ARTICLE

AAV-mediated *Sirt1* overexpression in skeletal muscle activates oxidative capacity but does not prevent insulin resistance

Laia Vilà[1,2,3], Carles Roca[1,2,3], Ivet Elias[1,2,3], Alba Casellas[1,3], Ricardo Lage[1,2], Sylvie Franckhauser[1,3] and Fatima Bosch[1,2,3]

Type 2 diabetes is characterized by triglyceride accumulation and reduced lipid oxidation capacity in skeletal muscle. SIRT1 is a key protein in the regulation of lipid oxidation and its expression is reduced in the skeletal muscle of insulin resistant mice. In this tissue, *Sirt1* up-regulates the expression of genes involved in oxidative metabolism and improves mitochondrial function mainly through PPARGC1 deacetylation. Here we examined whether *Sirt1* overexpression mediated by adeno-associated viral vectors of serotype 1 (AAV1) specifically in skeletal muscle can counteract the development of insulin resistance induced by a high fat diet in mice. AAV1-*Sirt1*-treated mice showed up-regulated expression of key genes related to β-oxidation together with increased levels of phosphorylated AMP protein kinase. Moreover, SIRT1 overexpression in skeletal muscle also increased basal phosphorylated levels of AKT. However, AAV1-*Sirt1* treatment was not enough to prevent high fat diet-induced obesity and insulin resistance. Although *Sirt1* gene transfer to skeletal muscle induced changes at the muscular level related with lipid and glucose homeostasis, our data indicate that overexpression of SIRT1 in skeletal muscle is not enough to improve whole-body insulin resistance and that suggests that SIRT1 has to be increased in other metabolic tissues to prevent insulin resistance.

Molecular Therapy — Methods & Clinical Development (2016) **5**, 16072; doi:10.1038/mtm.2016.72; published online 16 November 2016

INTRODUCTION

Type 2 diabetes is the most common metabolic disease worldwide,[1] and its incidence has increased dramatically in parallel with the increased prevalence of obesity.[2] Type 2 diabetes is characterized by the resistance of target tissues to insulin action.[3] Skeletal muscle is the major site for glucose disposal after a meal, taking up about 75% of circulating glucose.[4] Therefore, alterations in skeletal muscle metabolism can lead to insulin resistance and type 2 diabetes. It has been described that type 2 diabetic patients show a reduction in oxidation capacity and a decrease in the percentage of oxidative type 1 fibers together with lipid accumulation in skeletal muscle.[5,6] Moreover, in humans, there is a clear correlation between triglyceride accumulation in muscle cells (intramyocellular lipids) and insulin resistance.[7,8]

SIRT1, a member of the Sirtuins family, plays a pivotal role in skeletal muscle regulation of fatty acid oxidation.[9] Sirtuins are a family of protein deacetylases and ADP-ribosyltransferases involved in different processes such as cellular metabolism, response to stress or caloric restriction and lifespan.[10,11] In mammals, seven sirtuins (SIRT1–7) have been identified.[12] SIRT1 has NAD⁺-dependent deacetylase activity and is capable of modulating gene expression through deacetylation of proteins and transcription factors in several key metabolic tissues such as liver, skeletal muscle, and white adipose tissue.[13] In skeletal muscle, the role of SIRT1 in the regulation of lipid metabolism and glucose homeostasis has not

been fully elucidated yet, but reduced levels of SIRT1 have been reported in the skeletal muscle of mice[14] and humans[15] with insulin resistance. In the skeletal muscle of animals treated with SIRT1 activators, the expression of genes involved in oxidative metabolism is up-regulated and mitochondrial function improves, mainly through PPARGC1 deacetylation and activation; these changes are associated with prevention of insulin resistance development.[16–18] These observations have made SIRT1 a good candidate for the treatment of obesity and insulin resistance.

In vivo gene therapy offers the possibility of a one-time treatment with the prospect of lifelong beneficial effects. Adeno-associated viral (AAV) vector–mediated gene transfer, in particular, has shown promising results in several *in vivo* applications[19] driving multiyear expression of therapeutic transgenes for a variety of diseases.[20] Moreover, intramuscular administration of AAV vectors is a convenient approach to efficiently engineer the skeletal muscle. Specifically, AAV vector of serotype 1 (AAV1) is the serotype with high tropism for skeletal muscle upon intramuscular administration[21]; a single intramuscular injection of AAV1 to mice allows the transduction of ~80% of muscle fibers.[22]

Here, we examined the ability of the skeletal muscle-specific overexpression of SIRT1 to prevent insulin resistance induced by high fat (HF) diet in adult mice, as a potential gene therapy approach for the treatment of obesity and type 2 diabetes. We found that AAV vector-mediated skeletal muscle-specific *Sirt1* gene transfer led to the

¹Center of Animal Biotechnology and Gene Therapy, Universitat Autònoma de Barcelona, Barcelona, Spain; ²Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain; ³CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain. Correspondence: F Bosch (fatima.bosch@uab.cat)

up-regulation of genes key to β-oxidation and mitochondrial bio-genesis, and also to increased levels of phosphorylated AMP protein kinase (AMPK). Moreover, SIRT1 overexpression in skeletal muscle also increased the levels of phosphorylated AKT in basal conditions. However, treatment with AAV1-*Sirt1* vectors was insufficient to pre-vent the development of obesity and HF diet-induced whole-body insulin resistance.

RESULTS

Skeletal muscle *Sirt1* gene transfer

AAV1 vectors were used to obtain long-term expression of the *Sirt1* gene in skeletal muscle. Mice received a single total dose of 1×10^{11} vg/mice of either AAV1-CAG-Null (AAV1-Null) or AAV1-CAG-*Sirt1* (AAV1-*Sirt1*) distributed between the quadriceps, gastrocnemius, and tibialis cranealis muscles of each hindlimb (Figure 1a). Mice were analyzed 16 weeks after vector delivery and, as expected, SIRT1 protein levels were increased in all muscles injected with AAV1-*Sirt1*, whereas no increase was observed in other metaboli-cally relevant tissues such as liver and epididymal white adipose tis-sue (Figure 1b). In agreement with the increased expression of SIRT1 protein, the acetylated form of p53 (Ac-p53^{Lys379}) -a known target of SIRT1 (ref. [23])- was reduced by ~85% in nuclear extracts of quadri-ceps muscles from mice that received AAV1-*Sirt1* vectors (Figure 1c), providing evidence of increased SIRT1 activity in skeletal muscle after AAV1-*Sirt1* injection.

Increased oxidative capacity in skeletal muscle following AAV1-*Sirt1* administration

To determine whether SIRT1 overexpression increased the skel-etal muscle oxidative capacity under obesogenic conditions, mice overexpressing SIRT1 were fed a high fat (HF) diet for 15 weeks, and the local effects of SIRT1 overexpression on lipid metabolism were examined. The expression of the gene encoding for *Carnitine Palmitoyltransferase 1, muscular isoform* (*Cpt1b*), a key enzyme governing the entry of long-chain acyl-CoAs into mitochondria, was increased in gastrocnemius muscle (Figure 2a). Moreover, the expression of *Uncoupling protein 3* (*Ucp3*), a protein that uncouples oxidative phosphorylation and may promote the conversion of intracellular fat into heat, was increased in both the gastrocnemius and quadriceps muscles of AAV1-*Sirt1*-treated mice compared with AAV1-Null-injected mice (Figure 2a,b). Similarly, expression of *Pyruvate Dehydrogenase Kinase, isoenzyme 4* (*Pdk4*), a key inhibitor of the pyruvate dehydrogenase complex, was upregulated in gas-trocnemius and quadriceps muscles from AAV1-*Sirt1*-treated mice (Figure 2a,b). In addition, *Peroxisome proliferative activated recep-tor delta* (*PPARδ*) was slightly upregulated in quadriceps and gas-trocnemius muscles of AAV1-*Sirt1* mice, respectively (Figure 2a,b). Despite these changes, which altogether suggested an increase in the capacity for fat oxidation of the gastrocnemius and quadriceps muscles overexpressing *Sirt1*, the expression levels of *Ppargc1a*, a master regulator of lipid metabolism and one of the main targets of SIRT1,[24] remained unchanged (Figure 2a,b). However, the expres-sion levels of *Transcription factor A, mitochondrial* (*Tfam*) and *Sirtuin 3* (*Sirt3*), known targets of PPARGC1A,[25,26] were higher in gastrocne-mius muscle following AAV1-*Sirt1* treatment (Figure 2a). Although the mRNA levels of other sirtuins such as *Sirtuin 4* and *Sirtuin 5* were not changed in treated muscles (data not shown), the expression levels of *Sirtuin 6,* a potential target of SIRT1,[27] were significantly increased or showed a positive tendency in all injected muscles after AAV1-*Sirt1* treatment (Figure 2a–c). Since the increased expression

of oxidative genes observed in gastrocnemius and quadriceps may reflect a shift in the predominant fiber type in these muscles, the expression of myosin isoforms was examined. The gastrocnemius and quadriceps muscles from AAV1-*Sirt1*-treated mice showed a trend to express higher levels of the oxidative fiber marker *MyHC I* when compared with AAV1-Null mice, whereas no differences were observed in the levels of *MyHC IIa*, *MyHC IIb*, and *MyHC IIx* (Figure 2a,b). Furthermore, the content of MYHCI protein was also increased in quadriceps and gastrocnemius samples from mice that received AAV1-*Sirt1* vectors, reaching statistical significance in quadriceps muscle (Figure 3a). Although AAV-derived SIRT1 was also overexpressed in tibialis muscle, no significant changes were observed in the expression of genes involved in lipid oxidation and mitochondrial biogenesis in this muscle (Figure 2c). In accordance with this observation, the expression of *MyHC I* remained unchanged in the tibialis muscle of AAV1-*Sirt1*-treated mice (Figure 2c). Thus, these results suggest changes after AAV1-*Sirt1* delivery in the pat-tern of fiber type composition, associated with an increase in the oxidative capacity, in gastrocnemius and quadriceps but not in tibi-alis muscles. However, the increased expression of oxidation-related genes was not parallel with a decrease in the triglyceride content in gastrocnemius and quadriceps of AAV1-*Sirt1*-treated mice fed a HF diet compared with AAV1-Null-injected animals (Figure 3b).

AMPK activation after AAV1-*Sirt1* delivery

The activation by phosphorylation of AMPK controls β-oxidation activity and the switch toward type 1 fibers. Several reports have shown a strong relationship between SIRT1 and AMPK, indicat-ing that one could activate the other and *vice versa*.[28,29] To analyze whether *Sirt1* gene transfer to skeletal muscle could induce phos-phorylation of AMPK, western blot analysis was performed. Mice that received AAV1-*Sirt1* vectors and were fed a HF diet presented higher ratios of phosphorylated AMPK over total AMPK (AMPK-P/AMPKtot) in comparison with AAV1-Null-injected mice, reaching statistical significance in tibialis and quadriceps muscles (Figure 4a). These data indicate that *Sirt1* gene transfer to skeletal muscle acti-vates AMPK, which may lead to an increase in the oxidative capacity of this tissue.

SIRT1 and AMPK have been both shown to influence insulin sen-sitivity under either normal or high-fat diet-fed conditions.[16,30–32] In order to examine whether AAV1-*Sirt1* treatment also had effects on insulin signaling in skeletal muscle, the ratio between the phos-phorylated form of AKT (AKT-P) and total AKT was evaluated by Western blot in all injected muscles. As shown in Figure 4b, in basal conditions, the ratio AKT-P/AKT total was increased in all injected muscles, reaching statistical significance in gastrocnemius and tibi-alis muscles of mice that received AAV1-*Sirt1* vectors. These data suggest that SIRT1 overexpression in skeletal muscle improves insu-lin signaling in this tissue.

AAV1-*Sirt1*-treated mice were not protected against high fat diet-induced obesity and insulin resistance

Next, we examined whether skeletal muscle SIRT1 overexpression protected mice against the development of obesity induced by a high fat diet. After 15 weeks of HF diet feeding, both AAV1-Null and AAV1-*Sirt1*-treated mice increased their body weight by about 70% (Figure 5a) and no differences were observed in the food intake between groups (data not shown). In addition, the epididymal white adipose tissue and brown adipose tissue fat pads weighted the same in mice that received either AAV1-*Sirt1* or AAV1-Null
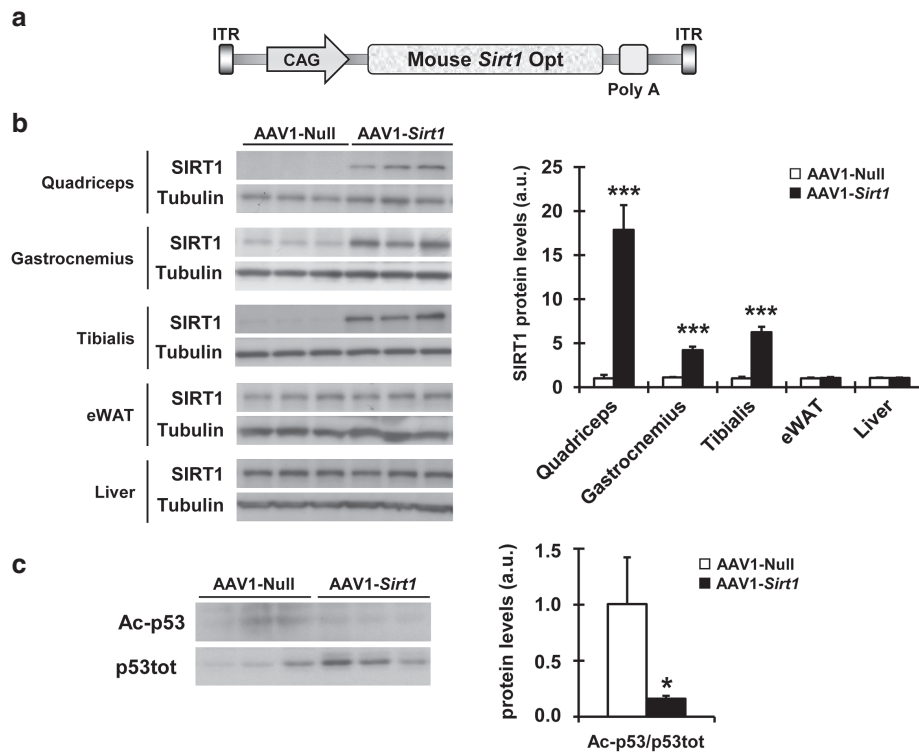
Figure 1   Intramuscular administration of AAV1-CAG-*Sirt1* vectors led to SIRT1 overexpression specifically in skeletal muscle. (**a**) Schematic representation of the AAV genome encoding the codon-optimized murine *Sirt1* sequence under the control of the CAG promoter. ITR, inverted terminal repeat. (**b**) Representative Western blots and their corresponding quantifications illustrating the content of SIRT1 in quadriceps, gastrocnemius, and tibialis muscles, in epididymal white adipose tissue (eWAT) and in liver of mice receiving AAV1-CAG-Null (AAV1-Null) or AAV1-CAG-*Sirt1* vectors (AAV1-*Sirt1*) at a dose of 1.6×10^{10} vg per muscle and fed a high fat (HF) diet for 15 weeks following vector delivery. (**c**) Representative Western blots and corresponding quantifications of the levels of total p53 protein (p53tot) and acetylated p53 (Ac-p53) in skeletal muscle of AAV1-Null and AAV8-*Sirt1* mice. a. u., arbitrary units. Data represent the mean ± SEM of at least four animals per group. *$P < 0.05$ and ***$P < 0.001$ versus AAV1-Null. AAV1, adeno-associated viral vectors of serotype 1; SEM, standard error of the mean.

vectors (Figure 5b,c). Hematoxylin-eosin staining also showed similar adipocyte size in epididymal white adipose tissues from both groups of animals and no differences were observed in the degree of lipid accumulation in the brown adipose tissue of AAV1-*Sirt1*-treated mice (Figure 5d). In addition, HF diet-induced fat deposition in the liver was very similar in both groups of mice (Figure 5d,e). Moreover, the serum lipid profile, which included triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol, and free fatty acids was similar in both groups of AAV-injected HF diet-fed mice (data not shown).

Finally, the effects of muscle SIRT1 overexpression on whole-body insulin sensitivity were also analyzed. The intraperitoneal insulin-tolerance test showed that the response to insulin was blunted to a similar extent in AAV1-Null-injected mice than in AAV1-*Sirt1*-treated mice, indicating that SIRT1 overexpression in skeletal muscle did not prevent the development of systemic insulin resistance under a HF diet (Figure 5f). Furthermore, glucose and insulin levels in fed conditions were similar in AAV1-*Sirt1* and AAV1-Null-injected mice (Figure 5g). All these results indicate that SIRT1 overexpression in skeletal muscle does not prevent the development of obesity and insulin resistance induced by high fat feeding.

DISCUSSION
Although several studies have demonstrated that whole-body activation of SIRT1 has beneficial effects on lipid and glucose metabolism, the tissue-specific actions of SIRT1 remain to be elucidated.[16–18] To address this type of scientific question, the most common

strategy is to generate transgenic animal models with tissue-specific expression of the protein of interest. In these cases, however, the phenotype observed in adult animals may not be the direct result of the effects of transgene expression in the adult tissue but the consequence of its expression during embryonic development.[33] In our study, we took advantage of AAV1, a vector serotype with high tropism for skeletal muscle upon intramuscular administration, to induce overexpression of SIRT1 in fibers of several muscles, allowing long-term expression of the transgene only in adulthood.

We show in HF diet-fed mice that SIRT1 overexpression in skeletal muscle leads to increased expression of genes related to lipid oxidation in gastrocnemius and quadriceps. AAV1-*Sirt1*-treated mice showed up-regulated levels of *Pdk4,* an enzyme that coordinates the shift from glucose metabolism toward fatty acid oxidation,[9] and *Cpt1b*, the enzyme controlling the entrance of fatty acids into the mitochondria to be oxidized.[34] These genes were also up-regulated in skeletal muscle of mice treated with the SIRT1 activator SRT1720.[16] Mice that received AAV1-*Sirt1* vectors also showed increased expression of *Ucp3* in all treated muscles, whose expression improves mitochondrial function, reducing the possibility of lipid-induced oxidative damage.[2] Accordingly, the SIRT1 activator resveratrol also induced higher levels of *Ucp3* transcripts in the gastrocnemius muscle of HF diet fed mice.[17] In contrast, no changes were observed in our study in the mRNA levels of *Ppargc1a*, one of the main target genes of SIRT1. However, AAV1-*Sirt1*-treated mice showed increased expression levels of two of PPARG1AC target genes in the gastrocnemius muscle, *Tfam* and *Sirt3*,[25,26] and these
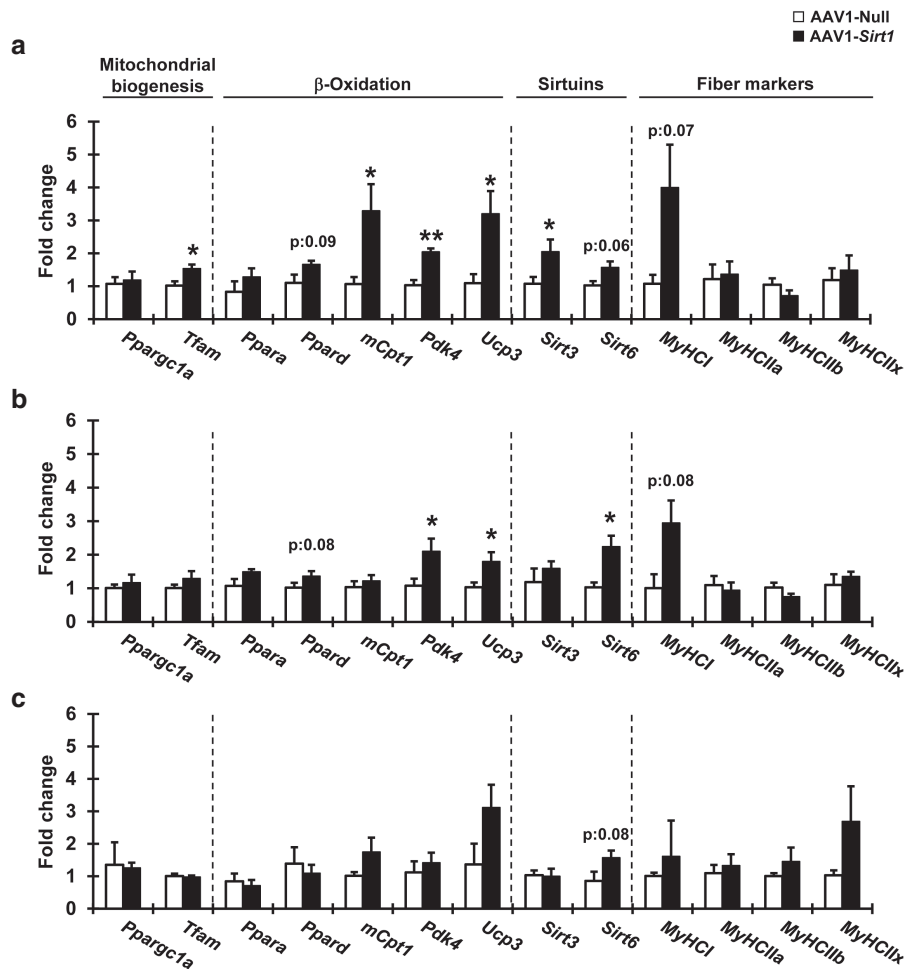
Figure 2 AAV1-*Sirt1*-treated mice showed increased expression of oxidative genes in skeletal muscle. (**a,b,c**) The expression levels of *Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha* (*Ppargc1a*), *Transcription factor A mitochondrial* (*Tfam*), *Peroxisome proliferative activated receptor alpha* (*Ppara*), *Peroxisome proliferative activated receptor delta* (*Ppard*), *Carnitine palmitoyltransferase 1, muscular isoform* (*Cpt1b*), *Pyruvate Dehydrogenase Kinase, isoenzyme 4* (*Pdk4*), *Uncoupling protein 3* (*Ucp3*), *Sirtuin 3* (*Sirt3*), *Sirtuin 6* (*Sirt6*), and markers of fiber types *MyHCI*, *MyHCIIa*, *MyHCIIb*, and *MyHCIIx* were quantified by RT-qPCR in gastrocnemius **a**, quadriceps **b**, and tibialis **c** of AAV1-Null and AAV1-*Sirt1*-injected mice. Data represent the mean ± SEM of at least four animals per group. *$P < 0.05$ and **$P < 0.01$ versus AAV1-Null. AAV1, adeno-associated viral vectors of serotype 1; RT-qPCR, real-time-quantitative polymerase chain reaction; SEM, standard error of the mean.

increases suggest that PPARG1A activity was higher after AAV1-*Sirt1* delivery, at least in gastrocnemius muscle. Moreover, AAV1-*Sirt1*-treated mice also presented increased mRNA levels of *Sirt6*, a potential target of SIRT1,[27] in all SIRT1 overexpressing muscles. Although the role of *Sirt6* in skeletal muscle metabolism remains uncertain, it seems to mediate the increase of fatty acid oxidation through *Hif1a* suppression.[35] In agreement with the observation of reduced levels of the acetylated form of p53, the expression data indicate that the *Sirt1* transgene is active in skeletal muscle of AAV1-*Sirt1*-treated mice, and suggest that lipid oxidation and mitochondrial biogenesis might be augmented in gastrocnemius and quadriceps muscles of AAV1-*Sirt1* mice. The increase in oxidative genes was accompanied by up-regulation, at mRNA and protein levels of MYHC I expression, a marker of the fiber type with highest oxidative capacity. A similar observation has been made in a mouse model of Duchenne Muscular Dystrophy which overexpresses a gain-of-function version of SIRT1 specifically in the muscle from birth[36] and by administration of the activator SRT1720,[16] suggesting that SIRT1 overexpression or activation induces fiber remodeling toward an oxidative phenotype. *Ppard*, *Sirt3*, and *Cpt1b* overexpression may also induce shift toward the oxidative fiber type,[5,37,38] and their up-regulation

in AAV1-*Sirt1*-treated mice may have contributed to the shift from type 2 to type 1 fibers and also to a more oxidative phenotype in gastrocnemius and quadriceps muscles of these mice.

The changes related to oxidative metabolism observed in the gastrocnemius and quadriceps muscles of AAV1-*Sirt1*-treated mice were, however, not detected in tibialis, despite the fact that this muscle also overexpressed SIRT1. Gastrocnemius, quadriceps and tibialis are all considered glycolytic muscles, but they differ in their fiber composition. It has been shown that the gastrocnemius and quadriceps muscles predominantly contain type 2 glycolytic fibers, but also a small proportion of type 1 oxidative fibers, whereas the tibialis muscle does not contain type 1 oxidative fibers.[39,40] The presence of some type 1 fibers in gastrocnemius and quadriceps muscles may confer more plasticity to these muscles allowing the shift from a glycolytic to a more oxidative phenotype.

SIRT1 and AMPK act as sensors of the cellular energy status and coordinately regulate the switch from glucose to fatty acid oxidation in the mitochondria.[11,41] We observed increased levels of AMPK phosphorylation in all injected muscles following AAV1-*Sirt1* treatment. As SIRT1 has been described as an AMPK activator,[29] these data reinforce the idea that SIRT1 is active in our animal model.

Moreover, although AMPK has been associated with the shift from muscle glycolytic fiber type 2 to oxidative type 1,[41,42] in our study the effect of SIRT1 on AMPK activation might be a direct effect, independent of the shift in the fiber type pattern, at least in tibialis
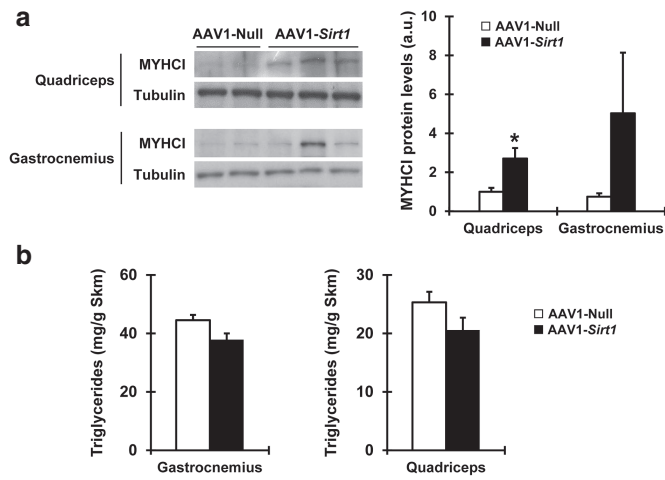

Figure 3 SIRT1 overexpression led to higher protein levels of oxidative fyber type. (**a**) Representative Western blots and corresponding quantifications of the MYHCI content in quadriceps and gastrocnemius muscles of AAV1-Null and AAV1-*Sirt1*-treated mice. (**b**) Triglyceride content in the gastrocnemius and quadriceps. All analyses were performed after 15 weeks of HF diet feeding. Data represent the mean ± SEM of at least three **a** or four **b** animals per group. *$P < 0.05$ versus AAV1-Null. AAV1, adeno-associated viral vectors of serotype 1; HF, high fat; SEM, standard error of the mean.

muscle. In addition, as AMPK activation stimulates lipid oxidation by reducing malonyl-CoA levels, an inhibitor of m-Cpt1,[43] these results reinforce the role of SIRT1 overexpression in activating oxidative capacity in skeletal muscle.

Therapeutic strategies aiming at increasing lipid oxidation and mitochondrial function in skeletal muscle may be of particular interest for the treatment of type 2 diabetes and obesity, since lipid accumulation in nonadipose tissues is effectively involved in the development of insulin resistance.[4] Moreover, skeletal muscle has key advantages as a target tissue for AAV-mediated gene transfer. Muscle is easily accessible by noninvasive procedures and intramuscular vector delivery leads to minimal systemic distribution of the vector.[22] SIRT1, which can control lipid oxidation, glucose metabolism, and mitochondrial function may thus be a good candidate in a gene therapy approach for diabetes treatment.[10,11]

Although several studies have demonstrated that whole-body activation of SIRT1 has beneficial effects on lipid and glucose metabolism, controversial results have been reported on the tissue-specific actions of SIRT1, in particular regarding insulin sensitivity.[16–18] It has been demonstrated in transgenic mice that overexpression of SIRT1 specifically in skeletal muscle does not improve insulin sensitivity when animals are fed either chow diet[44] or high fat diet.[45] However, previous observations in muscular cells[3,46] showed that SIRT1 overexpression enhances insulin-induced AKT phosphorylation. Consistent with this latter observation, in our model, insulin signaling was improved in the skeletal muscle after AAV1-*Sirt1* treatment, as indicated by the increase in the ratio AKT-P/AKTtotal; we did not observe, however, improvement in whole-body insulin sensitivity. In addition, the fact that in our mice, the increased levels of AKT-P were observed in both gastrocnemius and tibialis muscles
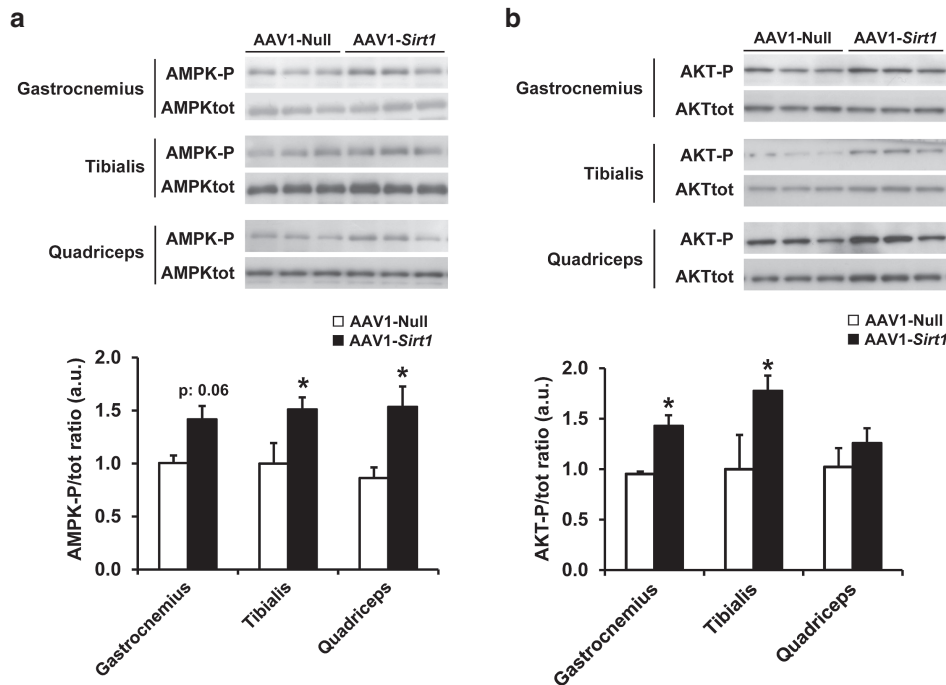

Figure 4 *Sirt1* gene transfer to skeletal muscle led to AMPK and AKT activation. (**a**) Representative Western blots and corresponding quantifications of the total AMP protein kinase (AMPKtot) and phosphorylated AMPK (AMPK-P) content in gastrocnemius, tibialis and quadriceps muscles of AAV1-Null and AAV1-*Sirt1*-treated mice. (**b**) Representative Western blots and corresponding quantifications of the content of total AKT (AKTtot) and phosphorylated AKT (AKT-P) in gastrocnemius, tibialis, and quadriceps muscles of AAV1-Null and AAV1-*Sirt1*-treated mice. Tubulin was used as a loading control. a.u., arbitrary units. All analyses were performed after 15 weeks of HF diet feeding. Data represent the mean ± SEM of at least four animals per group. *$P < 0.05$ versus AAV1-Null. AAV1, adeno-associated viral vectors of serotype 1; HF, high fat; SEM, standard error of the mean.
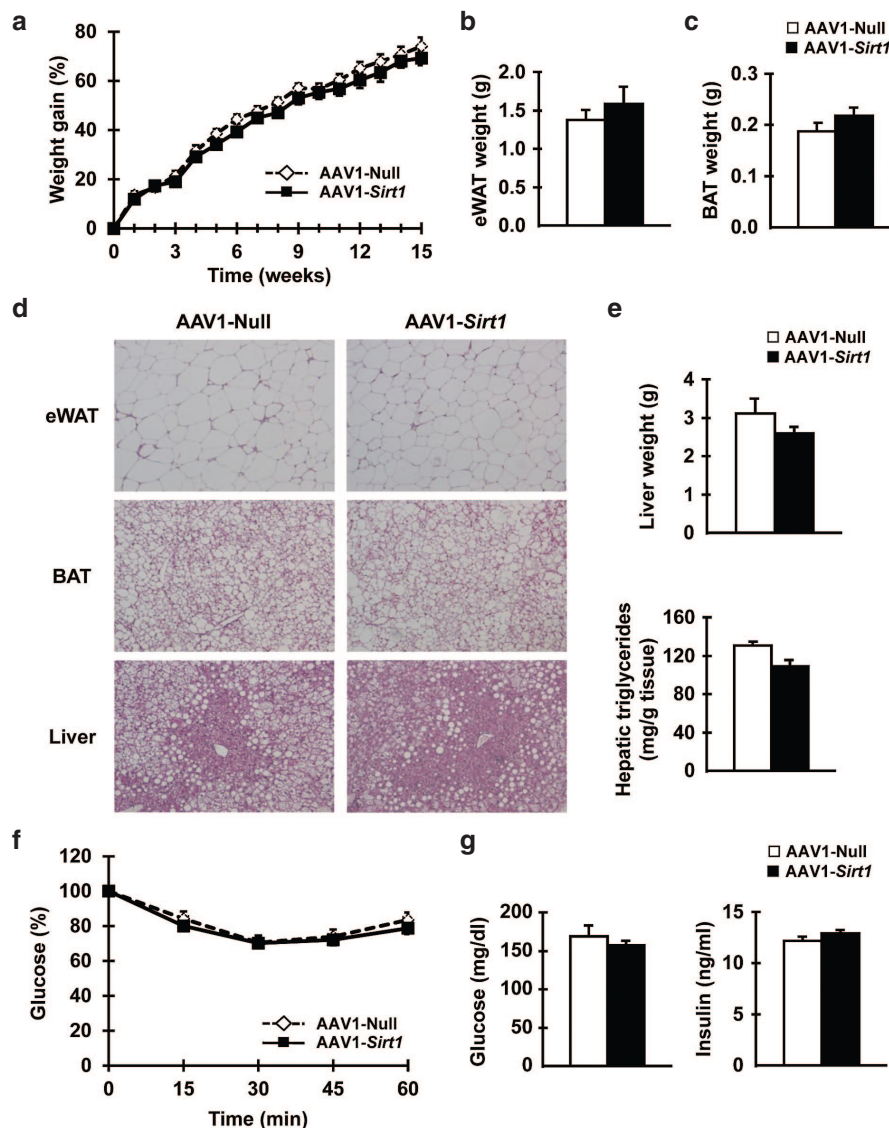
Figure 5 AAV-*Sirt1* treatment did not counteract the development of obesity and insulin resistance after a HF diet. (**a**) Body weight gain of AAV1-Null and AAV1-*Sirt1*-treated mice fed with a HF for 15 weeks. (**b,c**) Weight of the epididymal white adipose tissue (eWAT) **b** and brown adipose tissue (BAT) **c** fat pads. (**d**) Representative sections of eWAT, BAT and liver stained with hematoxylin-eosin. Original magnification ×100. (**e**) Liver weight and hepatic triglyceride content. (**f**) 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. (**g**) Blood glucose and insulin levels in fed conditions. All analyses were performed after 15 weeks of HF diet feeding. Data represent the mean ± SEM of at least 10 **a, f, g** or 4 **b-e** animals per group. AAV1, adeno-associated viral vectors of serotype 1; AAV, adeno-associated viral; HF, high fat; SEM, standard error of the mean.

indicates that AKT activation might be independent of the shift in fiber type and might be the result of a direct effect of SIRT1 overexpression, possibly mediated by AMPK activation as other authors have reported.[31]

The amelioration observed in insulin signaling, however, was not able to induce positive systemic effects, which is in agreement with what has been observed in HF diet fed transgenic mice overexpressing SIRT1 specifically in skeletal muscle.[45] Altogether, these results and the fact that the knockdown of SIRT1 activity in skeletal muscle did not impair *in vivo* insulin-stimulated AKT-phosphorylation and glucose uptake,[47] question the role of SIRT1 in skeletal muscle in the control of whole-body insulin sensitivity.

Our study also clearly demonstrates that SIRT1 overexpression only in skeletal muscle, despite inducing the expected metabolic changes, is not sufficient to improve insulin sensitivity, and that SIRT1 activation or overexpression in other metabolic tissues, such as liver or white and brown adipose tissue may be necessary to ameliorate whole-body insulin sensitivity.[16,30,48] In this regard, systemic delivery of SIRT1-expressing vectors could be considered an alternative. However, thinking from a translational point of view, the treatment of insulin resistance with a vector that mediated whole-body SIRT1 expression would not be the strategy of choice because of the possible deleterious effects that SIRT1 overexpression could have in certain organs, such as those described in heart[49,50] and brain.[51–53] On the other hand, our group has previously shown that AAV8-mediated liver specific SIRT1 overexpression in adult mice prevents the development of fatty liver and insulin resistance induced by a high carbohydrate diet.[54] Thus, this work demonstrates that

Table 1 Primer sequences for qPCR

Gene	Sequence
36b4-Fw	5'-TCCCACCTTGTCTCCAGTCT-3'
36b4-Rev	5'-ACTGGTCTAGGACCCGAGAAG-3'
mCpt1-Fw	5'-GCACACCAGGCAGTAGCTTT-3'
mCpt1-Rev	5'-CAGGAGTTGATTCCAGACAGGTA-3'
MyHC I-Fw	5'-GGCAAGCTCACATATACACAGC-3'
MyHC I-Rev	5'-ACAATCATGCCGTGCTGAC-3'
MyHC IIa-Fw	5'-CCAGGTGAGTGAGCTGAAGTC-3'
MyHC IIa-Rev	5'-AAGCTGCCTGGAAAATTCAC-3'
MyHC IIb-Fw	5'-CACCCAGGGAATGCTGAA-3'
MyHC IIb-Rev	5'-CGATCTCAGCCTGCATCA-3'
MyHC IIx-Fw	5'-GAGGACCAAGTGAGTGAGCTG-3'
MyHC IIx-Rev	5'-CTGGCGTGAGTATTCACCTG-3'
Pdk4-Fw	5'-CCTTCACCACATGCTCTTCG-3'
Pdk4-Rev	5'-CGGTTTTCTTGATGCTCGAC-3'
Ppargc1a-Fw	5'-ATACCGCAAAGAGCACGAGAAG-3'
Ppargc1a-Rev	5'-CTCAAGAGCAGCGAAAGCGTCACAG-3'
Ppara-Fw	5'-TCGGCGAACTATTCGGCTG-3'
Ppara-Rev	5'-GCACTTGTGAAAACGGCAGT-3'
Ppard-Fw	5'-TCCATCGTCAACAAAGACGGG-3'
Ppard-Rev	5'-ACTTGGGCTCAATGATGTCAC-3'
Sirt3-Fw	5'-TCCTCTGAAACCGGATGG-3'
Sirt3-Rev	5'-TCCCACACAGAGGGATATGG-3'
Sirt4-Fw	5'-TGATGTCCAAAGGCTGGAA-3'
Sirt4-Rev	5'-AGAGTTGGAGCGGCATTG-3'
Sirt5-Fw	5'-CCAGCTTTAGCAGGAAAAGG-3'
Sirt5-Rev	5'-GACTGGGATTCTGGCGTCT-3'
Sirt6-Fw	5'-GACCTGATGCTCGCTGATG-3'
Sirt6-Rev	5'-GGTACCCAGGGTGACAGACA-3'
Tfam-Fw	5'-TCGCATCCCCTCGTCTATCA-3'
Tfam-Rev	5'-CCACAGGGCTGCAATTTTCC-3'
Ucp3-Fw	5'-CTGCACCGCCAGATGAGTTT-3'
Ucp3-Rev	5'-ATCATGGCTTGAAATCGGACC-3'

qPCR, quantitative polymerase chain reaction.

targeting skeletal muscle to overexpress SIRT1 is not a valid therapeutic option and that hepatic-specific *Sirt1* gene transfer would be the strategy of choice for the treatment of type 2 diabetes. Our study underscores the challenge that developing gene therapies for complex metabolic diseases represents.

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 high fat diet (HF) (D12451, Research Diets, New Brunswick, NJ) for 15 weeks. The HF diet contained 45% of calories as fat. Animals were sacrificed under anesthesia and tissues were harvested and snap frozen or fixed in formalin. Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (UAB).

Recombinant AAV1 vector administration
AAV1 vectors encoding a codon-optimized murine *Sirt1* sequence under the control of the ubiquitous CAG promoter (containing the chicken β-actin promoter and cytomegalovirus enhancer) (AAV1-CAG-*Sirt1*) 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.[55,56] A noncoding plasmid carrying the CAG promoter was used to produce null particles (AAV1-CAG-Null). Vectors were administered by intramuscular injection at a dose of 1×10^{11} vg/mice in a total volume of 180 µl divided into six injection sites distributed in the quadriceps, gastrocnemius, and tibialis cranealis of each hindlimb (1.6×10^{10} vgs per muscle). One week later, animals began feeding a HF diet until their sacrifice (16 weeks postinjection of vector).

Gene expression analysis
For quantitative real-time polymerase chain reaction analysis, total RNA was extracted from different muscles using Tripure isolation reagent (Roche Molecular Biochemicals, Mannheim, Germany) and Rneasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 µg) was retrotranscribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time polymerase chain reaction was performed in a LightCycler 480 II (Roche) using LightCycler 480 SyBR Green I Master Mix (Roche). Primer sequences are described in Table 1.

Western blot analysis
For total protein extracts, tissues were homogenized in protein lysis buffer and centrifuged at 15,000*g* for 15 minutes at 4ºC. Nuclear extracts were isolated as previously described.[57] Proteins were separated by 8% or 10% wt/vol sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidenedifluoride (PVDF) membranes and probed overnight at 4°C. The primary antibodies used were: Anti-Sirt1 (07-131, Millipore, Billerica, MA), Anti-AMPK (2532, Cell Signaling, Danvers, MA), Anti-phospho-AMPK-Thr[172] (2531, Cell Signaling), Anti-AKT (9272, Cell Signaling), Anti-phospho-AKT-Ser[473] (9271, Cell Signaling), Anti-Acetyl-p53-Lys[379] (2570, Cell Signaling), Anti-Myosin Skeletal Slow (M8421, Sigma-Aldrich, St. Louis, MO), Anti-p53 (2524, Cell Signaling) and Anti-α-tubulin (ab4074, Abcam, Cambridge, UK). Detection was performed using the corresponding horseradish peroxidase-labeled secondary antibodies and western blotting detection reagent (ECL Plus; Amersham, Freiburg, Germany).

Immunohistochemical analysis
Tissues were fixed for 24 hours in formalin, embedded in paraffin and sectioned. For histological analysis, sections were deparaffinized and stained with hematoxylin/eosin.

Insulin tolerance test
Insulin tolerance test was performed as previously described.[58] Briefly, mice were given an intraperitoneal injection of insulin and glucose concentration was determined in blood samples at the indicated time points using a Glucometer Elite analyzer (Bayer, Leverkusen, Germany).

Metabolite and hormone assays
Liver and gastrocnemius triglyceride content was determined by extracting total lipids from the tissues with chloroform-methanol (2:1 vol/vol) as described by Carr *et al*.[59] Serum triglycerides, total cholesterol, LDL-cholesterol, and HDL-cholesterol were quantified spectrophotometrically using enzymatic assays kit (Horiba-ABX, Montpellier, France). Serum FFAs were measured by the acyl-CoA synthase and acyl-CoA oxidase method (Wako Chemicals GmbH, Neuss, Germany). All biochemical parameters were determined using a Pentra 400 Analyzer (Horiba-ABX). Glucose was determined using a Glucometer Elite analyzer (Bayer) and insulin levels were measured using the Rat Insulin ELISA Kit (Crystal Chemical, Chicago, IL).

Sirt1 increases oxidative capacity in muscle
L Vilà *et al*.

8

Statistical analysis

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

AUTHOR CONTRIBUTIONS

L.V., S.F., and F.B. conceived and designed the study and wrote the manuscript. L.V., C.R., I.E., and S.F. conducted experiments and supported the work with key suggestions. A.C. and R.L. conducted experiments. F.B. directed the project and secured funding.

REFERENCES

1. Chen, L, Magliano, DJ and Zimmet, PZ (2011). The worldwide epidemiology of type 2 diabetes mellitus–present and future perspectives. *Nat Rev Endocrinol* **8**: 228–236.
2. Schrauwen, P (2007). High-fat diet, muscular lipotoxicity and insulin resistance. *Proc Nutr Soc* **66**: 33–41.
3. Sun, C, Zhang, F, Ge, X, Yan, T, Chen, X, Shi, X *et al*. (2007). SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. *Cell Metab* **6**: 307–319.
4. Sesti, G (2006). Pathophysiology of insulin resistance. *Best Pract Res Clin Endocrinol Metab* **20**: 665–679.
5. Wang, YX, Zhang, CL, Yu, RT, Cho, HK, Nelson, MC, Bayuga-Ocampo, CR *et al*. (2004). Regulation of muscle fiber type and running endurance by PPARdelta. *PLoS Biol* **2**: e294.
6. Petersen, KF, Dufour, S, Befroy, D, Garcia, R and Shulman, GI (2004). Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* **350**: 664–671.
7. Perseghin, G, Scifo, P, De Cobelli, F, Pagliato, E, Battezzati, A, Arcelloni, C *et al*. (1999). Intramyocellular triglyceride content is a determinant of *in vivo* insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* **48**: 1600–1606.
8. Krssak, M, Falk Petersen, K, Dresner, A, DiPietro, L, Vogel, SM, Rothman, DL *et al*. (1999). Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* **42**: 113–116.
9. Gerhart-Hines, Z, Rodgers, JT, Bare, O, Lerin, C, Kim, SH, Mostoslavsky, R *et al*. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. *EMBO J* **26**: 1913–1923.
10. Yamamoto, H, Schoonjans, K and Auwerx, J (2007). Sirtuin functions in health and disease. *Mol Endocrinol* **21**: 1745–1755.
11. Lomb, DJ, Laurent, G and Haigis, MC (2010). Sirtuins regulate key aspects of lipid metabolism. *Biochim Biophys Acta* **1804**: 1652–1657.
12. Nogueiras, R, Habegger, KM, Chaudhary, N, Finan, B, Banks, AS, Dietrich, MO *et al*. (2012). Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. *Physiol Rev* **92**: 1479–1514.
13. Metoyer, CF and Pruitt, K (2008). The role of sirtuin proteins in obesity. *Pathophysiol* **15**: 103–108.
14. Coste, A, Louet, JF, Lagouge, M, Lerin, C, Antal, MC, Meziane, H *et al*. (2008). The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1{alpha}. *Proc Natl Acad Sci USA* **105**: 17187–17192.
15. Rutanen, J, Yaluri, N, Modi, S, Pihlajamäki, J, Vänttinen, M, Itkonen, P *et al*. (2010). SIRT1 mRNA expression may be associated with energy expenditure and insulin sensitivity. *Diabetes* **59**: 829–835.
16. Feige, JN, Lagouge, M, Canto, C, Strehle, A, Houten, SM, Milne, JC *et al*. (2008). Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. *Cell Metab* **8**: 347–358.
17. Lagouge, M, Argmann, C, Gerhart-Hines, Z, Meziane, H, Lerin, C, Daussin, F *et al*. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* **127**: 1109–1122.
18. Milne, JC, Lambert, PD, Schenk, S, Carney, DP, Smith, JJ, Gagne, DJ *et al*. (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* **450**: 712–716.
19. Mingozzi, F and High, KA (2011). Therapeutic *in vivo* gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* **12**: 341–355.
20. Daya, S and Berns, KI (2008). Gene therapy using adeno-associated virus vectors. *Clin Microbiol Rev* **21**: 583–593.
21. Chao, H, Liu, Y, Rabinowitz, J, Li, C, Samulski, RJ and Walsh, CE (2000). Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol Ther* **2**: 619–623.
22. Mas, A, Montané, J, Anguela, XM, Muñoz, S, Douar, AM, Riu, E *et al*. (2006). Reversal of type 1 diabetes by engineering a glucose sensor in skeletal muscle. *Diabetes* **55**: 1546–1553.
23. Deng, CX (2009). SIRT1, is it a tumor promoter or tumor suppressor? *Int J Biol Sci* **5**: 147–152.
24. Cantó, C and Auwerx, J (2009). PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr Opin Lipidol* **20**: 98–105.
25. Kelly, DP and Scarpulla, RC (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* **18**: 357–368.
26. Kong, X, Wang, R, Xue, Y, Liu, X, Zhang, H, Chen, Y *et al*. (2010). Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. *PLoS One* **5**: e11707.
27. Kim, HS, Xiao, C, Wang, RH, Lahusen, T, Xu, X, Vassilopoulos, A *et al*. (2010). Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab* **12**: 224–236.
28. Fullerton, MD and Steinberg, GR (2010). SIRT1 takes a backseat to AMPK in the regulation of insulin sensitivity by resveratrol. *Diabetes* **59**: 551–553.
29. Lan, F, Cacicedo, JM, Ruderman, N and Ido, Y (2008). SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. *J Biol Chem* **283**: 27628–27635.
30. Banks, AS, Kon, N, Knight, C, Matsumoto, M, Gutiérrez-Juárez, R, Rossetti, L *et al*. (2008). SirT1 gain of function increases energy efficiency and prevents diabetes in mice. *Cell Metab* **8**: 333–341.
31. Wang, P, Zhang, RY, Song, J, Guan, YF, Xu, TY, Du, H *et al*. (2012). Loss of AMP-activated protein kinase-α2 impairs the insulin-sensitizing effect of calorie restriction in skeletal muscle. *Diabetes* **61**: 1051–1061.
32. Fujii, N, Ho, RC, Manabe, Y, Jessen, N, Toyoda, T, Holland, WL *et al*. (2008). Ablation of AMP-activated protein kinase alpha2 activity exacerbates insulin resistance induced by high-fat feeding of mice. *Diabetes* **57**: 2958–2966.
33. Schiaffino, S (2010). Fibre types in skeletal muscle: a personal account. *Acta Physiol (Oxf)* **199**: 451–463.
34. Schreurs, M, Kuipers, F and van der Leij, FR (2010). Regulatory enzymes of mitochondrial beta-oxidation as targets for treatment of the metabolic syndrome. *Obes Rev* **11**: 380–388.
35. Houtkooper, RH, Pirinen, E and Auwerx, J (2012). Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* **13**: 225–238.
36. Chalkiadaki, A, Igarashi, M, Nasamu, AS, Knezevic, J and Guarente, L (2014). Muscle-specific SIRT1 gain-of-function increases slow-twitch fibers and ameliorates pathophysiology in a mouse model of duchenne muscular dystrophy. *PLoS Genet* **10**: e1004490.
37. Lin, L, Chen, K, Abdel Khalek, W, Ward, JL 3rd, Yang, H, Chabi, B *et al*. (2014). Regulation of skeletal muscle oxidative capacity and muscle mass by SIRT3. *PLoS One* **9**: e85636.
38. Hénique, C, Mansouri, A, Vavrova, E, Lenoir, V, Ferry, A, Esnous, C *et al*. (2015). Increasing mitochondrial muscle fatty acid oxidation induces skeletal muscle remodeling toward an oxidative phenotype. *FASEB J* **29**: 2473–2483.
39. Augusto V, Padovani CR, and Rocha Campos GE (2004). Skeletal muscle fiber types in C57BL6J mice. *Braz J morphol Sci* **21**: 89–94.
40. Izumiya, Y, Hopkins, T, Morris, C, Sato, K, Zeng, L, Viereck, J *et al*. (2008). Fast/Glycolytic muscle fiber growth reduces fat mass and improves metabolic parameters in obese mice. *Cell Metab* **7**: 159–172.
41. Fulco, M and Sartorelli, V (2008). Comparing and contrasting the roles of AMPK and SIRT1 in metabolic tissues. *Cell Cycle* **7**: 3669–3679.
42. Röckl, KS, Hirshman, MF, Brandauer, J, Fujii, N, Witters, LA and Goodyear, LJ (2007). Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. *Diabetes* **56**: 2062–2069.
43. Hegarty, BD, Turner, N, Cooney, GJ and Kraegen, EW (2009). Insulin resistance and fuel homeostasis: the role of AMP-activated protein kinase. *Acta Physiol (Oxf)* **196**: 129–145.
44. White, AT, McCurdy, CE, Philp, A, Hamilton, DL, Johnson, CD and Schenk, S (2013). Skeletal muscle-specific overexpression of SIRT1 does not enhance whole-body energy expenditure or insulin sensitivity in young mice. *Diabetologia* **56**: 1629–1637.
45. White, AT, Philp, A, Fridolfsson, HN, Schilling, JM, Murphy, AN, Hamilton, DL *et al*. (2014). High-fat diet-induced impairment of skeletal muscle insulin sensitivity is not prevented by SIRT1 overexpression. *Am J Physiol Endocrinol Metab* **307**: E764–E772.

46. Fröjdö, S, Durand, C, Molin, L, Carey, AL, El-Osta, A, Kingwell, BA *et al.* (2011). Phosphoinositide 3-kinase as a novel functional target for the regulation of the insulin signaling pathway by SIRT1. *Mol Cell Endocrinol* **335**: 166–176.

47. Schenk, S, McCurdy, CE, Philp, A, Chen, MZ, Holliday, MJ, Bandyopadhyay, GK *et al.* (2011). Sirt1 enhances skeletal muscle insulin sensitivity in mice during caloric restriction. *J Clin Invest* **121**: 4281–4288.

48. Boutant, M, Joffraud, M, Kulkarni, SS, García-Casarrubios, E, García-Roves, PM, Ratajczak, J *et al.* (2015). SIRT1 enhances glucose tolerance by potentiating brown adipose tissue function. *Mol Metab* **4**: 118–131.

49. Alcendor, RR, Gao, S, Zhai, P, Zablocki, D, Holle, E, Yu, X *et al.* (2007). Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* **100**: 1512–1521.

50. Kawashima, T, Inuzuka, Y, Okuda, J, Kato, T, Niizuma, S, Tamaki, Y *et al.* (2011). Constitutive SIRT1 overexpression impairs mitochondria and reduces cardiac function in mice. *J Mol Cell Cardiol* **51**: 1026–1036.

51. Wu, D, Qiu, Y, Gao, X, Yuan, XB and Zhai, Q (2011). Overexpression of SIRT1 in mouse forebrain impairs lipid/glucose metabolism and motor function. *PLoS One* **6**: e21759.

52. Libert, S, Pointer, K, Bell, EL, Das, A, Cohen, DE, Asara, JM *et al.* (2011). SIRT1 activates MAO-A in the brain to mediate anxiety and exploratory drive. *Cell* **147**: 1459–1472.

53. Kim, HD, Hesterman, J, Call, T, Magazu, S, Keeley, E, Armenta, K *et al.* (2016). SIRT1 mediates depression-like behaviors in the nucleus accumbens. *J Neurosci* **36**: 8441–8452.

54. Vilà, L, Elias, I, Roca, C, Ribera, A, Ferré, T, Casellas, A *et al.* (2014). AAV8-mediated Sirt1 gene transfer to the liver prevents high carbohydrate diet-induced nonalcoholic fatty liver disease. *Mol Ther Methods Clin Dev* **1**: 14039.

55. Ayuso, E, Mingozzi, F, Montane, J, Leon, X, Anguela, XM, Haurigot, V *et al.* (2010). High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. *Gene Ther* **17**: 503–510.

56. Matsushita, T, Elliger, S, Elliger, C, Podsakoff, G, Villarreal, L, Kurtzman, GJ *et al.* (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* **5**: 938–945.

57. Roglans, N, Vilà, L, Farré, M, Alegret, M, Sánchez, RM, Vázquez-Carrera, M *et al.* (2007). Impairment of hepatic Stat-3 activation and reduction of PPARalpha activity in fructose-fed rats. *Hepatology* **45**: 778–788.

58. Elias, I, Franckhauser, S, Ferré, T, Vilà, L, Tafuro, S, Muñoz, S *et al.* (2012). Adipose tissue overexpression of vascular endothelial growth factor protects against diet-induced obesity and insulin resistance. *Diabetes* **61**: 1801–1813.

59. Carr, TP, Andresen, CJ and Rudel, LL (1993). Enzymatic determination of triglyceride, free cholesterol, and total cholesterol in tissue lipid extracts. *Clin Biochem* **26**: 39–42.