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ARTICLE

AAV8-mediated *Sirt1* gene transfer to the liver prevents high carbohydrate diet-induced nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) is the most common hepatic disease worldwide, and evidence suggests that it promotes insulin resistance and type 2 diabetes. Caloric restriction (CR) is the only available strategy for NAFLD treatment. The protein deacetylase Sirtuin1 (SIRT1), which is activated by CR, increases catabolic metabolism and decreases lipogenesis and inflammation, both involved in the development of NAFLD. Here we show that adeno-associated viral vectors of serotype 8 (AAV8)-mediated liver-specific *Sirt1* gene transfer prevents the development of NAFLD induced by a high carbohydrate (HC) diet. Long-term hepatic SIRT1 overexpression led to upregulation of key hepatic genes involved in β-oxidation, prevented HC diet-induced lipid accumulation and reduced liver inflammation. AAV8-*Sirt1*–treated mice showed improved insulin sensitivity, increased oxidative capacity in skeletal muscle and reduced white adipose tissue inflammation. Moreover, HC feeding induced leptin resistance, which was also attenuated in AAV8-*Sirt1*–treated mice. Therefore, AAV-mediated gene transfer to overexpress SIRT1 specifically in the liver may represent a new gene therapy strategy to counteract NAFLD and related diseases such as type 2 diabetes.

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INTRODUCTION

Type 2 diabetes is a metabolic disease whose prevalence is increasing at an alarming rate.[1] The accumulation of fat, particularly in the liver, is a key factor in the onset of insulin resistance and type 2 diabetes.[2] Such accumulation, also known as nonalcoholic fatty liver disease (NAFLD), is a condition present in up to 50–75% of patients affected by type 2 diabetes, while it affects 20–30% of the general population.[3] NAFLD can even progress into nonalcoholic steatohepatitis (NASH) which is characterized by liver inflammation and damage.[4] Variations in dietary habits, such as increased consumption of carbohydrates, together with sedentary lifestyle are in part responsible for the increased frequency of metabolic syndrome and its hepatic component, NAFLD.[4,5]

To date, the only treatment capable of counteracting or ameliorating liver steatosis is based on lifestyle intervention by means of caloric restriction (CR).[6] However, the underlying mechanisms as well as the exact effects exerted by CR are largely unknown. One of the key genes involved in the numerous effects of CR is *Sirt1*, a homologue of the yeast silent information regulator 2 gene.[7,8] SIRT1 is a member of the Sirtuins, a family of protein deacetylases and ADP-ribosyltransferases involved in different pathways that regulate processes such as cellular metabolism, stress response, CR and lifespan.[9,10] In mammals, seven sirtuins (SIRT1-7) have been identified.[8] SIRT1 possesses a NAD^+-dependent deacetylase activity and is capable of modulating gene expression in several key metabolic tissues such as liver, skeletal muscle and white adipose tissue (WAT) by deacetylating proteins and transcription factors. As shown by transgenic animal models with SIRT1 overexpression or by animals treated with SIRT1 activators, SIRT1 upregulates the expression of genes that control oxidative metabolism and downregulates lipogenesis and inflammation, both involved in the development of NAFLD.[11–14] Moreover, the short-term, non-tissue specific overexpression of SIRT1 by adenoviral vectors also attenuated insulin resistance and hepatic steatosis.[15] In contrast, the hepatocyte-specific deletion of *Sirt1* results in hepatic steatosis and inflammation.[16]

To study the efficacy of long-term hepatocyte-specific *Sirt1* gene transfer in preventing high carbohydrate (HC) diet-induced NAFLD and insulin resistance in adult mice, we took advantage of the use of adeno-associated viral (AAV) vectors. AAV vectors have shown promising results in several *in vivo* applications[17] driving multi-year expression of therapeutic transgenes for a variety of diseases.[18] We found that long-term liver-specific *Sirt1* gene transfer led to upregulation of key β-oxidation genes, preventing HC diet-induced triglyceride accumulation, which in turn reduced macrophage infiltration and inflammation in the liver. Moreover, hepatic *Sirt1* gene transfer also attenuated the insulin resistant state induced by the HC diet. These data suggest that AAV8-mediated hepatic SIRT1 overexpression may be a new gene therapy approach to prevent NAFLD, insulin resistance and type 2 diabetes.

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RESULTS

Liver-specific *Sirt1* gene transfer

The aim of our study was to assess the ability of liver SIRT1 overexpression in preventing NAFLD induced by high carbohydrate consumption. We chose to use a model of NAFLD induction based on the feeding of animals with a diet enriched in sucrose (35% sucrose). The high consumption of fructose and/or sucrose for long periods of time has been associated with the increase in the incidence of NAFLD in the western society.[19] The 35% sucrose diet is in composition close to the human intake of sucrose and fructose in this population.[20] To achieve SIRT1 overexpression specifically in the liver, we used AAV vectors of serotype 8 and the human alpha 1-anti-trypsin (hAAT) promoter, a well-documented combination that restricts transgene expression to hepatocytes.[21,22] Using the same combination of vector serotype and promoter, a dose of 5×10^{11} vector genomes (vg)/mice of a *Gfp*-coding vector administered intravenously resulted, two weeks after vector delivery, in 55% specific hepatocyte transduction (Supplementary Figure S1a,b).

Mice were analyzed 15 weeks after intravenous injection of AAV8-hAAT-*Sirt1* and AAV8-hAAT-Null vectors at a dose of 5×10^{11} vg/mice to study the efficacy of vector transduction. The quantification of vector genomes showed values between 5.7 and 8.0 vg/cell in the livers of mice that received AAV8-*Sirt1* vectors. SIRT1 protein overexpression was detected in the liver of AAV8-*Sirt1*–treated animals at levels that were 2.3-fold higher than those of Null-treated mice ($P < 0.05$) (Figure 1a). Furthermore, the observed increase in hepatic SIRT1 protein levels was within the range reported in situations of caloric restriction (CR),[7] indicating that AAV-mediated *Sirt1* gene transfer was mimicking physiological conditions. Then, we evaluated the levels of SIRT1 activity in whole liver extracts of treated mice with two different approaches. In agreement with increased SIRT1 protein expression, SIRT1 activity showed higher (2.5-fold increase) velocity of SIRT1-mediated deacetylation of p53, a known target of SIRT1,[23] in AAV8-*Sirt1*–treated mice in comparison with AAV8-Null–treated animals (Figure 1b). Moreover, the levels of endogenous acetylated p53 (Ac-p53^{Lys379}) were significantly reduced by approximately 50% in liver nuclear extracts from mice that received AAV8-*Sirt1* vectors (Figure 1c), corroborating the increase in the activity of hepatic SIRT1 after AAV8-*Sirt1* injection.

Lower lipid accumulation in the liver of AAV8-*Sirt1*–treated mice fed with a HC diet

To examine whether an increase in hepatic SIRT1 expression may protect against the development of NAFLD, AAV8-*Sirt1*- and AAV8-Null–treated mice were fed a HC diet during 15 weeks. Histological analysis showed that the deposition of fat in the liver was increased in mice fed a HC diet compared with mice fed a standard (STD) diet (Supplementary Figure S2a). The injection of AAV8-Null vectors did not affect the degree of accumulation of lipids in the liver of animals fed a HC diet (Supplementary Figure S3a). However, this lipid accumulation was greatly attenuated following hepatic gene transfer with AAV8-*Sirt1* (Figure 2a). Indeed, the triglyceride content was reduced by 43% in the liver of AAV8-*Sirt1*–treated mice in comparison with AAV8-Null mice (Figure 2b), in parallel with a decrease of 17% in the weight of the organ (Figure 2c). HC diet induces lipid accumulation in the liver through increased lipogenesis and reduced β-oxidation (Supplementary Figure S2a,b).[24] No differences were observed in the hepatic expression levels of lipogenic genes such as *Sterol regulatory element binding transcription factor 1c* (*Srebf1*) and *Fatty acid synthase* (*Fasn*) in mice that received AAV8-*Sirt1* vectors (Data not shown). However, the liver expression of genes key to lipid oxidation and mitochondrial biogenesis such as *Peroxisome proliferative activated receptor, gamma, coactivator 1 α* (*Ppargc1α*), *Long-chain acyl-Coenzyme A dehydrogenase* (*Acadl*), *Very long chain acyl-CoA dehydrogenase* (*Acadvl*), *Sirtuin 6* (*Sirt6*) and *Sirtuin 3* (*Sirt3*) was increased in treated animals (Figure 2d). A slight upregulation of

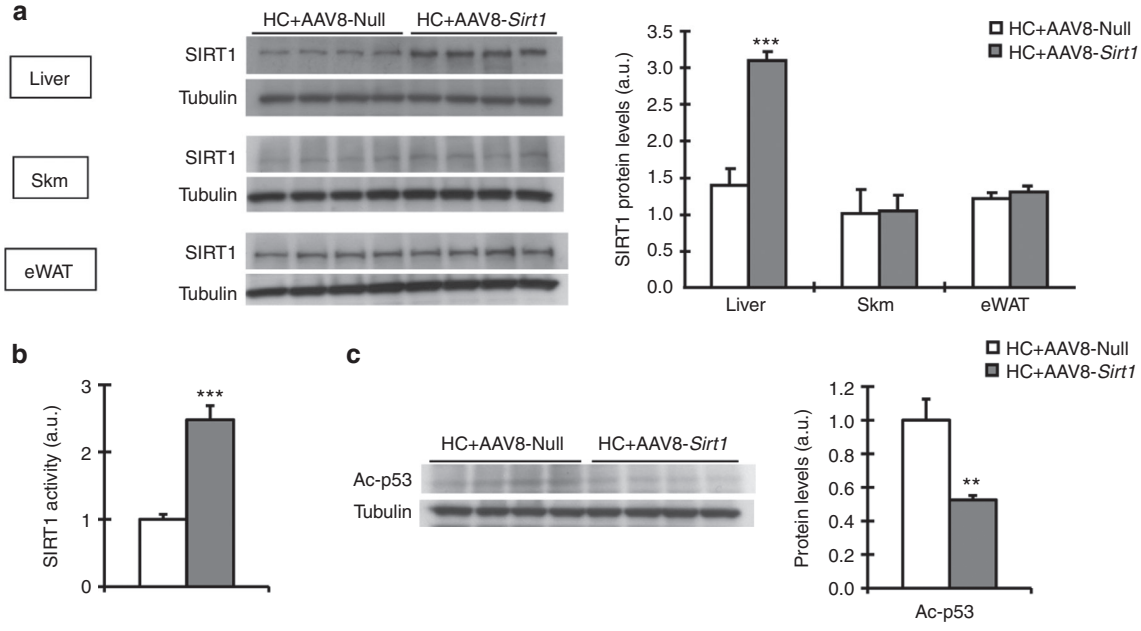

Figure 1 Systemic administration of AAV8-hAAT-*Sirt1* vectors led to specific SIRT1 overexpression in the liver. (**a**) A representative Western blot and its quantification are shown for SIRT1 in the liver, skeletal muscle (Skm), and epididymal white adipose tissue (eWAT) of mice fed with a high carbohydrate (HC) diet and treated with AAV8-hAAT-Null (AAV8-Null) or AAV8-hAAT-*Sirt1* vectors (AAV8-*Sirt1*) at a dose of 5×10^{11} vg/mice for 15 weeks. (**b**) SIRT1 deacetylation activity in liver crude nuclear extracts. (**c**) Representative western blot and quantification of Acetyl-p53-Lys^{379} protein levels in nuclear fractions from livers of AAV8-Null- and AAV8-*Sirt1*–treated mice, using tubulin as a loading control. All analyses were performed after 15 weeks on HC diet. Data represent the mean ± SEM of at least four animals per group. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus AAV8-Null. a.u., arbitrary units.

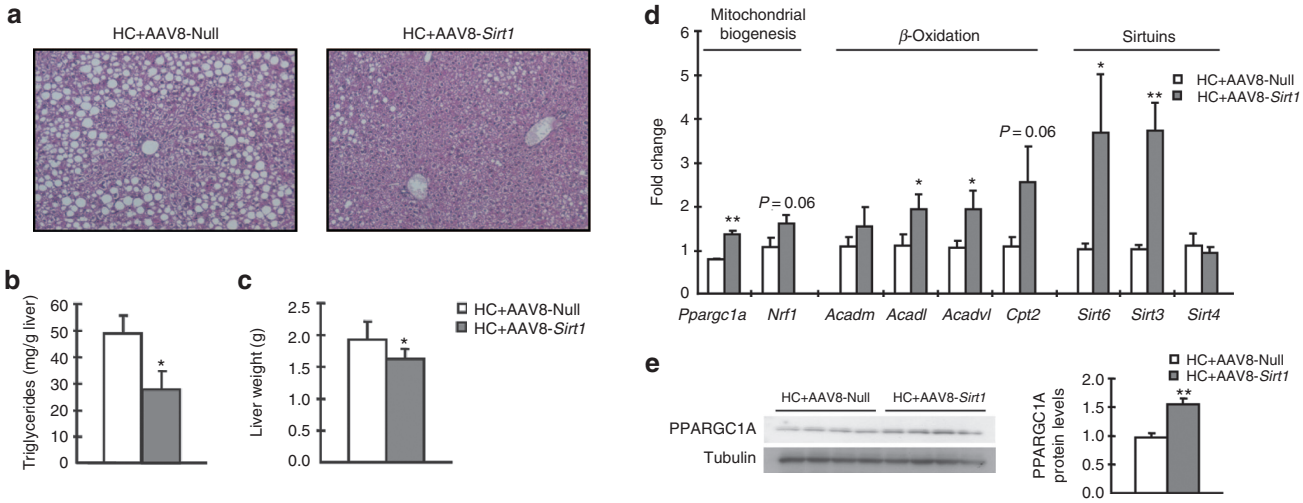

Figure 2 AAV8-*Sirt1*–treated mice showed reduced hepatic lipid accumulation when fed a high carbohydrate diet. (**a**) Representative sections of the liver from AAV8-Null– and AAV8-*Sirt1*–treated mice fed with a high carbohydrate (HC) diet stained with hematoxylin-eosin. Original magnification ×100. (**b**) Liver triglyceride content. (**c**) Liver weight (**d**) Expression levels of *Peroxisome proliferative activated receptor, gamma, coactivator 1 α* (*Ppargc1α*), *Nuclear respiratory factor 1* (*Nrf1*), *Medium-chain acyl-Coenzyme A dehydrogenase* (*Acadm*), *Long-chain acyl-Coenzyme A dehydrogenase* (*Acadl*), *Very long chain acyl-CoA dehydrogenase* (*Acadvl*), *Carnitinepalmitoyltransferase 2* (*Cpt2*), *Sirtuin 6* (*Sirt6*), *Sirtuin 3* (*Sirt3*), *and Sirtuin 4* (*Sirt4*) were analyzed in the liver of AAV8-Null and AAV8-*Sirt1* mice. (**e**) Representative western blot and quantification of hepatic PPARGC1a protein levels of AAV8-Null and AAV8-*Sirt1* mice, using tubulin as a loading control. All analyses were performed after 15 weeks on HC diet. Data represent the mean ± SEM of at least four animals per group. **P* < 0.05 and ***P* < 0.01 versus AAV8-Null.

Nuclear respiratory factor 1 (*Nrf1*) and *Carnitinepalmitoyltransferase 2* (*Cpt2*) mRNA was also observed in mice treated with AAV8-*Sirt1* and fed a HC diet (Figure 2d). Furthermore, the content of PPARGC1A protein was also increased in the liver following AAV8-*Sirt1* treatment (Figure 2e). Thus, these data suggest that the reduction in the hepatic accumulation of lipids could result from increased β-oxidation in the liver of AAV8-*Sirt1*–treated mice.

Mice treated with AAV8-*Sirt1* showed decreased macrophage infiltration in the liver

Immunostaining against the macrophage marker MAC-2 revealed a high degree of macrophage infiltration in the liver of AAV8-Null mice fed a HC diet, but not in that of AAV8-*Sirt1*–treated mice fed the same diet (Figure 3a). In agreement with a pro-inflammatory role of HC diet, AAV8-Null–treated mice fed this diet also showed hepatic upregulation of the macrophage marker *F4/80* and the proinflammatory cytokines *Interleukin 1*β (*Il1b*) and *Chemokine (C-C motif) ligand 5* (*Ccl5*) when compared with animals fed a STD diet (Supplementary Figure S4a). In contrast, HC-fed mice treated with AAV8-*Sirt1* showed downregulation of *F4/80* and *Cd68* as well as *Il1b* and *Ccl5* (Figure 3b). The serum levels of alanine transaminase (ALT) were also decreased in AAV8-*Sirt1*–treated mice in comparison with AAV8-Null–treated mice fed a HC diet (Figure 3c), but were not affected by the injection of the AAV8-Null vector per se (Supplementary Figure S3b). Altogether, these results suggest that SIRT1 overexpression protects the liver against HC diet-induced NAFLD-associated inflammation.

Serum lipid profile and glucose homeostasis

Despite having less fat accumulation in the liver, AAV8-*Sirt1*–treated mice had serum triglyceride, HDL-cholesterol, LDL-cholesterol and total-cholesterol levels similar to those of AAV8-Null–treated mice (Supplementary Figure S5a–d). However, mice receiving AAV8-*Sirt1* vectors presented a significant increase in serum free fatty acid levels, with no changes in glycerol levels, suggesting differences

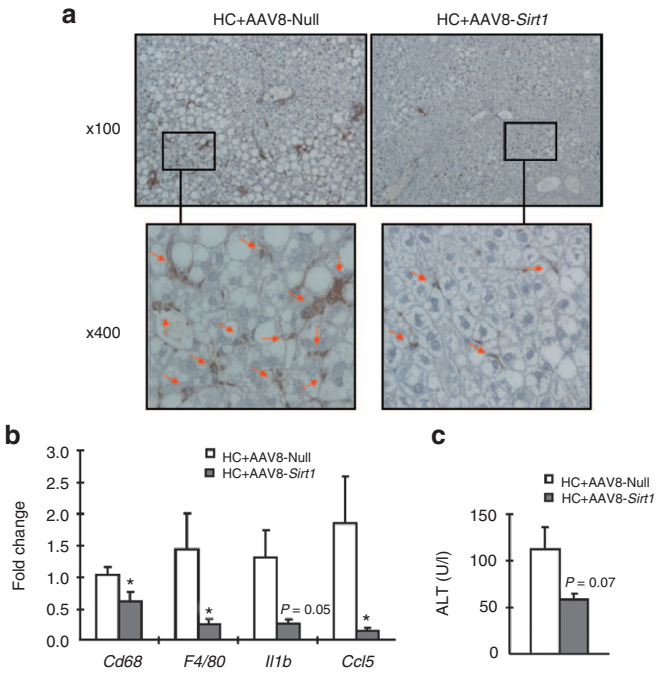

Figure 3 Lower macrophage infiltration and inflammation in the liver of AAV8-*Sirt1*–treated mice fed a high carbohydrate (HC) diet. (**a**) Immunostaining against MAC-2 (brown) in the liver of AAV8-Null and AAV8-*Sirt1* mice. Original magnification ×100 and ×400 (insets). Red arrows indicate MAC-2$^+$ cells. (**b**) *Cd68*, *F4/80*, *Interleukin 1*β (*Il1b*) and *Chemokine* (*C-C motif*) *ligand 5* (*Ccl5*) mRNA expression levels in the liver of AAV8-Null and AAV8-*Sirt1* mice. (**c**) Serum alanine transaminase (ALT) levels in AAV8-Null and AAV8-*Sirt1* mice. All analyses were performed after 15 weeks on HC diet. Data represent the mean ± SEM of at least four animals per group. **P* < 0.05 versus AAV8-Null.

in free fatty acid re-esterification between AAV8-Null and AAV8-*Sirt1*–treated mice (Supplementary Figure S5e,f). The effects of SIRT1 overexpression on whole body glucose homeostasis were

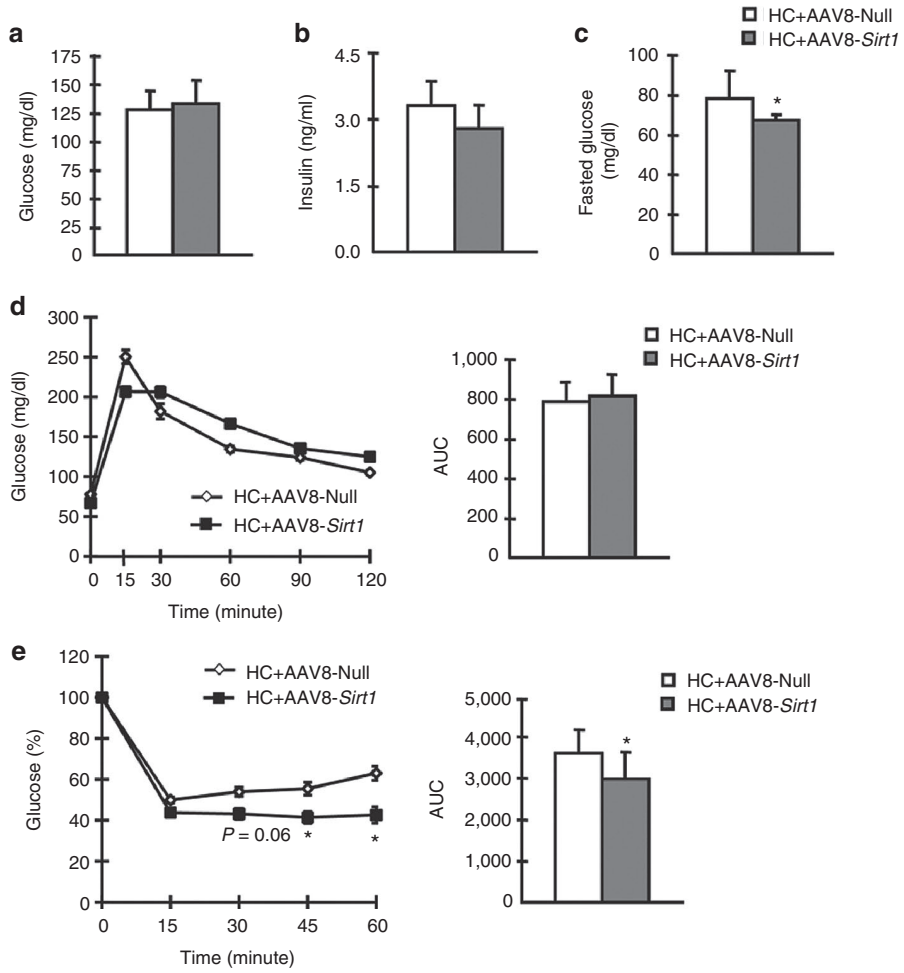

Figure 4 AAV8-*Sirt1*–treated mice were protected against insulin resistance induced by a high carbohydrate (HC) diet. (**a**) Blood glucose and (**b**) insulin levels in fed conditions are shown. (**c**) Fasted glucose levels. (**d**) Glucose tolerance was determined in fasted mice after an intraperitoneal injection of glucose (1 g/kg body weight), and blood glucose levels were measured at the indicated time points. On the right, area under the curve (AUC) of the glucose tolerance test was calculated. (**e**) Insulin sensitivity was determined after an intraperitoneal injection of insulin (0.75 units/kg body weight). Results are calculated as the percentage of initial blood glucose levels. Area under the curve (AUC) of the insulin tolerance test was calculated. All analyses were performed after 15 weeks on HC diet. Data represent the mean ± SEM of at least eight animals per group. *$P < 0.05$ versus AAV8-Null.

also examined. In fed conditions, no differences in glucose and insulin levels were observed between AAV8-Null and AAV8-*Sirt1*–treated mice (Figure 4a,b). However, AAV8-*Sirt1*–treated mice presented decreased fasting glucose levels (Figure 4c), suggesting a reduction in gluconeogenesis. Nevertheless, glucose tolerance was similar between both groups (Figure 4d). When an intraperitoneal insulin tolerance test (ITT) was performed, AAV8-*Sirt1*–treated mice showed an improved response to insulin, reducing basal glucose levels by 60%, whereas AAV8-Null mice reduced glucose levels by only 40% (Figure 4e,f).

Increased β-oxidation in skeletal muscle following AAV8-*Sirt1* treatment

AAV8-*Sirt1* mice showed a trend to accumulate less triglycerides in skeletal muscle than AAV8-Null mice (Figure 5a), suggesting that β-oxidation could be upregulated in this tissue following the treatment. In agreement with this hypothesis, the expression of key genes involved in lipid oxidation such as *Mitochondrial uncoupling protein 2* (*Ucp2*), *Peroxisome proliferator activated receptor δ* (*Ppard*) and *Pyruvate dehydrogenase kinase isoenzyme 4* (*Pdk4*) was increased in AAV8-*Sirt1*–treated mice (Figure 5b). In addition, the levels of phosphorylated AMP activated kinase (AMPK) were approximately 50% higher in mice receiving AAV8-*Sirt1* vectors versus those receiving AAV8-Null (Figure 5c). The phosphorylation of acetyl-CoA carboxylase, one of the targets of AMPK, was also augmented in AAV8-*Sirt1*–treated mice (Figure 5c). Thus, these results suggested that treatment with AAV8-*Sirt1* vectors increased β-oxidation in the skeletal muscle.

Reduced hypertrophy and inflammation in eWAT of AAV8-*Sirt1*–treated mice

After 15 weeks of HC diet, mice presented a greater degree of fat accumulation in adipose tissue, which was reflected by increased eWAT weight (data not shown) and mean area of adipocytes related to mice fed a STD diet (Supplementary Figure S6a,b). In AAV8-*Sirt1*–treated mice fed a HC diet, although body weight gain and food intake were similar to those of AAV8-Null mice (data not shown), a slight decrease in eWAT weight was observed, and this was associated to a 30% reduction in the mean area of adipocytes (Figure 6a–c). Moreover, AAV8-*Sirt1*–treated mice presented changes in the distribution of white adipocyte area frequencies in eWAT, showing reduced number of large-sized adipocytes and increased number of small and intermediate-sized adipocytes (Figure 6d).

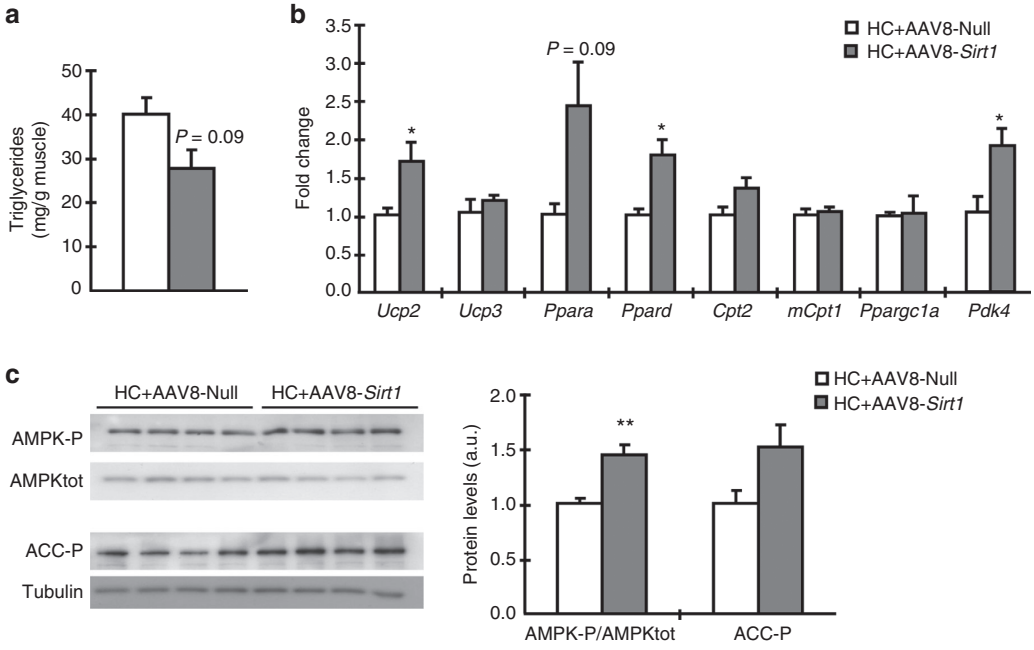

Figure 5 AAV8-*Sirt1*–treated mice showed increased β-oxidation capacity in skeletal muscle. (**a**) Triglyceride content in skeletal muscle. (**b**) Expression levels of *Mitochondrial uncoupling protein 2* (*Ucp2*), *Mitochondrial uncoupling protein 3* (*Ucp3*), *Peroxisome proliferator activated receptor α* (*Pparα*), *Peroxisome proliferator activated receptor δ* (*Ppard*), *Carnitinepalmitoyltransferase 2* (*Cpt2*), *Carnitinepalmitoyltransferase 1* (*mCpt1*), *Peroxisome proliferative activated receptor, gamma, coactivator 1 α* (*Ppargc1α*), and *Pyruvate deshydrogenase kinase isoenzyme 4* (*Pdk4*). (**c**) Representative western blots and quantifications are shown for total AMP protein kinase (AMPKtot), phosphorylated AMPK (AMPK-P), phosphorylated Acetyl-CoA Carboxylase (ACC-P) and tubulin protein levels in skeletal muscle of AAV8-Null and AAV8-*Sirt1* mice. Tubulin was used as a loading control. All analyses were performed after 15 weeks on HC diet. Data represent the mean ± SEM of at least four animals per group. **P* < 0.05 and ***P* < 0.01 versus AAV8-Null. a.u., arbitrary units.

WAT hypertrophy is usually linked to the development of inflammation and hypoxia.[25] Accordingly, HC diet induced upregulation of *Inducible nitric oxide synthase 2* (*Nos2*) and *Tumour necrosis factor α* (*Tnf*), as well as *Hypoxia inducible factor 1a* (*Hif1a*) in eWAT when compared with mice fed a STD diet (Supplementary Figure S6c). In contrast, in AAV8-*Sirt1*–treated mice fed with a HC diet, the eWAT expression levels of *Chemokine (C-C motif) Ligand 2* (*Ccl2*), *Nos2* and *Hif1a* were reduced, whereas no changes were observed in the anti-inflammatory cytokine *Interleukin 10* (Figure 6e). In agreement with the smaller size of the adipose tissue compartment in AAV8-*Sirt1*–treated mice, the eWAT expression and the serum levels of leptin were lower in these animals (Figure 6f). However, no differences in the expression or circulating levels of adiponectin were detected (Figure 6f). Thus, all these data suggest that inflammation and hypoxia were decreased in adipose tissue following the treatment with AAV8-*Sirt1*, although *Sirt1* gene transfer was restricted to the liver.

AAV8-*Sirt1*–treated mice were more sensitive to leptin action in peripheral tissues

Since fructose intake triggers leptin resistance in the liver, and this in turn may lead to a decrease in lipid oxidation,[24,26] we examined leptin signaling in AAV8-*Sirt1*–treated mice fed a HC diet. The levels of hepatic mitogen-activated protein kinase Erk1/2, which is a target of leptin activated by phosphorylation,[26] were increased in AAV8-*Sirt1*–treated mice (Figure 7a). In addition, the amount of the nuclear, fully activated form of Signal Transducer and Activator of Transcription 3 (STAT3), which is phosphorylated in response to leptin (STAT3-PSer^{727}),[27] was increased in the liver of mice that received AAV8-*Sirt1* vectors (Figure 7a). The hepatic levels of STAT3-PSer^{727}, which are an indicator of leptin sensitivity, correlated negatively with serum leptin levels (Figure 7b), suggesting that AAV8-*Sirt1*–treated mice remained sensitive to leptin. In skeletal muscle, leptin acts mainly via AMPK activation. Thus, the observation of an increase in skeletal muscle AMPK-P/AMPK total ratio in AAV8-*Sirt1*–treated mice (Figure 5c) together with the negative correlation between the AMPK-P/AMPK total ratio and serum leptin levels (Figure 7c), also suggested an improvement of leptin sensitivity in this tissue following treatment.

DISCUSSION

The excess of carbohydrate consumption for prolonged periods of time, mainly in the form of fructose or sucrose intake, is one of the main causes of NAFLD development.[5,28] The only available treatment for NAFLD is based on lifestyle intervention by decreasing nutrient consumption.[29] One of the molecular mechanisms by which CR improves hepatic metabolism is SIRT1 activation.[30] Thus, the overexpression of SIRT1 *in vivo* is an attractive approach to mimic CR. Although several studies have demonstrated that whole-body activation of SIRT1 has beneficial effects on metabolism,[11,12,14] the tissue-specific actions of SIRT1 remain to be elucidated. Here we evaluated the therapeutic efficacy of the long-term overexpression of SIRT1 in the liver mediated by AAV8 vectors in which a hepatocyte-specific promoter drives transgene expression. We report for the first time that long-term liver-specific overexpression of SIRT1 improves NAFLD in adult mice fed with a HC diet. Moreover, our study has shed light on the mechanisms by which SIRT1 activation counteracts the effects of HC. Liver SIRT1 overexpression increased lipid oxidation and reduced inflammation in the liver, improved insulin sensitivity, increased oxidative capacity in skeletal muscle and reduced white adipose tissue inflammation and counteracted the development of leptin resistance.

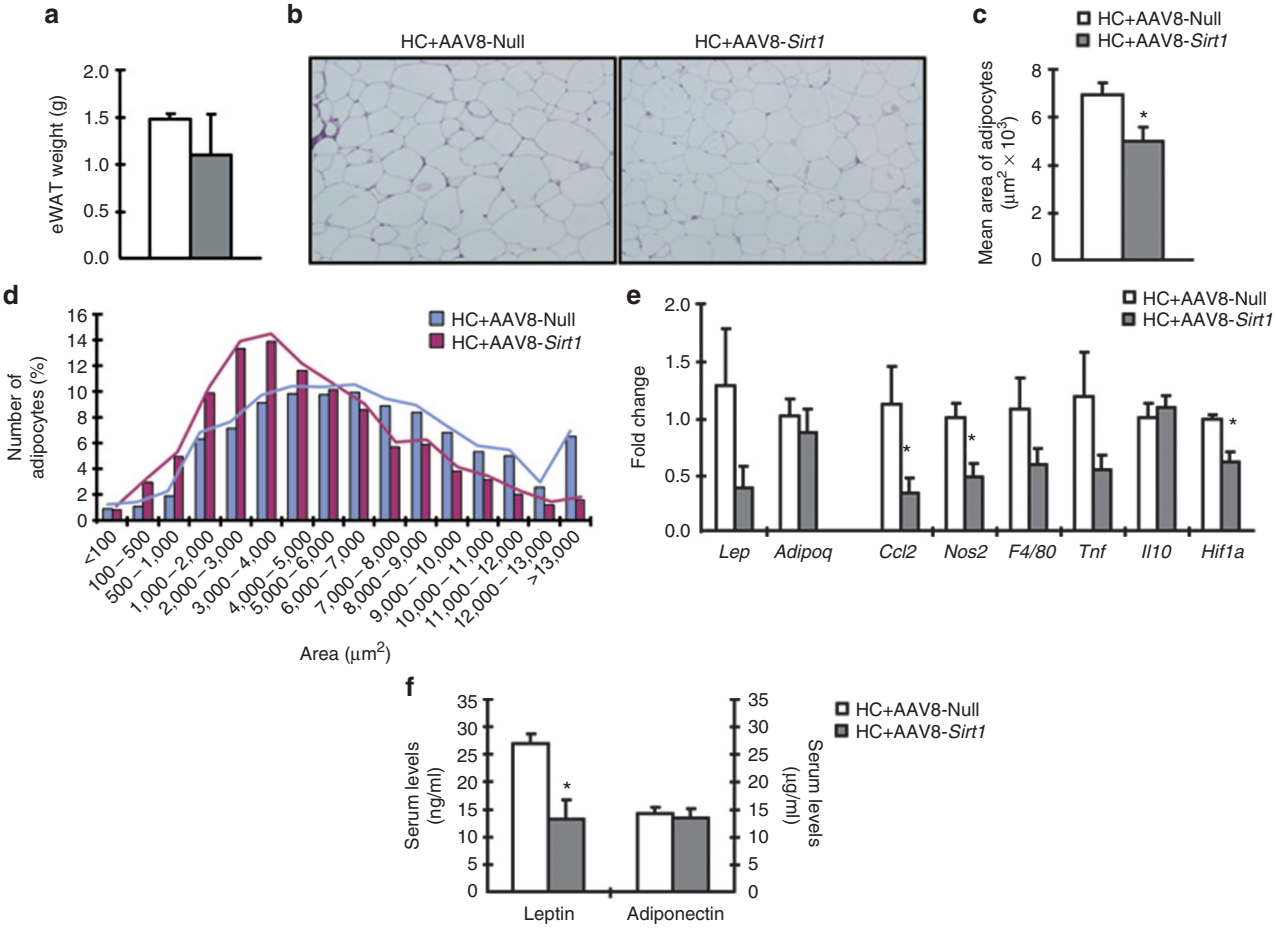

Figure 6 Reduced adipocyte size and inflammation in epididymal white adipose tissue of AAV8-*Sirt1*–treated mice. (**a**) Epididymal white adipose tissue (eWAT) weight. (**b**) Representative sections of eWAT, stained with hematoxylin-eosin. Original magnification ×100. (**c**) Mean adipocyte area. (**d**) Frequency distribution of adipocyte area. (**e**) *Leptin* (*Lep*), *Adiponectin* (*Adipoq*), *Chemokine* (*C-C motif*) *Ligand 2* (*Ccl2*), *Inducible Nitric Oxide Synthase 2* (*Nos2*), *F4/80*, *Tumour necrosis factor α* (*Tnfα*), *Interleukin 10* (*Il10*), and *Hypoxia Inducible Factor 1a* (*Hif1a*) expression levels in eWAT. (**f**) Serum leptin and adiponectin levels. All analyses were performed after 15 weeks on HC diet. Data represent the mean ± SEM of at least four animals per group. *$P < 0.05$ versus AAV8-Null.

The levels of SIRT1 overexpression observed following AAV8-mediated gene transfer were unexpectedly low for the dose of *Sirt1*-coding vectors used in the study. However, the quantification of vector genomes in the liver of these mice or the percentage of liver cells transduced using an equal dose of an AAV8-hAAT-*Gfp* vector obtained by the same manufacturing process, were within the range reported for AAV8-mediated gene transfer to the liver at doses similar to the ones used in the present study.[31,32] It is worth noticing that similar levels of SIRT1 overexpression have been reported in studies in which the SIRT1 gene is delivered by systemic administration of adenoviral vectors,[15] a vector type with high tropism for the liver, and after caloric restriction,[7] suggesting the existence of regulatory mechanisms that control the levels of SIRT1 protein. In fact, mammalian SIRT1 is regulated by endogenous proteins involved in signal transduction and transcription, as well as by microRNAs such as miRNA-34a.[8] Although SIRT1 overexpression was not too high, the measurement of SIRT1 activity and Ac-p53^{Lys379} levels indicated that the activity of SIRT1 was clearly increased in mice following AAV8-*Sirt1* delivery.

AAV8-*Sirt1*–treated mice showed lower liver triglyceride content without changes in total body weight gain. A similar reduction in hepatic lipid accumulation has been observed in transgenic mice overexpressing SIRT1 ubiquitously or in mice treated with SIRT1 activators.[11,14] The observed upregulation of genes involved in β-oxidation could account for the reduced lipid accumulation in the liver of AAV8-*Sirt1*–treated mice. In particular, we documented an increase in the expression and protein levels of PPARGC1A, a master regulator of mitochondrial biogenesis and substrate utilization.[33] In this regard, SIRT1 has been shown to upregulate PPARGC1A transcription and activity by direct deacetylation of this factor.[34] In contrast, lower *Ppargc1a* expression levels were reported in mice with hepatocyte-specific deletion of SIRT1.[16] AAV8-*Sirt1*–treated mice also showed increased expression of *Sirt3,* a target of PPARGC1A, which has been reported to activate *Acadl*[35,36] a fatty-acid oxidation enzyme also upregulated in the liver following the treatment with AAV8-*Sirt1* vectors. Collectively, all these data suggest that SIRT1 treatment increases the liver capacity to oxidate lipids, which could explain the reduced lipid accumulation in this tissue.

HC intake is associated with increased hepatic inflammation,[29] a process that results from lipid peroxidation.[4,37] In this situation, macrophages and monocytes are important sources of pro-inflammatory cytokines in the liver and key players in the progression to NASH and its resolution.[38] HC diet feeding induced vast hepatic macrophage infiltration in AAV8-Null mice, but this was prevented by AAV8-*Sirt1*

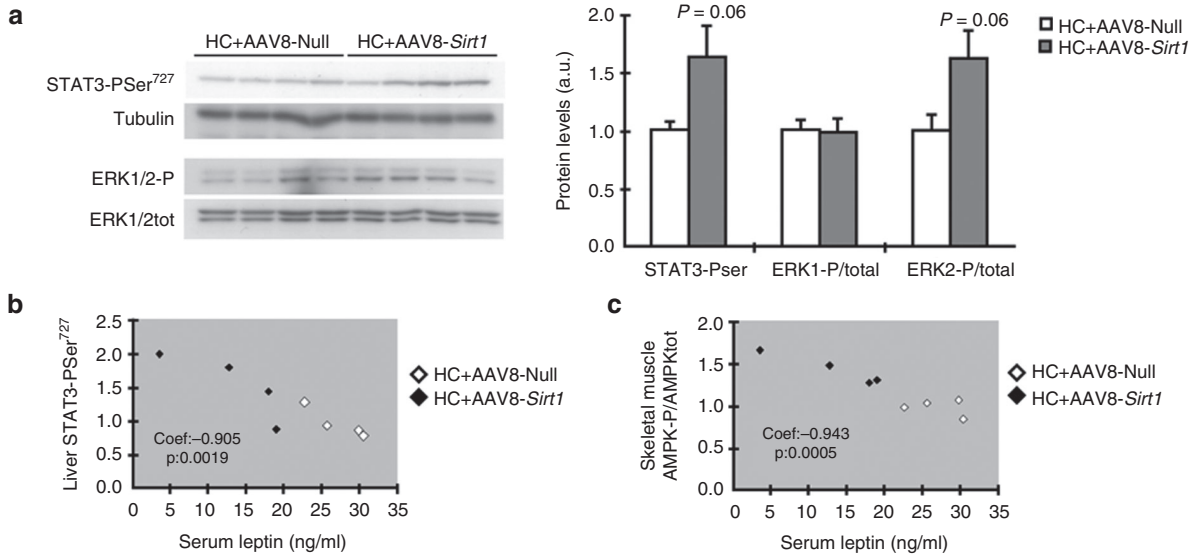

Figure 7 Hepatic SIRT1 overexpression counteracts the development of leptin resistance induced by a high carbohydrate diet. (**a**) Representative western blots and quantifications are shown for the active and phosphorylated form of Signal Transducer and Activator of Transcription 3 at the Serine 727 residue (STAT3-PSer^{727}), phosphorylated mitogen-activated protein kinase ERK1/2 (ERK1/2-P), total ERK1/2 (ERK1/2tot) and tubulin protein levels in liver of AAV8-Null and AAV8-*Sirt1* mice. Tubulin was used as a loading control. Data represent the mean ± SEM of at least four animals per group. Correlation analyses between serum leptin levels and hepatic protein levels of STAT3-PSer^{727} (**b**) and serum leptin levels and the ratio AMPK-P/AMPKtot in skeletal muscle (**c**) in AAV8-Null and AAV8-*Sirt1* mice. All analyses were performed after 15 weeks on HC diet. a.u., arbitrary units.

treatment as confirmed by the detection of decreased Mac-2 immunostaining and the downregulation of *Cd68*, *F4/80*, *Il1b*, and *Ccl5* expression levels. This was in agreement with the reduction in the levels of proinflammatory cytokines observed in SIRT1 transgenic mice fed a HFD,[11] while whole-body or hepatic disruption of the *Sirt1* gene resulted in increased hepatic inflammation.[16,39] Moreover, *Ccl5* has been described as one of the first inflammatory markers that are upregulated when hepatic steatosis develops.[40] Therefore, the observation of decreased *Ccl5* expression in AAV8-*Sirt1*–treated mice further highlights that these mice are effectively protected against HC diet-induced NAFLD.

AAV8-*Sirt1*–treated mice did not only show improved liver metabolism, but also amelioration of insulin sensitivity. This improvement in glucose metabolism may result from the reduction in inflammation observed both in liver and adipose tissue, since insulin signaling is well described to be impaired by inflammation.[41] Moreover, lipid accumulation in tissues that are key to glucose disposal such as liver and skeletal muscle is also associated with insulin resistance.[42,43] Therefore, the lower degree of inflammation together with the decreased triglyceride content detected in these tissues could play a pivotal role in the peripheral amelioration of insulin sensitivity in AAV8-*Sirt1*–treated mice. Regarding insulin sensitivity, similar beneficial effects have been reported in SIRT1 transgenic mice or mice treated with SIRT1 activators.[12,44]

Liver-specific SIRT1 overexpression was also associated with positive effects in extra-hepatic tissues, likely due to the reduced levels of leptin observed in serum following AAV8-*Sirt1* treatment. Leptin is a hormone that controls whole-body metabolism and exerts its effects in metabolic tissues.[27] HC or fructose-supplemented diets have been shown to provoke downregulation of lipid oxidation in the liver, which in turn increases leptin secretion to counteract this reduction in fat oxidation.[24,26] Increased leptin levels are often associated with a state of peripheral leptin resistance due to reduced STAT3-P-Ser^{727}.[24,26] In our study, HC diet feeding caused an increased in the levels of serum leptin in AAV8-Null mice and this increase was partially counteracted by AAV8-*Sirt1* treatment, suggesting that AAV8-*Sirt1*–treated mice were not leptin resistant, as confirmed by the higher hepatic STAT3-P-Ser^{727} protein content. The preservation of leptin sensitivity in mice treated with AAV8-*Sirt1* vectors, possibly due to the increased lipid oxidation in the liver, may have prevented the increase in leptin secretion in these mice. As a consequence, leptin sensitivity in skeletal muscle and adipose tissue would have been maintained, contributing to the higher β-oxidation in skeletal muscle and adipose lipolysis[27] observed in AAV8-*Sirt1*–treated mice. The latter may have led to the reduction in adipocyte size and to the increase in serum free fatty acids levels, which may have been oxidized in other tissues. Thus, one of the mechanisms by which hepatic SIRT1 overexpression led to systemic effects could be through the maintenance of leptin sensitivity primarily in the liver, but also in other metabolic tissues.

Although overexpression of SIRT1 or its pharmacological activation reproduces the beneficial effects of caloric restriction, such as reduction of tissue lipid accumulation and improved insulin sensitivity, SIRT1 is a deacetylase that also regulates many transcription factors involved in numerous biological processes such as the regulation of cell cycle and apoptosis.[8] In this regard, SIRT1 was originally considered to be a potential tumor promoter by its inhibitory effect on p53,[23] raising the concern that overexpressing SIRT1 long-term in humans could lead to the development of hepatic tumors. However, studies in transgenic animal models in which SIRT1 is overexpressed in the whole body or specifically in a particular tissue such as liver, muscle or adipose tissue have not shown any evidence of increased tumorigenesis.[11,44–46] Indeed, new evidence suggests that SIRT1 activation and caloric restriction act as tumor suppressors in different laboratory models of ageing- and metabolic syndrome-related cancers.[23,47] Similarly, caloric restriction delays the onset of age-associated pathologies like diabetes and cancer in nonhuman primates.[48] The only adverse events reported for prolonged calorie restriction in humans are associated with the malnourishment caused by the chronic deficiency of nutrients.[49] In this regard, SIRT1 overexpression in the liver could represent an alternative to achieve the same beneficial effects of calorie restriction without exposing

patients to the risk of malnourishment. Further studies in longer-lived mammals, such as dogs, are warranted to assess the long-term safety of the approach.

In summary, our work demonstrates that an AAV8-mediated gene transfer approach that allows long-term hepatic SIRT1 overexpression counteracts HC diet-induced NAFLD and improves whole-body metabolism in adult mice. Given that NAFLD also predicts the predisposition to develop diabetes mellitus, hypertension and dyslipidemia,[4,29] the development of new approaches that can potentially delay or even block the progression of NAFLD is currently a public health priority. Thus, gene therapy strategies based on the gene transfer of the *Sirt1* gene to the liver may hold great potential to prevent the development of NAFLD, insulin resistance and type 2 diabetes.

MATERIALS AND METHODS

Animals

Three-month-old C57Bl/6 male mice were obtained from Harlan laboratories (Teklad, Madison, WI). Mice were kept in a specific pathogen-free facility (SER-CBATEG) and maintained under a light-dark cycle of 12 hours. Mice were fed *ad libitum* with a standard (STD) (2018S Harlan) or a HC diet (D12450B Research Diets, New Brunswick, NJ) during 15 weeks. The HC diet contained 70% of calories as carbohydrates and, specifically, 35% as sucrose, whereas the standard diet contained 60% of calories as carbohydrates but only 5% come from sugars. When stated, mice were fasted for 16 hours. Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (UAB).

Recombinant AAV8 vectors

AAV8 vectors encoding codon-optimized murine *Sirt1* or green fluorescent protein (*Gfp*) under the control of the human alpha 1-anti-trypsin (hAAT) promoter (AAV8-hAAT-*Sirt1* and AAV8-hAAT-*Gfp* vectors) were generated in our laboratory by triple transfection of human embryonic kidney 293 cells and purified using an optimized cesium chloride gradient–based purification protocol.[50] A noncoding plasmid carrying the hAAT promoter was used to produce null particles (AAV8-hAAT-Null).

In vivo administration of AAV vectors

AAV8 vectors were administered by tail vein injection at a dose of 5×10^{11} vg/mice in a total volume of 200 µl. One week after treatment feeding with a HC diet began and was maintained for four months.

Vector genome copy number

Total DNA was isolated with a MasterPureDNA Purification Kit (Epicentre Biotechnologies, Madison, WI) from overnight tissue digestions in proteinase K (0.2 mg/ml). The vector genome copy number in 20 ng of total genomic DNA was determined by quantitative PCR with primers and probe specific for the SV40 element in the AAV8-hAAT-*Sirt1* vector. Forward primer: 5′- AGC AAT AGC ATC ACA AAT TTC ACA A -3′; reverse primer: 5′- CAG AC A TGA TAA GAT ACA TTG ATG AGT T -3′; probe: 5′- AGC ATT TTT TTC ACT GCA TTC TAG TTG TGG TTT GTC -3′. The final values were determined by comparison with a reference standard curve built from serial dilutions of a linearized plasmid bearing the hAAT- *Sirt1* expression cassette used in the study supplemented with 20 ng/µl of irrelevant mouse genomic DNA.

Quantification of liver transduction

Three male mice were injected intravenously with 5×10^{11} vg/mice of AAV8-hAAT-*Gfp* vectors and immunohistochemistry against GFP was performed two weeks later in the liver of these mice. Detection of GFP expression was performed in five random fields per section at an original magnification ×20. The percentage of transduced hepatocytes was calculated by dividing the number of GFP^+ nuclei by total nuclei of each liver section.

Gene expression analysis

For quantitative real-time (RT)-PCR analysis, total RNA was extracted from different tissues using isolation reagent (Tripure; Roche Molecular Biochemicals, Mannheim, Germany) and Rneasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1–2 µg) was retrotranscribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-PCR was performed in a LightCycler 480 II (Roche) using LightCycler 480 SyBR Green I MasterMix (Roche). The sequences of the sense and antisense oligonucleotide primers used were: 36b4, 5′TCCCACCTTGTCTCCAGTCT3′, 5′ACTGGTCTAGGACCCGAGAAG3′; Acadl, 5′CCCATGGCATTAGCCTCTT3′, 5′AGAATAGTTCTGCTGTGTCCTGAG3′; Acadm, 5′AGTACCCTGTGGAGAAGCTGAT3′,5′TCAATGTGCTCACGAGCTATG3′; Acadvl, 5′GGTGGTTTGGGCCTCTCTA3′, 5′GGGTAACGCTAACACCAAGG3′; Adipoq, 5′TGTTCCTCTTAATCCTGCCCA3′, 5′CCAACCTGCACAAGTTCCCTT3′; Ccl2, 5′CCCAATGAGTAGGCTGGAGA3′, 5′TCTGGACCCATTCCTTCTTG3′; Ccl5, 5′CCTCACCATCATCCTCACTGCA3′, 5′TCTTCTCTGGGTTGGCACACAC3′; Cd68, 5′GGGGCTCTTGGGAACTACAC3′, 5′CAAGCCCTCTTTAAGCCCCA3′; Cpt2, 5′CCAAAGAAGCAGCGATGG3′, 5′TAGAGCTCAGGCAGGGTGA3′; F4/80, 5′CTTTGGCTATGGGCTTCCAGTC3′, 5′GCAAGGAGGACAGAGTTTATC3′; Hif1a, 5′GCACTAGACAAAGTTCACCTGAGA3′, 5′CGCTATCCACATCAAAGCAA3′; Il1b, 5′TGTAATGAAAGACGGCACACC3′, 5′TCTTCTTTGGGTATTGCTTGG3′; Il10, 5′CTATGCTGCCTGCTCTTACTG3′, 5′AACCCAAGTAACCCTTAAAGTC3′; Lep, 5′GAGACCCCTGTGTCGGTTC3′, 5′CTGCGTGTGTGAAATGTCATTG3′; mCpt1, 5′GCACACCAGGCAGTAGCTTT3′, 5′CAGGAGTTGATTCCAGACAGGTA3′; Nos2, 5′CTTTGCCACGGACGAGAC3′, 5′TCATTGTACTCTGAGGGCTGAC3′; Nrf1, 5′CTGCAGGTCCTGTGGGAATG3′, 5′ACTCGCGTCGTGTACTCATC3′; Pdk4, 5′CCTTCACCACATGCTCTTCG3′,5′CGGTTTTCTTGATGCTCGAC3′;Ppargc1a,5′ATACCGCAAAGAGCACGAGAAG3′,5′CTCAAGAGCAGCGAAAGCGTCACAG3′;Ppara,5′TCGGCGAACTATTCGGCTG3′, 5′GCACTTGTGAAAACGGCAGT3′; Ppard, 5′TCCATCGTCAACAAAGACGGG3′,5′ACTTGGGCTCAATGATGTCAC3′;Sirt3,5′TCCTCTGAAACCGGATGG3′, 5′TCCCACACAGAGGGATATGG3′; Sirt4, 5′TGATGTCCAAAGGCTGGAA3′, 5′AGAGTTGGAGCGGCATTG3′; Sirt6, 5′GACCTGATGCTCGCTGATG3′, 5′GGTACCCAGGGTGACAGACA3′;Tnfa, 5′CATCTTCTCAAAATTCGAGTGACAA3′,5′TGGGAGTAGACAAGGTACAACCC3′; Ucp2, 5′ACTTTCCCTCTGGATACCGC3′, 5′ACGGAGGCAAAGCTCATCTG3′; Ucp3, 5′CTGCACCGCCAGATGAGTTT3′, 5′ATCATGGCTTGAAATCGGACC3′.

Western blot analysis

For total protein extracts, tissues were homogenized in protein lysis buffer and centrifuged at 15,000*g* during 15 minutes at 4 °C. Nuclear extracts were isolated as previously described.[24] The primary antibodies used were: Anti-Sirt1 (Millipore 07-131, Billerica, MA), Anti-phospho-ACC-Ser^{79} (Millipore 07-303), Anti-AMPK (Cell Signalling 2532, Danvers, MA), Anti-phospho-AMPK-Thr^{172} (Cell Signalling 2531), Anti-phospho-STAT3-Ser^{727} (Cell Signalling 9134), Anti-ERK1/2 (Cell Signalling 9102), Anti-phospho-ERK1/2-Thr^{202}/Tyr^{204} (Cell Signalling 9101), Anti-Acetyl-p53-Lys379 (Cell Signalling 2570), Anti-PGC1 (Santa Cruz H-300, Dallas, TX), Anti-α-tubulin (Abcam ab4074, Cambridge, UK). Detection was performed using the corresponding horseradish peroxidase-labeled secondary antibodies and Western blotting detection reagent (ECL Plus; Amersham, Freiburg, Germany).

Isolation and extraction of nuclei for SIRT1 deacetylase assay

Aliquots of liver homogenate (without protease inhibitors) were spun through 4 ml of sucrose 30%, 10 mmol/l Tris HCl (pH 7.5), 10 mmol/l NaCl and 3 mmol/l $MgCl_2$ at 1,300 *g* for 10 minutes at 4°C; the pellet was washed with cold 10 mmol/l Tris–HCl (pH 7.5) and 10 mmol/l NaCl. The nuclei were suspended in 200 µl of extraction buffer containing 50 mmol/l Hepes KOH (pH 7.5), 420 mmol/l NaCl, 0.5 mmol/l EDTA Na_2, 0.1 mmol/l EGTA, and glycerol 10%, sonicated for 30 seconds, and stood on ice for 30 minutes. After centrifugation at 20,000*g* for 10 minutes, the supernatant (crude nuclear extract) was stored at −80 °C until use.

SIRT1 deacetylase assay

SIRT1 deacetylase activity was evaluated using 50 µg of crude nuclear extract from livers of AAV8-Null and AAV8-*Sirt1*–treated mice. SIRT1 activity was measured using a deacetylase fluorometric assay kit (SIRT1 Activity Assay Kit, Abcam, ab156065). The fluorescence intensity at 440 nm (exc. 340 nm) was measured every 2 minutes for a total of 60 minutes immediately after the addition of fluorosubstrate peptide. The results are reported as relative fluorescence/µg of protein (AU).

Immunohistochemical and morphometrical analysis

Tissues were fixed for 24 hours in formalin, embedded in paraffin and sectioned. For histological analysis, sections were deparaffinised and stained with haematoxylin/eosin. For immunohistochemical detection, sections were deparaffinised and incubated overnight at 4 °C with antibody against

Mac-2 (Cederlane-CL8942AP) or GFP (Abcam ab6673), washed with PBS three times for 5 minutes, incubated with the corresponding secondary antibody and revealed with ABC Complex (Vector Laboratories, UK). Sections were counterstained in Mayer's haematoxylin.

Measurement of adipocyte area

The morphometric study of adipocytes' size was performed as previously described.[25] Four animals per group were used and at least 400 adipocytes per animal were analyzed.

Glucose and insulin tolerance tests

For the glucose tolerance test, awake mice, fasted overnight (16 hours) with free access to water, were given an intraperitoneal injection of glucose (1 g/kg body weight) and glucose concentration was determined in blood samples at indicated time points using a Glucometer Elite analyzer (Bayer, Leverkusen, Germany). For the insulin tolerance test, insulin (0.75 IU/kg body weight; Humulin regular, Eli Lilly, Indianapolis, IN) was injected intraperitoneally into awake fed mice and glucose concentration was determined at indicated time points.[25]

Metabolite and hormone assays

The content of triglycerides in liver and quadriceps and the serum levels of triglycerides, total-cholesterol, HDL-cholesterol, FFAs, glycerol, insulin, leptin, and adiponectin were measured as previously described.[25]

Statistical analysis

All values are expressed as the mean ± SEM (standard error of the media). Differences between groups were compared by Student's *t*-test. Statistical significance was considered if $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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