation (C) was set as fixed modification, and oxidation (M) and acetyl (K) were set as variable modifications. Trypsin and GluC were set as the digestion enzymes, with the max number of missed cleavages set to five. DDA runs from both the mitochondrial and cytosolic fractions were run to make one combined library. Heavy acetyl fragment ion pairs were generated in silico, such that the spectral library would contain both the light (endogenous) acetylation peaks and the heavy (chemical) acetylation peaks. The experimental samples were processed using Spectronaut (v10) using the generated spectral library. The subcellular fraction experimental samples were processed separately. The data were processed using an in-house R script, which can be accessed through the GitHub link: https://doi.org/10.5281/zenodo.3471182. The Stoichiometry of acetylation was calculated from the ratio of the heavy-labeled peak to remove any contribution from naturally occurring iso- topes from the light labeled peak. All fractions were combined for downstream analysis; proteins that were P < 0.05 combined with VIP > 1 were filtered as significant changes (see Statistics section for processing. The raw data, processed data, spectral library, and the analysis logs describing the settings for the Spectronaut analyses have been deposited to the ProteomeXchange Consortium via the MassIVE partner repository with the data set identifier PXD014013.

Metabolomics. Hepatocytes were plated at 900,000 cells per 35 mm collagen-coated plate. All cells received a media change of pre-media (10% FBS, 1% penicillin/streptomycin/glutamine in the Dulbecco’s modified Eagle medium (DMEM)) 2 h before 13C media was added. After the 2 h pre-incubation, cells were either immediately snap-frozen in liquid nitrogen or given 13C media (10% FBS, 1 mM 13C labeled glutamine, 25 mM 13C labeled glucose, 99% Cambridge Isotope Laboratories) in DMEM for 30 min and then snap-frozen with liquid nitrogen. Plates of cultured hepatocytes and media were kept frozen at −80 °C until time of extraction. Media (500 μL) was lyophilized by vacuum centrifugation. Metabolites were extracted directly from plated hepatocytes or from lyophilized media on ice with 4 °C 7:2 HPLC grade methanol/water/formic acid. After addition of extraction solvent, cells were immediately scraped from the plate using a cell scraper; media samples were vortexed and probe sonicated for 10 s. Resulting extracts were transferred to microcentrifuge tubes and incubated at 4 °C for 5 min, then centrifuged at 10000 × g for 10 min at 4 °C to pellet precipitated protein. Extracts were aliquoted into glass autosampler vials and dried by using a speedvac. For GC-MS analysis, the dried extract was derivatized for 90 min with 20 mg/ml methoxyamine hydrochloride in pyridine at 20 °C (25 μL) and then with MSTFA for 30 min at 37 °C (25 μL). Samples were analyzed by GC-Orbitrap; 1 μL of sample, split 1:10, was injected onto a TraceGOLD TG-SILMS GC column (cat. no. 26096–1420, Thermo Scientific). Temperature was held at 50 °C for 1 min, then ramped to 320 °C at a rate of 11 °C/min, then held at 320 °C for 4.40 min. Molecules were analyzed with positive electron-impact (EI)-Orbitrap full scan of 50–650 m/z range. For data analysis, selected m/z and retention times were used to quantify metabolites and their degradation products; peak areas were quantified using the xcms package R3. Metabolomics raw MS files are available on the public repository MassIVE with the accession code MSV000083885 (https://massive.ucsd.edu/MSV000083885). Additional information can be found in Supplementary Table 3 and Supplementary Data 3.

Statistics. Data analysis was performed in Graphpad Prism v 7.02 (GraphPad Software, Inc) and R v3.5.1. Unless otherwise specified, data are expressed as mean ± standard deviation. For the proteomics, fold changes were computed within each DiLeu batch experiment, an F-test was used to test for equivalent variance among groups, and a Student’s t test was performed assuming equal or unequal variance according to the results of the F-test. A final fold change was calculated by averaging the two experiments together, and the p-values of the two separate DiLeu experiments were combined using Fisher’s method as implemented in the R package metaP (R version 3.5.1). For the stoichiometry of acetylation, a one-way ANOVA test was performed. Comparison of the peptides and acetyl proteome distribution was performed using a Kolmogorov–Smirnov test. For all other analyses, comparison of the means was performed using a Student’s t test. The following statistical significance was used: *P<0.05; **P<0.01; ***P<0.005.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The mass spectrometry proteomics data that support the findings of this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the accession code PXD013736. The acetyl-proteomics data that support the findings of this study have been deposited to the ProteomeXchange Consortium via the MassIVE partner repository with the accession code PXD014013. The metabolomics data that
support the findings of this study have been deposited in the MassIVE repository with the accession code MSV000083885 (https://www.ebi.ac.uk/ msdss/MSV000083885). The R script that was used to process the acetyl-proteomics data have been deposited on Github with the identifier (search terms: AT1 Acetylation Stoich) (https://doi.org/10.5281/zenodo.3238525). The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary tables.

Code availability

The in house R script that was used to process the acetyl-proteomics data have been deposited on Github with the identifier (search terms: AT1 Acetylation Stoich) (https://doi.org/10.5281/zenodo.3238525). The README file found on Github describes how the input data for the scripts can be accessed through ProteomeXchange accession code PXD014013.

Received: 1 March 2019 Accepted: 8 August 2019
Published online: 02 September 2019

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Acknowledgements

We thank Dr. John Sivars for critical reading of an early version of this paper. We thank Karl Miller for technical help. This work was supported by the NIH (NS094154 and AG053973 to L.P.; AG057408 to L.P. and R.M.A.; GM65386 to J.M.D.; R01DK071801, R01AG052324, and P41GM108538 to L.L.; P41 GM108538 to J.J.C.); a core grant to the Waisman Center from NICHD-U54 HD900526; I.A.D. was supported by T32 AG000213 and T32 GM000213 and T32 GM007507. SIM imaging was performed at the Biochemistry Optical Core of the University of Wisconsin-Madison (Madison, WI). The Orbitrap instruments were purchased through the support of an NIH shared instrument grant (NIH-NCRR S10RR029531) and Office of the Vice Chancellor for Research and Graduate Education at the University of Wisconsin-Madison. This work was also supported using resources and facilities of the William S. Middleton Memorial Veterans Hospital (Madison, WI, USA).

Author contributions

I.A.D. wrote the paper with input from all authors. L.P. designed the overall study. L.P. and T.W.R. analyzed the data. M.S.B., L.L., J.M.D., J.J.C., R.M.A. and L.P. provided critical advice for the experiments. L.P. designed the overall study. L.P. and I.A.D. wrote the paper with input from all authors.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11945-9.

Competing interests: J.M.D. is a co-founder of Galilei BioScience Inc, devoted to the development of small-molecule effectors for SIRT6. Remaining authors have no conflict of interests to disclose.

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Peer review information: Nature Communications would like to thank Suzanne Jackowski and other, anonymous, reviewers for their contributions to the peer review of this work. Peer review reports available.

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