Nutritional stress exacerbates hepatic steatosis induced by deletion of the histidine nucleotide binding (Hint2) mitochondrial protein.

Juliette Martin¹, Maria L. Balmer¹, Saranya Rajendran¹, Olivier Maurhofer¹, Jean-François Dufour¹,², Marie V. St-Pierre¹,

Department of Clinical Research¹, University of Bern and Department of Hepatology, Gastroenterology and Visceral Surgery², University Hospital, Inselspital, Bern, Switzerland.

Running title: Hint2 deletion and nutritional stress.

Corresponding author: Jean-François Dufour MD, +41 31 6329762
Department of Hepatology, Gastroenterology and Visceral Surgery, Inselspital, Bern, Switzerland, CH-3010.
jean-francois.dufour@dkf.unibe.ch
Abstract

The histidine nucleotide binding protein, Hint2, is a mitochondrial phosphoramidase expressed in liver, brown fat, pancreas and muscle. The livers of Hint2 knockout (Hint2\(^{-/-}\)) mice accumulate triglycerides and show a pattern of mitochondrial protein lysine hyperacetylation. The extent and nature of the lysine acetylation changes and the response of Hint2\(^{-/-}\) mice to nutritional challenges that elicit a modification of protein acetylation have not been investigated. To compare the adaptation of Hint2\(^{-/-}\) and control (Hint2\(^{+/+}\)) mice to episodes of fasting and high fat diet, we subjected animals to either feeding *ad libitum* or fasting for 24 h, and to either a high fat diet (HFD) or control diet for 8 weeks. Triglyceride content was higher in Hint2\(^{-/-}\) than in Hint2\(^{+/+}\) livers, whereas plasma triglycerides were 4-fold lower. Malonyl-CoA levels were increased two-fold in Hint2\(^{-/-}\) livers. After 24 h fasting, Hint2\(^{-/-}\) displayed a decrease in body temperature, commensurate with a decrease in mass of brown fat and downregulation of uncoupling protein 1. HFD treated Hint2\(^{-/-}\) livers showed more steatosis, and plasma insulin and cholesterol were higher than in Hint\(^{+/+}\) mice. Several proteins identified as substrates of sirtuin 3 and 5 and active in intermediary and ketone metabolism were hyperacetylated in liver and brown fat mitochondria after both HFD and fasting regimens. Glutamate dehydrogenase activity was downregulated in fed and fasted livers, and this was attributed to an increase in acetylation and ADP-ribosylation. The absence of Hint2 deregulates the post-translational modification of several mitochondrial proteins, which impedes the adaptation to episodes of nutritional stress.
Key words: Histidine nucleotide binding protein, Hint2, lysine acetylation, hepatic steatosis, fasting, high fat diet
The histidine triad nucleotide-binding protein, Hint2, belongs to the histidine triad family of enzymes that share the sequence motif, H-Φ-H- Φ-H- Φ- Φ, where Φ is a hydrophobic amino acid. Hint2 catalyzes the hydrolysis of phosphoramidate bonds, which yield a nucleotide product (19, 25). Hint2 has been crystallized in its homodimeric form bound to AMP (25). Hint2 is a mitochondrial protein expressed in the liver, pancreas, brown fat (27), skeletal muscle and heart (26). We have shown previously that the genetic deletion of Hint2 in mice (Hint2−/−) is associated with an age-dependent accumulation of hepatic triglycerides and structural anomalies in liver mitochondria, as well as a decrease in activity of the mitochondrial matrix enzyme, glutamate dehydrogenase (GDH). Moreover, Hint2−/− mice display an increase in lysine acetylation of several mitochondrial proteins, including GDH (27).

Lysine acetylation is a reversible, posttranslational modification that regulates the activity of selected enzymes mediating the intermediary metabolism of carbohydrates, fat and proteins. Hence, lysine acetylation permits a targeted, tissue specific response to changes in energy supply. A low energy supply resulting from acute fasting or an extended caloric restriction regulates acetylation of certain mitochondrial enzymes that govern fatty acids metabolism and electron transport in the liver, but not in brown fat or heart (33) (21). An oversupply of energy resulting from a chronic high-fat diet also promotes global changes in hepatic lysine acetylation (18). One basis for a reversible change in acetylation patterns relates to the upregulation or downregulation of sirtuin 3, a mitochondrial deacetylase (34) (16) (18). However, in some instances, such as after long standing caloric restriction (33), sirtuin 3 upregulation occurs concomitantly with an increase in acetylated proteins. A second physiological basis for acetylation changes, independent of sirtuin 3 expression, relates to the availability of acetyl-coenzyme A (acCoA), the co-substrate required by acetyltransferases, and NAD⁺, the
co-substrate used by sirtuin deacetylases. Intracellular concentrations of acCoA increase during nutritional excess, whereas NAD$^+$ concentrations increase under conditions of fasting or caloric restriction (39). A third basis for changes in global protein acetylation could be linked to the actions of acetyltransferases. The GCN5L1 protein, whose genetic knockdown reduces protein acetylation, was identified as a mitochondrial acetyltransferase (35), although a regulatory role has yet to be confirmed. It is likely that several factors interact to reversibly regulate protein acetylation and thereby influence the physiological response to changes in nutritional status. On the basis of the changes in mitochondrial lysine acetylation observed so far in Hint2$^{-/-}$ mice, the Hint2 protein should join the list of agents capable of influencing protein acetylation.

The extent and nature of the acetylation changes in mitochondrial proteins in the absence of Hint2 has not been studied. Moreover, the response of Hint2$^{-/-}$ mice to nutritional changes that elicit a modification of protein acetylation has not been investigated. We postulated that the absence of Hint2 protein perturbs the normal metabolic response to changes in the nutritional status and renders the Hint2 knockout mice more vulnerable than control mice to episodes of fasting and high fat diet. Therefore, we subjected age-matched groups of Hint2$^{+/+}$ and Hint2$^{-/-}$ mice to dietary changes. To generate nutrient deficit, we either fed mice ad libitum or imposed a 24 h fasting period. To generate nutrient excess, we fed mice either a standard control diet or a high fat diet (HFD) for 8 weeks. The metabolic parameters and acetylation profiles were compared. Our findings show that the Hint2 protein wields influence in the overall adaption to nutritional stress. Moreover, our findings support the notion that Hint2 modulates the post-translational modification of several proteins by an indirect mechanism.
Methods

Animals
As described previously, the genetic deletion of Hint2 by homologous recombination in embryonic stem cells generated Hint2^-/- mice in a mixed C57Bl6J/129Sv background (27). Mice were housed and cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee, University of Bern. For the fed/fasting experiments, adult male (20-25 weeks) Hint2^+/+ and Hint2^-/- mice were housed individually, and either fed a Teklad Global Rodent 2918® diet (Harlan, Madison WI) ad libitum or fasted for 24 h. The 20-25 week age group was chosen because hepatic steatotic changes had previously been detected in Hint2^-/- mice of this age (27). Mice were euthanized between 0900 and 1200 h. Brown adipose tissue (BAT) and plasma were frozen immediately at -80°C. Livers were removed and processed immediately to collect a fraction enriched for heavy mitochondria, as described (17). Aliquots of mitochondrial protein were frozen at -80°C. For the control diet/high fat diet (HFD) experiments, 8 week-old Hint2^+/+ and Hint2^-/- mice were fed a control diet (CD, #TD.04541, 3.8 kcal/g, 12.3% kcal from fat, n=10) or a high fat diet (HFD, #TD.99249, 5.5 kcal/g, 59.6% kcal from fat, n=10) (Harlan Laboratories) for 8 weeks. Younger mice were chosen for the control/HFD studies to avoid the phenotypic changes of increased body weight and hepatic lipids that appear in Hint2^-/- mice aged ≥ 20 weeks. Livers from the control/HFD groups were routinely frozen at -80°C. Mitochondria were isolated from 200 mg frozen tissue by means of a mitochondrial isolation kit (Thermo Fisher Scientific, Rockford IL).

Immunoprecipitation
Isolated mitochondria (650 µg) were lysed in 1% n-dodecyl-β-D-maltoside (Sigma, St. Louis, MO), 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM nicotinamide, 50 nM trichostatin A and a protease inhibitor cocktail (Roche,
Germany). The lysate was diluted to a final detergent concentration of 0.5% and incubated with an anti-acetylated-lysine rabbit antibody (10 µl) (Cell Signaling Technology, Beverly, MA) overnight at 4°C. The antibody complexes were captured with Dynabeads M-280 sheep anti-rabbit IgG (Dynal AS, Norway) for 2 hours at 4°C, and then washed 4 times in 0.1% detergent. The bound proteins were extracted with SDS-Page loading buffer (Thermo Fisher Scientific, Rockford IL) and subjected to immunoblotting.

**Quantification of triglycerides, free fatty acids, ammonia, glycogen**

For triglyceride measurements, frozen liver and BAT tissues were minced, lysed in buffer containing 5% Triton-X 100 and homogenized. For free fatty acids (FFA) quantification, both liver and BAT tissues were homogenized in chloroform-Triton X-100 then centrifuged. The organic phase was dried under N₂ and suspended in assay buffer. Triglycerides and FFA in liver, BAT and plasma were measured colorimetrically by means of a quantification kit (Biovision, Mountainview, CA). Ammonia was measured in 20 µl plasma by means of a colorimetric assay (Abcam, Cambridge, UK). Glycogen in liver homogenate was quantified by means of a colorimetric assay (Glycogen Assay Kit II; Biovision). Protein quantities of 50-60 µg from fasted livers and 1-2 µg from fed livers were used to fall within the linear range of the glycogen assay.

**Isolation of mitochondria from brown adipose tissue**

Interscapular BAT from 5 mice were pooled and homogenized in 250 mM sucrose. A mitochondrial enriched fraction was isolated, as described (4).

**Sirtuin 3 activity**

Sirtuin 3 activity was measured fluorometrically in Hint2⁻/⁻ and Hint2⁺/+ mitochondrial fractions. The assay buffer contained 25 mM Tris, 150 mM NaCl, 1mM DTT, 2 mM NAD⁺ and the fluorogenic peptide substrate, Ac-Arg-Gly-Lys(Ac) -7amino-4-
methylcoumarin (R&D Systems Inc., Minneapolis, MN). The reactions were
terminated in a stop solution (50 mM Tris, 100 mM NaCl, 30% isopropanol, 4 mM
nicotinamide) containing 0.2 ng/µl recombinant mouse trypsin 3. To test whether
sirtuin 3 activity was affected by the presence Hint2 protein, we resuspended
recombinant human His-sirtuin 3 (1 µg) (R&D Systems) in assay buffer containing
equimolar recombinant GST-Hint2 or GST, the fluorogenic peptide substrate and
varying concentrations of NAD⁺ (0.1 mM – 2 mM). The reaction was terminated after
30 min.

Quantification of coenzyme A esters
CoA and its short-chain esters were quantified by HPLC-MS. The method described
by Demoz *et al.* was adapted (8). Liver pieces (100 mg), previously frozen in liquid N₂
and stored at -150°C, were pulverized in liquid N₂, then homogenised at 4°C in 5%
sulfosalicylic acid containing 50 µM dithioerythritol. After centrifugation (16000 x g),
the protein-free supernatant was passed through a 0.45 µm PTFE filter then injected
onto an Atlantis® T3-3 µm column (Waters, Milford MA). The protein pellet was
dissolved in 40 mM Tris:10% SDS for estimation by means of the Lowry method. The
separation of acetyl-CoA, malonyl-CoA, methylmalonyl-CoA, succinyl-CoA, propionyl-
CoA and free CoA was achieved by means of a gradient elution with ammonium
formate 100 mM, pH 6.38, and methanol (95:5 to 45:55).

Liver Histology
Liver specimens from 10 *Hint2*⁺⁺ and 10 *Hint2*⁻⁻ from each diet group were
cryosectioned, fixed in 4% buffered formaldehyde solution, and stained with
hematoxylin and eosin. The pathological features of the liver were assessed
microscopically and coded (0-2, 3-4, >4) according to the nonalcoholic fatty liver
disease (NAFLD) Activity Score (NAS) criteria described by Kleiner et al. (22). NAS values for the HFD groups were compared by means of the $2 \times 3$ Fisher's exact probability test with the Freeman-Halton extension and two-tailed P-values (13).

Additional sections were incubated in 100% propylene glycol then in Oil Red O 0.5%, which stains the neutral lipids. Images were captured at 100x magnification by means of a Nikon DN100 camera mounted on Olympus BHS-PC microscope. The areas positive for Oil Red O were quantified with the imaging software, MetaMorph (Molecular Devices Corporation, Sunnyvale, CA). Data from individual sections (three fields on each section) were pooled, and are presented as means ($\pm$ SD) expressed as pixels/mm$^2$. For statistical comparison, a one-way ANOVA and Bonferroni's multiple comparison tests were applied.
Results

Response of Hint2+/+ and Hint2−/− mice to 24 h fasting.

In 25 week old mice, the mean body weight of Hint2−/− was 23% higher than that of Hint2+/+ (P < 0.05) (Table 1). Likewise, the ratio of liver weight to body weight after fasting was higher in Hint2−/− than in Hint2+/+ mice (P < 0.05). These differences were not evident in younger mice. The loss of body weight after 24 h of fasting was not different between the groups. No significant differences in blood glucose or plasma ketone levels were detected between Hint2+/+ and Hint2−/− mice (Table 1).

We have shown previously that as Hint2−/− mice age, their livers exhibit steatotic changes (27). To determine whether steatosis persisted after fasting, we measured triglyceride levels in the liver and plasma of 25 week-old Hint2+/+ and Hint2−/− mice. Triglyceride content was 2.9-fold higher in Hint2−/− than in Hint2+/+ livers (Fig. 1A). The 24 h fast induced triglyceride accumulation in both groups. Commensurate with the increase in liver triglycerides, plasma triglycerides were decreased in Hint2−/− under fed conditions (Fig. 1B). Free fatty acid levels in fasting livers were not different between Hint2−/− and Hint2+/+ (Fig. 1C). Liver glycogen content was depleted by the 24 h food deprivation in both groups (Fig. 1D).

In addition to the liver, Hint2 is also expressed in BAT, but not white fat (27). BAT is a thermogenic tissue, by virtue of the actions of the highly expressed uncoupling protein (UCP1). The body temperatures of 20-week old mice were compared under fed and fasting conditions. After fasting, body temperature was significantly lower in Hint2−/− than in Hint2+/+ mice (Fig. 2A). The weight of interscapular BAT was equal between the fed groups but tended to decrease after fasting in Hint2−/− mice (Fig. 2B). The UCP1 protein and mRNA levels in mitochondria isolated from pooled BAT were compared. The UCP1 protein was downregulated in fasting Hint2−/− BAT (Fig. 2C),
without a corresponding decrease in mRNA (Fig. 2D). Levels of FFA decreased in BAT of fasted Hint2/− mice (Fig. 2E), which implies an acceleration of fatty acid mobilization. This was supported by the increase in phosphorylated perilipin in the fasted BAT (Fig. 2F).

**Response of Hint2+/+ and Hint2−/− mice to high-fat diet.**

Excess dietary fat constitutes a nutritional stress that can promote hepatic steatosis, a manifestation of the metabolic syndrome. To gauge the response of Hint2−/− mice to this stress, we imposed an 8-week course of either a HFD or a control diet. Regardless of regimen, Hint2−/− mice gained more body weight than did Hint2+/+ mice, without a commensurate increase in food consumption (Table 2). However, the HFD provoked a higher weight gain in Hint2+/+ (210% increase over control diet) than in Hint2−/− (130% increase). Fasting insulin concentrations were higher in Hint2−/− mice than in Hint2+/+ mice under both feeding regimens, although fasting blood glucose concentrations were not different. Plasma cholesterol tended to be higher in Hint2−/− than in Hint2+/+. The HFD provoked an increase in alanine transaminase in both groups (Table 2).

After HFD, steatosis was more severe and more frequent in Hint2−/− livers (Fig. 3A). Hint2−/− livers presented steatotic changes in 70% of cases, whereas fewer Hint2+/+ livers were affected. Although plasma triglycerides were not different between the groups (data not shown), the oil Red-O staining of liver specimens confirmed that excess lipid accumulated in Hint2−/− livers (Figure 3B, 3C). When HFD treated livers were evaluated according to the histological scoring system for NAFLD, the features of hepatocyte ballooning, lobular inflammation, microgranulomas and megamitochondria were occasionally detected in both Hint2−/− and Hint2+/+ groups.
However, the NAS values were not significantly different between the groups (Fig. 3D).

**Changes in protein acetylation after fasting and high-fat diet.**

Since mitochondrial lysine acetylation changes in response to nutritional status, but the global acetylation pattern in *Hint2*<sup>−/−</sup> livers is upregulated even without nutritional stress (27), we asked whether the hyperacetylation patterns of *Hint2*<sup>−/−</sup> mitochondria would increase further when exposed to nutrient excess or deprivation. The global lysine acetylation patterns of mitochondrial protein under control, fasting and high-fat diet were compared (Fig. 4). Under conditions of control diet and HFD (Fig. 4A), as well as after 24h fasting (Fig. 4B), the mitochondrial proteins in *Hint2*<sup>−/−</sup> livers were hyperacetylated. The greatest differences in global acetylation were observed between the *Hint2*<sup>−/−</sup> and *Hint2*<sup>+/+</sup> groups and these differences were not further amplified or attenuated by the dietary manipulation. A range of liver proteins were affected, primarily those between 30 and 150 kDa (Fig. 4A, B). To determine whether acetylation was affected in tissues other than the liver, we examined BAT mitochondria in fed and fasted groups. BAT mitochondrial proteins from *Hint2*<sup>−/−</sup> mice showed a similar pattern of hyperacetylation, under both fed and fasted conditions (Fig. 4C). The Hint2 status was a greater determinant of the lysine acetylation pattern than was the dietary manipulation.

To determine whether the upregulation of lysine acetylation affected proteins associated with hepatic intermediary metabolism, we adopted the candidate approach. Lysine-acetylated proteins were immunoprecipitated from liver mitochondria of control/HFD groups (Fig. 5) and fed/fasted groups of mice (Fig. 6A). Proteins previously described as regulating the adaptation to nutritional status were screened. The extent of acetylation of carbamoyl phosphate synthase 1 (CPS1),
which catalyses the first step of the urea cycle and is a substrate for sirtuin 5 (28), was higher in Hint2−/− than in Hint2+/− mitochondria under all conditions (Fig. 5) (Fig. 6A). Acyl-CoA dehydrogenase long-chain (ACADL), a substrate of sirtuin 3 and active in the oxidation of fatty acids (16), showed increased acetylation in both control and HFD treated Hint2−/− mice (Fig. 5) but not in the fasted Hint2−/− group (not shown). The acyl-CoA dehydrogenase medium-chain (ACADM) showed a marginal increase in control, HFD and fasted Hint2−/− mice (Fig. 5, 6A). The 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCoAs), which catalyzes the conversion of acetyl-CoA and acetoacetyl-CoA into HMG-CoA and participates in ketogenesis, was hyperacetylated in Hint2−/− mice from the control and HFD groups (Fig. 5). A slight increase was observed after fasting in Hint2−/− mice compared to Hint2+/+ mice (Fig. 6A). Acetylation of HMG-CoA lyase was increased in control/HFD Hint2−/− mitochondria (Fig. 5). Pyruvate carboxylase was hyperacetylated in Hint2−/−, more so after HFD. The chaperone protein, HSP-60, showed an increase in acetylation in Hint2−/− in both control and HFD (Fig. 5), although no difference was detected in fasted mice (not shown). The peptidylprolyl cis-trans isomerase, cyclophilin D, which is a substrate for sirtuin 3 (37), showed an increase in acetylation under both HFD (Fig. 5) and fasting conditions (Fig. 6A). The acetylation of cyclophilin D increases its isomerase activity and its interaction with adenine nucleotide translocase (ANT), which in turn regulates the opening of the mitochondrial permeability transition pore (PTP) (11). The acetylation of ANT in Hint2−/− was not changed (not shown). The increase in acetylation of cyclophilin D can perhaps explain the augmented response to repetitive Ca2+ spikes with premature opening of mPTP exhibited previously in mitochondria from isolated Hint2−/− hepatocytes (31).

The acetylation of ATP synthase α, one subunit of the ATP-synthase Complex V, was upregulated in Hint2−/− liver mitochondria after feeding ad libitum, but not after
fasting (Fig. 6A). Similarly, the acetylation of urate oxidase, which catalyzes uric acid to allantoin in rodents and is a substrate for sirtuin 5 (30), was upregulated in Hint2−/− mitochondria from fed but not fasted mice (Fig. 6A). The expression of sirtuin 5 itself did not differ between Hint+/+ and Hint2−/− mitochondria (Fig. 6B). To determine whether increased acetylation of uric acid oxidase resulted in concomitant metabolite changes, we measured liver and plasma uric acid levels. Uric acid levels were higher in liver but lower in plasma of Hint2−/− mice fed ad libitum, although the differences did not reach statistical significance (data not shown).

Cyclophilin D interacts with p53 protein that has translocated to the mitochondria and this interaction can regulate the mitochondrial PTP (38). P53 itself is acetylated by the acetyltransferase, p300/CBP. We tested whether differences in p53 acetylation could be detected on immunoblots probed with an anti-acetyl (Lys389) p53 antibody. Acetylated p53 was detected only in Hint2−/− mitochondrial fractions of fed and fasting mice, but not after HFD (Fig. 6C). Because p53 is deacetylated by sirtuin 1, which in turn translocates to the mitochondria (2), we tested whether differences in expression of sirtuin 1 could account for the observed changes in acetyl-p53. Sirtuin 1 was expressed similarly in Hint2+/+ and Hint2−/− mitochondria (Fig. 6C).

To determine whether a similar set of proteins was affected in BAT, we isolated and pooled BAT mitochondria from four fasting Hint2+/+ and Hint2−/− mice and immunoprecipitated the acetylated proteins. The UCP1 protein was absent from the input of fasted Hint2−/− mitochondria (Fig. 7A). An acetylated version of UCP1 at the requisite size of 33 kDa was pulled down in Hint2+/+ but not Hint2−/− mitochondria. An acetylated product of a lower size was pulled down in both Hint2+/+ and Hint2−/− preparations. Since BAT functions as a site of fatty acid β-oxidation, the acetylation status of ACADL and 3-hydroxyacyl coenzyme A dehydrogenase short chain
(Hadhsc) was investigated. Both showed increased acetylation in BAT mitochondria from fasted Hint2\(^{-/-}\) mice. Changes in the acetylation status of glutamate dehydrogenase (GDH) and Complex V ATP synthase \(\beta\) and \(\alpha\), were also detected (Fig. 7A). No change in the expression of the deacetylase, sirtuin 3, was detected (Fig. 7B).

**Mitochondrial sirtuins 3 and 4 in Hint2\(^{-/-}\) mice.**

Unlike sirtuin 3, sirtuin 4 is not considered to exert its major function as a deacetylase, but rather as an NAD\(^+\) requiring ADP-ribosyltransferase of GDH (15) (1). We have previously shown that the activity of GDH is lower in Hint2\(^{-/-}\) than in Hint2\(^{+/+}\) livers, due in part to an increase in its lysine acetylation (27). We have re-examined GDH activity and its regulation by ADP-ribosylation in mitochondria under fed and fasted conditions (Fig. 8A, 8B). Mitochondrial GDH activity was lower in both fed and fasted Hint2\(^{-/-}\) mitochondria (Fig. 8A). The addition of purified phosphodiesterase I (PDE) unmasked significantly more latent GDH activity in Hint2\(^{-/-}\) than in Hint2\(^{+/+}\) mitochondria (Fig. 8B). This suppressed latent activity could not be accounted for by a difference in GDH protein expression (Fig. 8C) or sirtuin 4 expression (Fig. 8D). Since a low cellular energy state induced by fasting should promote the GDH-mediated conversion of glutamate to ammonia and \(\alpha\)-ketoglutarate, we measured plasma ammonia levels. The plasma ammonia tended to be lower in Hint2\(^{-/-}\) fasted animals (Fig. 8E).

Since sirtuin 3 is the predominant mitochondrial deacetylase, we asked whether changes in the expression or activity of sirtuin 3 could have accounted for the global hyperacetylation pattern. The level and enzymatic activity of sirtuin 3 in mitochondria under fed and fasted conditions were quantified. Sirtuin 3 expression tended to be higher in Hint2\(^{-/-}\) than in Hint2\(^{+/+}\) mitochondria (Fig. 9A), commensurate with an
increase in activity (Fig. 9B). Since Hint2 is a nucleotide binding protein, and sirtuin 3 requires NAD\(^+\) as a co-substrate, we asked whether the affinity of sirtuin 3 for NAD\(^+\) changes in the presence of excess Hint2 protein. No change in activity \textit{in vitro} was detected when recombinant GST-Hint2 was added to the sirtuin 3 assay (Figure 9C).

In addition to the deacetylase sirtuin enzymes, the acetylation status of proteins is also a function of the acetylation reaction, which in mitochondria is partly mediated by the GCN5L1 protein. GCN5L1 binds to acetyl-CoA, likely resides in the matrix and intermembrane space fractions of the mitochondria and interacts with and mediates the acetylation of complex V ATP5α (35). To determine whether the expression of GCN5L1 differed between \textit{Hint2}\(^{+/+}\) and \textit{Hint2}\(^{-/-}\) mice, we probed immunoblots of mitochondria from control and HFD groups with an anti-GCN5L1 antibody. No differences were detected (data not shown).

**The mechanism of hepatic steatosis in \textit{Hint2}\(^{-/-}\) mice.**

We reasoned that the additive consequences of a moderate increase in ACADL and ACADM (Fig. 5, 6) acetylation were insufficient to produce the degree of hepatic steatosis observed in \textit{Hint2}\(^{-/-}\) mice, since neither is the rate-limiting step in fatty acid β oxidation. Therefore we examined the rate-limiting carnitine palmitoyl transferase 1α (Cpt1α), which is allosterically regulated by intracellular concentrations of malonyl-CoA, and is subject to lysine acetylation. Mice from the fasted group were chosen for this comparison because their hepatic triglyceride levels were highest. \textit{Hint2}\(^{-/-}\) mitochondria showed a slight increase in Cpt1α acetylation (Fig. 10A). Moreover, the total malonyl-CoA concentration was two-fold higher in \textit{Hint2}\(^{-/-}\) livers, whereas acetyl-CoA concentrations were not significantly different (Fig. 10B). Therefore, a decrease in the acyl-carnitine transferase step likely contributed, in part, to the accumulation of triglycerides in the \textit{Hint2}\(^{-/-}\) livers. To determine whether a change in the expression or
The acetylation state of the mitochondrial or cytosolic form of the malonyl-CoA decarboxylase (MLYCD) enzyme is responsible for the two-fold increase in malonyl-CoA, we examine the level of acetylation of MLYCD in immunoprecipitated preparations of liver mitochondria and cytoplasm. No change was detected (Fig. 10A). No significant changes were detected in the cytoplasmic levels of ACC, p-ACC or p-ACC/ACC ratio (Fig. 10C). However, a minor increase in the level of fatty acid synthase (FAS) was detected, implicating an increase in fatty acid synthesis (Fig. 10D).

The elevated liver triglyceride levels coupled with the reduced plasma triglycerides in Hint2−/− mice raises the possibility that a reduction in the export of triglycerides from the liver could have contributed to the hepatic steatosis. To determine whether the export of lipids was affected in Hint2−/− livers, we measured the activity of the microsomal triglyceride transfer protein (MTTP) under fed conditions. MTTP activity was reduced by 20% in Hint2−/− livers (Figure 11A). This decrease in triglyceride transfer activity was not related to a reduction in expression of either the MTTP protein or its heterodimeric partner, protein disulfide isomerase (Figure 11B).
Discussion

The major phenotype exhibited by our mice bearing a constitutive deletion of Hint2 was the increased accumulation of hepatic lipids along with the hyperacetylation of mitochondrial proteins in liver and brown fat. When the Hint2\(^{-/-}\) mice were stressed with a nutritional challenge, the hepatic steatosis was aggravated. Hence, the underlying propensity for steatosis rendered Hint2\(^{-/-}\) mice more vulnerable than control mice to episodes of fasting and high fat diet.

Several proteins whose acetylation was modified by the absence of Hint2 likely contributed to the accumulation of triglycerides in the liver. The additive effects of an increase in acetylation of ACADL and ACADM predict a reduction in fatty acid oxidation capability. Likewise, the increase in acetylation of CPT1\(\alpha\), coupled with a two-fold increase in its allosteric inhibitor, malonyl-CoA, could reasonably be expected to alter CPT1\(\alpha\) function. Several acetylation sites on CPT1\(\alpha\) have been reported previously but the link between acetylation and activity has not been studied (21, 40). The two-fold increase in malonyl-CoA could not be attributed to a decrease in phosphorylation of ACC or to a change in the acetylation of malonyl-CoA decarboxylase (Fig. 10). In skeletal muscle and white adipose tissue, sirtuin 4 deacetylates and represses the activity of malonyl-CoA decarboxylase thereby favouring an increase in malonyl-CoA levels and lipogenesis de novo (23). However, the actions of sirtuin 4 are tissue specific. No changes in hepatic malonyl-CoA levels or hepatic lipogenesis were observed in sirtuin 4 knockout mice (23), which supports the notion that the increase in lipids of Hint2\(^{-/-}\) livers was not mediated by sirtuin 4 or malonyl-CoA decarboxylase. Malonyl-CoA levels can be influenced by the activity of FAS. In mice, the liver-specific knockout of FAS leads to a three-fold increase in hepatic malonyl-CoA levels (6). However FAS is thought to make a minor contribution to liver triglyceride storage since FAS knockout mice show normal levels
of liver lipids and remain subject to fasting-induced hepatic steatosis (20). In Hint2−/−
livers, FAS expression tended to increase, which is consistent with a decrease rather
than increase in malonyl-CoA (Fig. 10), although FAS activity was not measured.
The increase in FAS could have been secondary to the higher insulin levels in Hint2−/−
mice (Table 2) (27), since FAS is transcriptionally upregulated by insulin (32).

An experimental disturbance in microsomal triglyceride transfer activity, either by
pharmacological inhibition or by liver-specific knockout of the MTTP protein produces
changes in triglyceride levels similar to our Hint2−/− mice (9). However, the marginal
decrease in MTTP activity observed in Hint2−/− livers, coupled with a slight increase
rather than decrease in plasma cholesterol (Table 2) discounts a dysfunction in
MTTP as primary determinant of lipid accumulation in Hint2−/− livers.

Despite the exacerbation of steatosis and the decrease in body temperature, Hint2−/−
mice could withstand a fasting period of 24 h. An extended fasting period of 48 h was
previously shown to provoke the complete deacetylation of CPS1 with a consequent
increase in enzymatic activity (28). In our studies, Hint2−/− mice responded to the 24 h
fasting protocol by an incomplete deacetylation of CPS1 (Fig. 6A), but this did not
impair urea production (data not shown) nor increase the circulating ammonia levels.
In fact, the plasma ammonia levels decreased after fasting in the Hint2−/− group
(Figure 8E). Likewise, HMG-CoA synthase, which should respond to fasting by
deacetylation (36), showed residual acetylation in the Hint2−/− fasted group. However,
the production of ketones, as estimated by plasma levels of β-hydroxybutyrate, was
not significantly different between Hint2−/− and Hint2+/+ mice. The Hint2−/− mouse was
also able to elicit the expected response to fasting of a decrease in acetylation of
urate oxidase (30).
The BAT tissue in $\text{Hint2}^{-/-}$ but not $\text{Hint2}^{+/+}$ mice responded to fasting by a downregulation of UCP1, which can explain the decrease in body temperature. Previous reports have described starvation-mediated BAT atrophy and UCP1 downregulation in mice and have emphasized that changes are time and age dependent and occur in specific mitochondrial subfractions (3). Since mRNA levels of UCP1 remained constant, a posttranslational modification leading to an increase in UCP1 turnover in $\text{Hint2}^{-/-}$ is the most likely explanation. UCP1 is posttranslationally modified by acetylation (Fig. 7) and ubiquitination and degraded by the cytoplasmic proteosome (7). In the BAT of 24 h fasted rats, genes related to the ubiquitin and proteasome system are upregulated (29). We speculate that fasting could have upregulated a relevant E3 ubiquitin ligase that targets UCP1, and that the absence of Hint2 may have increased the accessibility of UCP1 to subsequent ubiquitination and degradation. In doing so, Hint2 would mimic the actions of another Hint family member, Hint1, which is expressed in cytosolic and nuclear compartments and interacts with the Scf-Skp2 E3 ubiquitin ligase complex to regulate the levels of the cyclin-dependent kinase inhibitor, p27 (5). Alternatively, the accelerated downregulation of UCP1 in $\text{Hint2}^{-/-}$ may have occurred indirectly. UCP1 is activated upon direct interaction with FFA (12), then undergoes a conformational change, which increases its susceptibility to proteolysis (10). The levels of FFA in the BAT of fasted $\text{Hint2}^{-/-}$ mice were decreased, which implies an acceleration of fatty acid mobilization. Mobilized fatty acids in BAT are destined for local oxidation as an energy source or for release to other tissues (14). The higher acetylation state of the fatty acid oxidation enzymes Hadhsc and ACADL (Fig. 7) in $\text{Hint2}^{-/-}$ BAT predicts a decrease rather than an increase in local beta-oxidation but we have not further investigated the fate of BAT mobilized fatty acids. Nevertheless, we speculate that
the mobilization of fatty acids was accelerated in fasted $\text{Hint2}^{-/-}$ BAT, which in turn was linked to the shortened half-life of the UCP1 protein.

The proteins whose acetylation status is affected by the loss of Hint2 include confirmed substrates both of sirtuin 3 (ACADL, ACADM, ATP synthase α, HSP60, pyruvate carboxylase, HMG-CoAs, HMG-CoAl, GDH) and sirtuin 5 (CPS, urate oxidase). Moreover, the sirtuin 4 substrate, GDH, was affected by an increase in ADP-ribosylation, which shows that post-translation modifications other than acetylation are affected in the absence of Hint2. Taken together, our findings show that Hint2 influences the state of protein acetylation and ADP-ribosylation likely by means of an indirect mechanism. Experimental evidence supports an interaction between the sirtuin 3 and 4 within the mitochondria (1). If Hint2 were to disturb such an interaction, downstream modification of substrates for both enzymes could be affected. In support of this is the observation that GDH, a substrate for both sirtuins, is the enzyme whose activity is most affected in $\text{Hint2}^{-/-}$ livers.

Like Hint2, the ubiquitous Hint1 protein present in the cytosol and nucleus of cells influences the acetylation of specific proteins. Unlike Hint2, a Hint1 deficiency impairs rather than augments the acetylation of ataxia telangiectasia mutated and the histone gamma-H2AX (24). Since Hint1 has been identified as a component of the TIP60 histone acetyltransferase complex, an indirect mechanism secondary to the disruption of protein-protein interactions can be inferred (41). Hint2 may operate by means of a similar mechanism.

An alternative explanation for the indirect effect of Hint2 is an alteration of the mitochondrial milieu to promote auto-acetylation of proteins. Scott et al. showed that denaturation of proteins by boiling was sufficient to cause global hyperacetylation, regardless of the presence of the GCN5L1 acetyltransferase (35). Moreover, non-
enzymatic lysine acetylation increases as a function of acetyl-CoA concentration and pH (39). The Hint2−/− mice showed no increase in acetyl CoA when total liver extracts were tested although this does not exclude a change at the mitochondrial level. Hint2−/− mice have not yet been studied with respect to status and regulation of intramitochondrial pH.

In summary, the deletion of Hint2 elicits changes in the post translational modification of substrate proteins of sirtuin 3, 4 and 5 in both liver and brown fat. Hint2−/− mice are subject to a perpetual state of hyperacetylation of metabolic enzymes in the mitochondria, which is characteristic of nutritional stress. The ensuing disturbance of fatty acid oxidation renders Hint2−/− mice susceptible to hepatic lipid accumulation, which is aggravated when mild experimental regimens of short-term HFD and 24 h fasting are imposed. Hint2−/− mice may lack the metabolic capacity to adapt to more severe nutritional challenges such as long-standing HFD or starvation because of the inability to fine tune physiological responses to the burden of nutritional excess or deprivation. Our findings show that the Hint2 status is an even more important determinant of the pattern of lysine acetylation of mitochondrial proteins than is the nutritional state.
Acknowledgements

We thank Philipp Kellmann and Jürg Müller for technical assistance. We thank Dr. Gisèle Ferrand at the animal facility, EPFL, Lausanne, Switzerland for the breeding and diet management of the animals.

This work was funded in part by grant 31003A_160001 awarded by the Swiss National Fonds and a Swiss Federation Scholarship 2012.0933.
Table 1. Comparison of body and liver weights, blood glucose and ketones in \(Hint2^{+/+}\) and \(Hint2^{-/-}\) mice.

<table>
<thead>
<tr>
<th></th>
<th>20 weeks</th>
<th>25 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+/+)\</td>
<td>(-/-)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>34.2±1.9</td>
<td>38.9±5.3</td>
</tr>
<tr>
<td>Loss body weight after 24 h fast (%)</td>
<td>10.3±1.5</td>
<td>10.5±2.1</td>
</tr>
<tr>
<td>Fasting liver weight (% BW)</td>
<td>3.7±0.5</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>Interprandial blood glucose (mM)</td>
<td>8.5±1.6</td>
<td>10.2±2.6</td>
</tr>
<tr>
<td>Fasting blood glucose (mM)</td>
<td>3.5±0.6</td>
<td>3.7±0.8</td>
</tr>
<tr>
<td>Fasting plasma β-OH-butyrate (mM)</td>
<td>1.0±0.3</td>
<td>0.9±0.3</td>
</tr>
</tbody>
</table>

Groups (n=10; age 20 weeks) (n=5; age 25 weeks) of \(Hint2^{+/+}\) and \(Hint2^{-/-}\) mice were fed ad libitum or fasted for 24 h. Values are means ± SD.

* \(P < 0.05\), unpaired t-test, \(Hint2^{+/+}\) vs. \(Hint2^{-/-}\).
Table 2. Effect of high fat diet on body/liver weights and metabolic parameters in *Hint2*+/+ and *Hint2*−/− mice.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th></th>
<th></th>
<th></th>
<th>High-fat diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+/+</td>
<td></td>
<td>−/−</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>3.2 ± 0.9</td>
<td>6.4 ± 2.0</td>
<td>9.9 ± 3.5</td>
<td>15.5 ± 3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food consumption (kcal)</td>
<td>5091 ± 537</td>
<td>4806 ± 20</td>
<td>9543 ± 400</td>
<td>8297 ± 1590</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight (% BW)</td>
<td>4.6 ± 0.4</td>
<td>4.0 ± 0.4</td>
<td>5.1 ± 0.5</td>
<td>4.2 ± 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose (mM)</td>
<td>5.4 ± 1.0</td>
<td>4.9 ± 1.3</td>
<td>4.1 ± 1.1</td>
<td>4.1 ± 1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting plasma insulin (ng/ml)</td>
<td>0.5 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>1.8 ± 0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma cholesterol (mM)</td>
<td>2.4 ± 0.2</td>
<td>3.1 ± 0.5</td>
<td>4.0 ± 0.7</td>
<td>5.2 ± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine transaminase (U/l)</td>
<td>12.3 ± 4.3</td>
<td>17.0 ± 5.5</td>
<td>53.3 ± 38.9</td>
<td>46.3 ± 20.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Groups (n=10) of *Hint2*+/+ and *Hint2*−/− mice (aged 8 weeks) were fed a control diet or high-fat diet for 8 weeks. Values are means ± SD.

* P < 0.05, unpaired t-test, *Hint2*+/+ vs. *Hint2*−/−

* P < 0.05, unpaired t-test, high-fat vs. control diet
References

protein hyperacetylation accelerate the development of the metabolic syndrome. Mol Cell 44: 177-190, 2011.


Figure legends

**Figure 1. Metabolic effects of fasting in $Hint2^{-/-}$ and $Hint2^{+/+}$ mice.**  
A. Hepatic triglycerides were measured in fed and 24 h fasted (n=5) mice (25 weeks of age). The 24 h fast induced triglyceride accumulation in both groups. Triglyceride levels were significantly higher in $Hint2^{-/-}$ than in $Hint2^{+/+}$ livers. B. Plasma triglyceride concentrations were significantly lower in $Hint2^{-/-}$ than in $Hint2^{+/+}$ fed mice. The 24 h fast induced a significant decrease only in $Hint2^{+/+}$ mice. C. Free fatty acids levels in fasting livers were not significantly different. D. Glycogen content was not different between $Hint2^{-/-}$ and $Hint2^{+/+}$ livers under fed conditions. Glycogen was depleted by the 24 h food deprivation. Values are mean ± SD. Panels A, B and D display significant changes tested by one-way ANOVA and Bonferroni’s multiple comparisons, $P< 0.05$. (*, $Hint2^{+/+}$ vs. $Hint2^{-/-}$ fed; **, $Hint2^{+/+}$ vs. $Hint2^{-/-}$ fasting; #, $Hint2^{+/+}$ fed vs. fasted; ##, $Hint2^{-/-}$ fed vs. fasted).

**Figure 2. Comparison of temperature and brown fat (BAT) in $Hint2^{-/-}$ and $Hint2^{+/+}$ mice.**  
A. Body temperature of $Hint2^{-/-}$ and $Hint2^{+/+}$ mice after fasting. Body temperature decreased after 24 h fasting. Fasted $Hint2^{-/-}$ mice (black bars) maintained a lower body temperature than did $Hint2^{+/+}$ mice (white bars). Mice (n=16 fed group, n=19 fasted group) were aged 20 weeks. Kruskal-Wallis test with Dunn’s multiple comparisons, $P< 0.05$. (*, $Hint2^{+/+}$ vs. $Hint2^{-/-}$ fasted; #, $Hint2^{+/+}$ fed vs. fasted; ##, $Hint2^{-/-}$ fed vs. fasted). Means ± SD are shown. B. The ratio of interscapular BAT to body weight (BATW/BW). The ratio was equal in the two groups under fed conditions. The decrease in BAT weight in fasting $Hint2^{-/-}$ mice was not statistically significant (Kruskal-Wallis test). Mice (n = 6-8 per group) were aged 20 weeks. C. Immunoblotting of the UCP1 protein in BAT mitochondria. BAT mitochondria isolated from 3 mice were pooled and tested by SDS-PAGE. CoxIV was
used as a loading control. After fasting, the expression of UCP1 relative to CoxIV increased in $\text{Hint2}^{-/-}$ (white bars) but decreased in $\text{Hint2}^{-/-}$ BAT (black bars). D. Quantitative PCR of UCP1 mRNA in BAT. RNA was extracted from 3 pools of BAT tissue harvested from fed and fasted $\text{Hint2}^{-/-}$ and $\text{Hint2}^{+/+}$ mice and analyzed in triplicate. UCP1 mRNA was 2.6-fold higher in fed $\text{Hint2}^{-/-}$ BAT (black bars) than in $\text{Hint2}^{+/+}$ BAT (white bars). (*, $P<0.05$, unpaired t-test). E. Free fatty acids (FFA) in BAT lysate. FFA was lower in fasted $\text{Hint2}^{-/-}$ than in $\text{Hint2}^{+/+}$ BAT (n=4) (*, $P<0.05$, unpaired t-test). F. Immunoblotting of phosphorylated perilipin in $\text{Hint2}^{-/-}$ and $\text{Hint2}^{+/+}$ BAT. Phosphorylation of perilipin was higher in fasted $\text{Hint2}^{-/-}$ mice. Actin was used as a loading control.

**Figure 3. Effect of high fat diet on lipid accumulation in $\text{Hint2}^{-/-}$ and $\text{Hint2}^{+/+}$ livers.** A. Microscopy of liver sections stained with H&E. Mice were fed a control diet (CD) or high fat diet (HFD) for 8 weeks. Shown is one representative image from each group. The HFD triggered steatotic changes in $\text{Hint2}^{-/-}$ livers. B. Oil Red-O staining of liver sections from mice fed a CD or HFD. Neutral lipids accumulated in both $\text{Hint2}^{-/-}$ and $\text{Hint2}^{+/+}$ livers after HFD. Shown is one representative image from each group. C. Quantification of Oil Red-O staining. The areas positive for Oil Red O were quantified with imaging software. Data from three fields per section were pooled and are presented as means (±SD) expressed as pixels/mm$^2$. $\text{Hint2}^{-/-}$ livers accumulated more lipids than did $\text{Hint2}^{+/+}$ livers. (n=10 per group) (ANOVA with Bonferroni’s multiple comparison test, $P<0.05$; #, $\text{Hint2}^{+/+}$ control vs. HFD; ##, $\text{Hint2}^{-/-}$ control vs. HFD; **, $\text{Hint2}^{-/-}$ vs. $\text{Hint2}^{+/+}$, HFD). D. Contingency table showing histological scoring of $\text{Hint2}^{-/-}$ and $\text{Hint2}^{+/+}$ livers after high-fat diet. Microscopic liver sections were classified into three NAFLD activity score categories. The groups were
not significantly different (2×3 Fisher's exact probability test with Freeman-Halton extension).

**Figure 4. Comparison of lysine acetylation patterns of mitochondrial proteins isolated from Hint2⁻/⁻ and Hint2⁺/⁺ mice.** A. Immunoblot of liver mitochondrial proteins isolated from Hint2⁻/⁻ and Hint2⁺/⁺ mice fed a control diet or high fat diet for 8 weeks. Acetylation was detected by means of an anti-acetylated lysine antibody. A range of mitochondrial proteins in Hint2⁻/⁻ livers were hyperacetylated. Voltage-dependent anion channel (VDAC) served as the loading control. Shown are representative samples of 3 individual mitochondrial isolations. B. Immunoblot of liver mitochondrial proteins isolated from Hint2⁻/⁻ and Hint2⁺/⁺ mice after 24 h fasting. A range of mitochondrial proteins in fasted Hint2⁻/⁻ livers were hyperacetylated. Glutamate dehydrogenase (GDH) served as the loading control. Shown are representative samples of 3 individual mitochondrial isolations. C. Immunoblot of mitochondrial proteins isolated from the BAT of fed and fasted Hint2⁻/⁻ and Hint2⁺/⁺ mice. Mitochondrial proteins of Hint2⁻/⁻ BAT showed a pattern of hyperacetylation, under both fed and fasted conditions. HSP60 served as the loading control. Shown are representative samples of 2 individual BAT mitochondrial isolations.

**Figure 5. Identification of hyperacetylated mitochondrial proteins from control and high fat diet fed mice.** Lysine-acetylated proteins from liver mitochondria of control and HFD Hint2⁻/⁻ and Hint2⁺/⁺ mice were immunoprecipitated with an anti-acetylated lysine antibody. Under control and high fat diet conditions, the extent of acetylation was higher in Hint2⁻/⁻ than in Hint2⁺/⁺ for carbamoyl phosphate synthase 1 (CPS1), acyl-CoA dehydrogenase long-chain (ACADL), acyl-CoA dehydrogenase medium-chain (ACADM), pyruvate carboxylase (PC), 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCoA), 3-hydroxy-3-methylglutaryl CoA lyase (HMGCoA)}
Acetylation of the peptidylprolyl cis-trans isomerase, cyclophilin D, was slightly increased after HFD. Shown are representative immunoprecipitations of mitochondrial proteins isolated from two mice. For HMGCo-A lyase, the mitochondria from two mice were pooled before immunoprecipitation.

**Figure 6. Identification of hyperacetylated mitochondrial proteins from fed and fasted Hint2−/− and Hint2+/+ livers.** A. Immunoprecipitation of lysine acetylated proteins from liver mitochondria of fed and fasted Hint2−/− and Hint2+/+ mice. Fed Hint2−/− mitochondria showed slightly higher acetylation of carbamoyl phosphate synthase 1 (CPS1), pyruvate carboxylase (PC), the α subunit of ATP synthase and urate oxidase. Fasted Hint2−/− mitochondria showed higher acetylation of CPS1, acyl-CoA dehydrogenase medium-chain (ACADM), 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCoAs), HMG-CoA lyase (HMGCoAl), cyclophilin D. Shown are representative immunoblots of two pooled mitochondrial preparations (upper panel) and one pooled preparation (lower panel). B. Immunoblot of sirtuin 5 expression in Hint2−/− and Hint2+/+ mitochondria. Hsp60 was used as loading control. Shown are two representative samples from individual mice. C. Immunoblots of mitochondrial protein isolated from Hint2+/+ and Hint2−/− livers. Acetylated p53 was detected in mitochondrial fractions of fed and fasting Hint2−/− livers. Sirtuin1 expression was not different between Hint2+/+ and Hint2−/− mitochondria. VDAC served as the loading control. Shown are four representative samples from individual mice.

**Figure 7. Identification of hyperacetylated mitochondrial proteins isolated from Hint2+/+ and Hint2−/− BAT.** A. Pooled BAT mitochondria from four fasting Hint2+/+ and Hint2−/− mice were immunoprecipitated with anti-acetylated lysine antibody. Acetylated UCP1 protein at 33 kDa was detected in Hint2+/+ but not Hint2−/−.
mitochondria. Increased acetylation was detected in Hint2−/− for ACADL, 3-hydroxyacyl Coenzyme A dehydrogenase short chain (Hadhsc), glutamate dehydrogenase (GDH) and Complex V ATPsynthase β and α. B. Immunoblot of sirtuin 3 expression in BAT mitochondria. The expression of sirtuin 3 was not different between Hint2−/− and Hint2+/+ BAT mitochondria. Hint2 protein was expressed only in Hint2+/+ BAT mitochondria. CoxIV served as the loading control.

Figure 8. Glutamate dehydrogenase (GDH) activity and sirtuin 4 expression in Hint2−/− and Hint2+/+ mitochondria. A. Mitochondrial GDH activity in Hint2−/− and Hint2+/+ mice. GDH activity was lower in Hint2−/− mice under both fed and fasting conditions. (n = 4 fed group, n = 5 fasted group). B. Mitochondrial GDH activity after stimulation with purified phosphodiesterase I (PDE). The difference in GDH activity in the presence and absence of PDE was normalized to basal activity and is reported as percentage. More latent GDH activity was recovered in Hint2−/− than in Hint2+/+ mitochondria. C. Immunoblot of GDH in liver mitochondria. GDH expression was not different between Hint2+/+ and Hint2−/− mitochondria. Hint2 protein was expressed only in Hint2+/+ mitochondria. CoxIV served as the loading control. Shown are representative samples of two individual preparations. D. Immunoblot of sirtuin 4 in liver mitochondria. The expression of sirtuin 4 was similar in mitochondria of Hint2+/+ and Hint2−/− livers. Hsp60 served as the loading control. E. Ammonia concentrations in plasma. The plasma ammonia concentration decreased in fasted Hint2−/− mice. In panels A, B, E, values are mean ± SD. (P< 0.05, one way ANOVA with Bonferroni’s multiple comparison test; *, fed Hint2+/+ vs. Hint2−/−; **, fasted Hint2+/+ vs. Hint2−/−; ###, Hint2−/− fed vs. fasted).

Figure 9. Expression and activity of sirtuin 3 in Hint2−/− and Hint2+/+ mitochondria. A. Immunoblot of sirtuin 3 protein levels in liver mitochondria. Sirtuin
3 expression tended to be higher in Hint2<sup>−/−</sup> than in Hint2<sup>+/+</sup> mitochondria under fed and fasted conditions. Hint2 was expressed only in Hint2<sup>+/+</sup> mitochondria. CoxIV served as the loading control. Shown are representative samples from three fed mice and four fasted mice. **B.** Sirtuin 3 activity in Hint2<sup>−/−</sup> and Hint2<sup>+/+</sup> liver mitochondrial preparations. Activity was measured in triplicate in pooled mitochondria from four mice per group. Activity was higher in Hint2<sup>−/−</sup> than in Hint2<sup>+/+</sup> mitochondria under fed and fasted conditions. Values are mean ± SD. (P< 0.05, one way ANOVA with Bonferroni’s multiple comparison test; *, fed Hint2<sup>+/+</sup> vs. Hint2<sup>−/−</sup>; **, fasted Hint2<sup>+/+</sup> vs. Hint2<sup>−/−</sup>). **C.** Effect of Hint2 protein on sirtuin 3 activity in Hint2<sup>−/−</sup> mitochondria. Sirtuin 3 activity was measured after addition of increasing concentrations of the co-substrate NAD<sup>+</sup>. Either recombinant GST or GST-Hint2 was added. No change in sirtuin 3 activity was detected. Measurements were made in triplicate from one pooled mitochondrial preparation.

**Figure 10. Expression of lipogenic enzymes in livers of Hint2<sup>−/−</sup> and Hint2<sup>+/+</sup> mice.** **A.** Immunoprecipitation of lysine acetylated proteins from mitochondria. Proteins were immunoprecipitated from Hint2<sup>−/−</sup> and Hint2<sup>+/+</sup> liver mitochondria with an anti-acetylated lysine antibody. An increase in the acetylation of carnitine palmitoyl transferase 1α (Cpt1α) was detected in Hint2<sup>−/−</sup> mitochondria. The acetylation of cytosolic or mitochondrial malonyl-CoA decarboxylase (MLYCD) was not different. Shown are representative samples from two individual mice. **B.** Malonyl-CoA and acetyl-CoA levels in Hint2<sup>+/+</sup> and Hint2<sup>−/−</sup> livers. The malonyl-CoA levels were higher in Hint2<sup>−/−</sup> (black bars) than in Hint2<sup>+/+</sup> (white bars) liver homogenate, whereas acetyl-CoA levels were not different. Values are mean ± SD. (n = 4) (P<0.05, unpaired t-test; *, Hint2<sup>+/+</sup> vs. Hint2<sup>−/−</sup>. **C.** Immunoblot of acetyl-CoA carboxylase (ACC) in Hint2<sup>−/−</sup> and Hint2<sup>+/+</sup> livers. The expression of ACC and phosphorylated ACC (pACC) were
not different between the groups. Actin served as the loading control. D. Immunoblot of fatty acid synthase (FAS) in Hint2^-/- and Hint2^+/+ livers. An increase in the level of fatty acid synthase (FAS) was detected in Hint2^-/- mice. β-Tubulin served as the loading control. Shown are two representative samples.

**Figure 11. Microsomal triglyceride transfer protein (MTTP) activity in Hint2^+/+ and Hint2^-/- livers.** A. MTTP fluorometric based assay. Livers from 20 - 25 week old Hint2^+/+ and Hint2^-/- mice were homogenized and 100 µg were used for MTTP activity. Data are expressed as a percentage of the mean value in Hint2^+/+ livers and are the average of three experiments. MTTP activity was reduced in Hint2^-/- (P<0.05, unpaired t-test). B. Immunoblot of MTTP in liver. The expression of MTTP and its heterodimeric partner, protein disulfide isomerase (PDI) was not different in Hint2^+/+ and Hint2^-/- livers. Actin was used as the loading control.
Fig 1

(A) Hepatic Triglycerides (nmol/mg protein)

(B) Plasma Triglycerides (nmol/ml)

(C) Free Fatty Acids (nmol/mg liver)

(D) Glycogen (µg/mg protein)
Fig. 2
Fig 3
**Fig 4**  

**A**  

<table>
<thead>
<tr>
<th>Control Diet</th>
<th>High Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hint</strong>2+/+</td>
<td><strong>Hint</strong>2+/+</td>
</tr>
<tr>
<td><strong>Hint</strong>2--</td>
<td><strong>Hint</strong>2--</td>
</tr>
</tbody>
</table>

**B**  

<table>
<thead>
<tr>
<th>Fasted</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hint</strong>2+/+</td>
<td><strong>Hint</strong>2+/+</td>
</tr>
<tr>
<td><strong>Hint</strong>2--</td>
<td><strong>Hint</strong>2--</td>
</tr>
</tbody>
</table>

**C**  

<table>
<thead>
<tr>
<th>Fed</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hint</strong>2+/+</td>
<td><strong>Hint</strong>2+/+</td>
</tr>
<tr>
<td><strong>Hint</strong>2--</td>
<td><strong>Hint</strong>2--</td>
</tr>
</tbody>
</table>

- **VDAC (33kD)**
- **GDH (55kD)**
- **Hsp60 (60kD)**
Fig 5

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control Diet</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunoprecipitate</td>
<td>+/+</td>
<td>-/-</td>
<td>Input</td>
<td>+/+</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>CPS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGCoAs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGCoAl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>High Fat Diet</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunoprecipitate</td>
<td>+/+</td>
<td>-/-</td>
<td>Input</td>
<td>+/+</td>
<td>-/-</td>
<td></td>
</tr>
<tr>
<td>CPS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGCoAs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGCoAl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 6

A  
Fed  
Fasted  
<table>
<thead>
<tr>
<th>Immunoprecipitate</th>
<th>Input</th>
<th>Immunoprecipitate</th>
<th>Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>−/−</td>
<td>+/+</td>
<td>−/−</td>
</tr>
<tr>
<td>CPS1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mHMGCoaS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGCoaI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophilin D</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Fed  
Fasted  
<table>
<thead>
<tr>
<th>Hint2+/+</th>
<th>Hint2−/−</th>
<th>Hint2+/+</th>
<th>Hint2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sirt5 30kD</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hsp60 60kD</td>
<td></td>
</tr>
</tbody>
</table>

C

High Fat Diet  
<table>
<thead>
<tr>
<th>Hint2+/+</th>
<th>Hint2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl p53</td>
<td>VDAC</td>
</tr>
</tbody>
</table>

Control Diet  
<table>
<thead>
<tr>
<th>Hint2+/+</th>
<th>Hint2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl p53</td>
<td>VDAC</td>
</tr>
</tbody>
</table>

Fasting  
<table>
<thead>
<tr>
<th>Hint2+/+</th>
<th>Hint2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl p53</td>
<td>Sirt1</td>
</tr>
<tr>
<td></td>
<td>VDAC</td>
</tr>
</tbody>
</table>
Fig 7
Figure 8
Fig 10
Fig 11