

Specific SIRT1 Activation Mimics Low Energy Levels and Protects against Diet-Induced Metabolic Disorders by Enhancing Fat Oxidation

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SUMMARY

The NAD⁺-dependent deacetylase SIRT1 controls metabolic processes in response to low nutrient availability. We report the metabolic phenotype of mice treated with SRT1720, a specific and potent synthetic activator of SIRT1 that is devoid of direct action on AMPK. SRT1720 administration robustly enhances endurance running performance and strongly protects from diet-induced obesity and insulin resistance by enhancing oxidative metabolism in skeletal muscle, liver, and brown adipose tissue. These metabolic effects of SRT1720 are mediated by the induction of a genetic network controlling fatty acid oxidation through a multifaceted mechanism that involves the direct deacetylation of PGC-1 α , FOXO1, and p53 and the indirect stimulation of AMPK signaling through a global metabolic adaptation mimicking low energy levels. Combined with our previous work on resveratrol, the current study further validates SIRT1 as a target for the treatment of metabolic disorders and characterizes the mechanisms underlying the therapeutic potential of SIRT1 activation.

INTRODUCTION

The prevalence of metabolic disorders has been increasing over the past decades with the adoption of a sedentary lifestyle combined with excessive caloric intake. Increased physical activity and better feeding habits are clearly a requisite to limit or reverse weight excess and its deleterious metabolic consequences. However, dietary management and exercise are not usually successful as an intervention, underscoring the need for more efficient medication to treat metabolic disorders. Intense drug discovery efforts in the metabolic field currently focus on enhancing energy expenditure in organs specialized in energy consumption.

Integrated metabolic networks, which are governed at the transcriptional level by transcription factors and coregulators, enable the organism to adapt the metabolic state of different

organs to nutrient availability (Desvergne et al., 2006; Feige and Auwerx, 2007; Spiegelman and Heinrich, 2004). Sirtuins, a family of NAD⁺-dependent deacetylases, have recently emerged as integral components of these metabolic networks, which are particularly important for energy homeostasis (Guarente, 2006; Michan and Sinclair, 2007; Yamamoto et al., 2007). Sirtuins modulate gene expression according to the energetic state of the cell, which they sense through NAD⁺ levels, by deacetylating histones as well as transcription factors and coregulators (Feige and Auwerx, 2008). The founding member of the family, SIRT1, promotes longevity in response to caloric restriction in species ranging from yeast to mammals, and it is believed that these protective actions may result, at least in part, from the regulation of energy homeostasis (Guarente, 2006). Consistently, SIRT1 is an important regulator of metabolic processes such as lipolysis, fatty acid oxidation (FAO), mitochondrial activity, and gluconeogenesis (Baur et al., 2006; Gerhart-Hines et al., 2007; Lagouge et al., 2006; Picard et al., 2004; Rodgers et al., 2005; Rodgers and Puigserver, 2007), which occur in response to an intracellular rise in the NAD⁺/NADH ratio when energy supply is low. As SIRT1 is activated by caloric restriction, the ability to allosterically induce its activity opens the possibility to pharmacologically mimic low energetic levels and, thereby, stimulate fat utilization to prevent diet-induced obesity and its associated disorders. This concept has been validated in mice treated with the natural polyphenol resveratrol, which activates SIRT1 and protects from obesity by inducing oxidative mitochondrial metabolism through deacetylation of the PPAR γ coactivator 1 α (PGC-1 α) (Baur et al., 2006; Lagouge et al., 2006). The observation that resveratrol can activate both SIRT1 and the AMP-activated protein kinase (AMPK) leaves open the question of what degree of efficacy is due to the direct activation of SIRT1 (Baur et al., 2006). To address this issue, we have used a selective synthetic SIRT1 activator with improved on-target selectivity, potency, and efficacy, and we have tested its metabolic actions for protection against diet-induced obesity and metabolic disorders in mouse models. Our results demonstrate that SRT1720 administration protects from diet-induced obesity and its negative consequences on glucose homeostasis by primarily promoting fat consumption in skeletal muscle, liver, and brown adipose tissue (BAT).

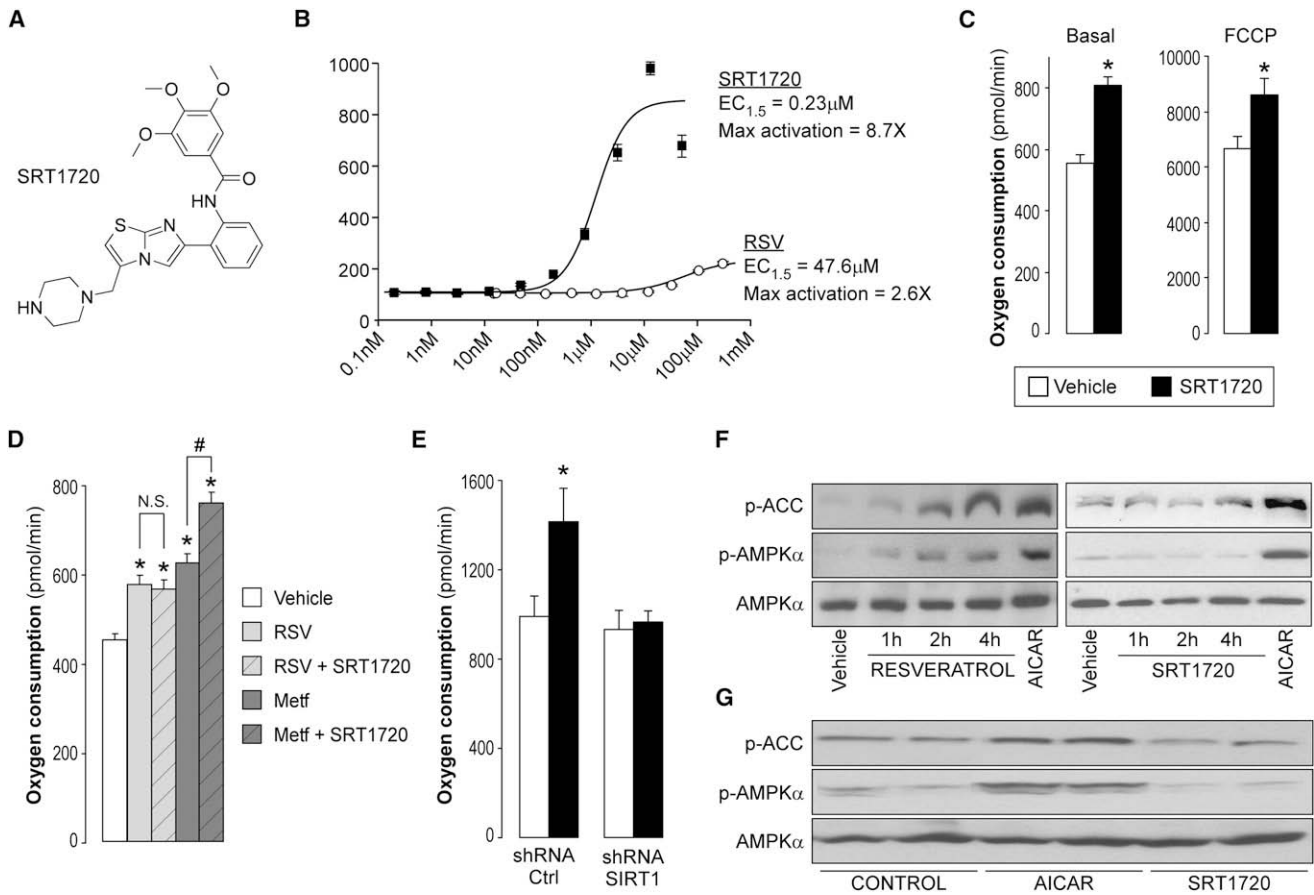


Figure 1. SRT1720 Is a High-Affinity Specific Activator of SIRT1

(A) Structure of the SRT1720 compound.

(B) The action of resveratrol (RSV) and SRT1720 on SIRT1 activity was assessed in a mass spectrometry-based deacetylation assay of a 20 residue peptide.

(C–E) Oxygen consumption was measured in C2C12 myotubes treated for 24 hr with 10 nM SRT1720 before or after a 1 hr treatment with the uncoupler FCCP at 500 nM (C), in combination with 5 μM resveratrol (RSV) or 2 mM metformin (Metf) (D), or 48 hr following an adenoviral infection with control or SIRT1 shRNAs (E).

(F) The activation of the AMP-activated protein kinase (AMPK) was assessed in C2C12 myotubes treated with vehicle, vehicle and 500 μM AICAR (15 min), 5 μM resveratrol, or 50 nM SRT1720 by evaluating the phosphorylation state of AMPKα and its target acetylCoA carboxylase (ACC) by western blotting.

(G) The acute action of SRT1720 on AMPK activation was evaluated after a single administration at 500 mg/kg in wild-type male C57BL/6J 4 hr prior to sacrifice. AICAR was administered as a positive control 4 hr prior to sacrifice at 500 mg/kg. AMPKα and ACC phosphorylation was analyzed by western blot in gastrocnemius muscle.

Error bars represent SEM, and significant differences to untreated controls ($p < 0.05$) are indicated by an asterisk. Other significant differences are indicated by a pound symbol.

RESULTS

SRT1720 Is a Potent and Specific Activator of SIRT1

Given the beneficial metabolic actions of the naturally occurring SIRT1 activator resveratrol, we selected a more potent and efficacious SIRT1 activator identified in a small molecule screen for SIRT1 agonists (Milne et al., 2007; Figure 1A). In a mass spectrometry deacetylation assay, SRT1720 induced the activity of SIRT1 by 8.7-fold, whereas the activation by resveratrol was limited to a 2.6-fold increase (Figure 1B). Importantly, this increased efficacy was also associated with enhanced potency, as the $EC_{1.5}$ for SIRT1, which represents the concentration required to reach a 50% increase over basal, was 0.23 μM for SRT1720 but 47.6 μM for resveratrol.

Since resveratrol protects against metabolic disorders by inducing energy expenditure, the action of SRT1720 was validated

in a cellular model utilizing oxygen consumption as a readout of energy expenditure. In C2C12 myotubes, SRT1720 significantly stimulated oxygen consumption both in basal conditions and after stimulation with the chemical uncoupler FCCP (Figure 1C). Interestingly, this effect was observed after 24 hr of treatment, but not after a short 1 hr treatment (data not shown), a timing suggesting that the actions of SRT1720 on oxygen consumption are mediated by transcriptional mechanisms. In addition, the SRT1720-mediated stimulation of oxygen consumption was totally blocked by the ATP synthase inhibitor oligomycin (Figure S1A available online). To evaluate the specificity of SRT1720, we first analyzed whether it could synergize with other stimulators of cellular respiration in C2C12 cells (Figure 1D). The actions of SRT1720 were additive to those of the AMPK activator metformin, but not to those of resveratrol, suggesting that SRT1720 induces oxygen consumption via SIRT1. The direct involvement of SIRT1 in the

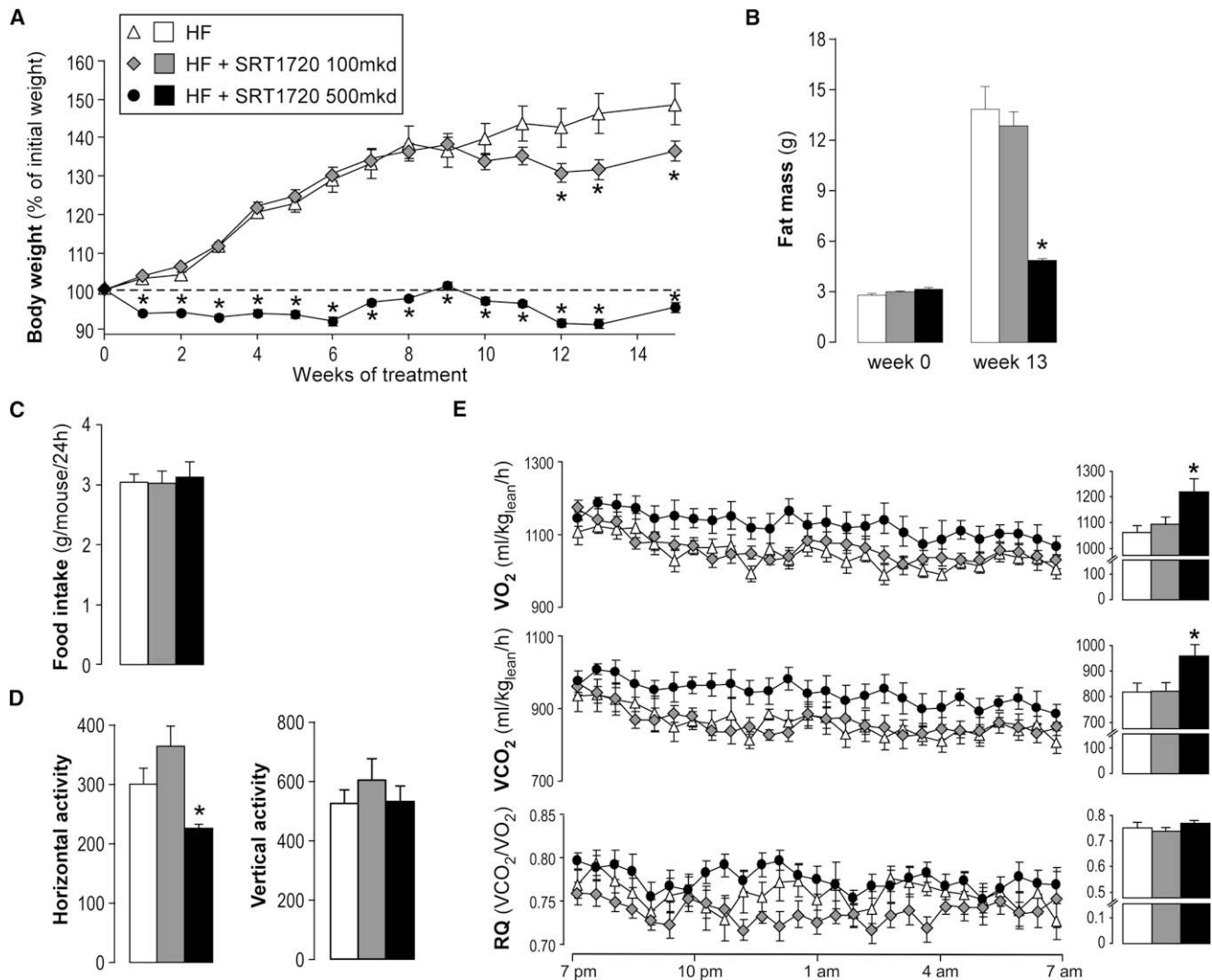


Figure 2. SRT1720 Prevents Diet-Induced Obesity in Male C57BL/6J Mice

(A) Body weight evolution of animals fed with a HF diet alone or supplemented with SRT1720 at 100 or 500 mg per kg of body weight per day (mkd) ($n = 10$). (B) Fat mass was measured by dexascan before and after 13 weeks of treatment ($n = 10$). (C) Average daily food intake ($n = 10$). (D) Spontaneous activity of mice represented by the average horizontal and vertical activities monitored over a 24 hr period after 13 weeks of treatment ($n = 8$). (E) Energy expenditure was evaluated by the measurement of oxygen consumption (VO_2), of carbon dioxide release (VCO_2), and by the calculation of the respiratory quotient (RQ) over a 12 hr period after 10 weeks of treatment. The adjacent bar graphs represent the average for each group ($n = 8$). Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

response to SRT1720 was formally demonstrated using a shRNA knockdown of SIRT1, which ablated the SRT1720-mediated increase of oxygen consumption (Figure 1E). In addition, we evaluated the action of SRT1720 on AMPK, which has been demonstrated to be activated by resveratrol independently of SIRT1 (Baur et al., 2006; Dasgupta and Milbrandt, 2007). Unlike resveratrol and the synthetic AMPK activator AICAR, SRT1720 did not induce a significant level of phosphorylation of the α subunit of AMPK and of its downstream target acetylCoA carboxylase (ACC) in C2C12 myotubes at various time points and concentrations (Figures 1F and S1B). Furthermore, and perhaps most importantly, acute SRT1720 administration *in vivo* did not enhance AMPK signaling either (Figure 1G). Altogether, these results

demonstrate that SRT1720 is a potent and specific SIRT1 activator that does not directly stimulate AMPK.

SRT1720 Protects from Diet-Induced Diabetes

The metabolic actions of SRT1720 were then evaluated by administering the compound by food admixture to wild-type C57BL/6J male mice challenged with a high-fat (HF) diet. The incorporation of the compound to the diet was adjusted weekly to food intake and body weight in order to achieve average exposures of 100 and 500 mg/kg/day (mkd). While the low dose only partially protected from weight gain after ~ 10 weeks of treatment, the high dose totally prevented diet-induced obesity by inhibiting fat accumulation (Figures 2A and 2B). Importantly,

this effect on body weight did not result from altered feeding behavior or increased locomotor activity (Figures 2C, 2D, and S2A). Enzymes associated with hepatic injury or tissue breakdown were normal during the study, indicating that SRT1720 was well tolerated and that the efficacy observed was not through a toxic response (Figure S2B). In addition, despite slightly elevated fecal lipid content, the histology of the gut was not altered by the treatment (Figures S2C and S2D), suggesting that SRT1720 did not significantly alter intestinal nutrient uptake.

The lean phenotype of SRT1720-treated mice fed HF diet correlated with enhanced energy expenditure, as both oxygen consumption and carbon dioxide release were increased by the treatment (Figure 2E). This stimulation of the metabolic rate occurred without changes in the respiratory quotient (RQ), which reflects the relative use of carbohydrates versus lipids as a source of energy. Since mice on HF diet use fatty acids as the main source of energy, the RQ of untreated animals was close to 0.7, the theoretical lower limit of this parameter corresponding to lipid utilization solely. To evaluate the action of SRT1720 on fuel preference in a setting where RQ variations were not masked by HF content in the diet, we treated mice on regular chow diet and repeated indirect calorimetry either in fed animals or in animals where endogenous fat utilization was stimulated by short-term fasting (Figure S3B). The RQ was higher on chow than on HF diet (0.95 versus 0.75, respectively) and was only slightly lowered by SRT1720 in fed conditions. However, the RQ of chow-fed mice decreased robustly upon fasting, demonstrating that SRT1720 can shift the fuel preference toward fatty acids.

Since diet-induced obesity exerts detrimental actions on plasma lipid profiles and glucose homeostasis, we analyzed whether SRT1720 could normalize these parameters. Lipid profiles in the plasma of SRT1720-treated mice were improved as both triglyceride and cholesterol levels were reduced on HF diet (Figure 3A). Reduced cholesterol concentrations resulted mainly from a decrease in LDL cholesterol (Figure 3A). On chow diet, cholesterol levels were unaffected by SRT1720 (data not shown), but triglyceride and VLDL levels were lowered by SRT1720 in chow-fed mice (Figure S3C). SRT1720 also prevented the negative impact of HF feeding on glucose homeostasis as fasting blood glucose and insulin were both reduced (Figure 3B). In addition, mice treated with SRT1720 had a better tolerance to a glucose load, which was particularly prominent in the late time points of an intraperitoneal glucose tolerance test (IP-GTT) (Figure 3C). Together with the reduced fasting insulin levels, this observation suggested that SRT1720 improves glucose homeostasis by enhancing insulin sensitivity. This hypothesis was confirmed in a hyperinsulinemic-euglycemic clamp in which the glucose infusion rate, which reflects the sensitivity of peripheral tissues to insulin, was significantly enhanced by SRT1720 treatment (Figure 3D). Since obesity and insulin resistance are intricately linked, we demonstrated that improved glucose tolerance at 500 mkd was not an indirect consequence of reduced adiposity since glucose tolerance was also improved in an IP-GTT in chow-fed mice treated with SRT1720 for 3 weeks, in which body weight was not substantially affected (Figure S3E). In addition, the direct action of SRT1720 on insulin sensitivity was confirmed by the increased glucose infusion rate in a hyperinsulinemic-euglycemic clamp in mice treated for 3 weeks on HF

diet, for which the average body weight difference between groups does not exceed 3 g (Figure 3E). In this experiment, tritiated glucose was used as a tracer to evaluate whole-body glucose turnover before and after insulin perfusion (Figure 3F). Although the glucose turnover only tended to increase in SRT1720-treated mice, the ability of insulin to suppress endogenous glucose production was significantly enhanced upon SRT1720 administration, demonstrating that SRT1720 improves hepatic insulin sensitivity. Furthermore, insulin sensitivity was also enhanced in the skeletal muscle, as insulin-stimulated glucose uptake, measured through ^{14}C -2-deoxy-glucose uptake, was higher in the gastrocnemius muscle of SRT1720-treated mice (Figure 3G).

Altogether, these results demonstrate that activating SIRT1 by SRT1720 in a context of caloric excess strongly protects from fat accretion. Furthermore, our data show that SRT1720 improves glucose tolerance and insulin sensitivity both in the context of HF and chow feeding.

SRT1720 Promotes Energy Expenditure in Metabolic Tissues

A key factor controlling energy homeostasis is the balance between caloric intake and expenditure, which modulates fat accumulation and peripheral insulin sensitivity. Therefore, we analyzed the actions of SRT1720 on lipid accumulation and energy expenditure in organs that integrate the metabolic response of the entire body. The mass of epididymal white adipose tissue (WAT) from mice, where HF feeding was thwarted by 500 mkd SRT1720, was three times lower than that of control mice on HF diet only (Figure S4A). In addition, this reduced fat accumulation was associated with smaller adipocyte size (Figure S4B), a feature that correlates with leanness and insulin sensitivity. Since smaller adipose tissue could be linked to altered adipogenesis or enhanced lipolysis, we verified the expression of well-established markers of these processes in WAT. SRT1720 did not induce major impairments in the ability of adipocytes to differentiate and store lipids, as despite a slight induction of peroxisome proliferator-activated receptor (PPAR) γ expression, the levels of its downstream targets C/EBP α , aP2, and CD36 were not affected (Figure S4C). Consistent with the previous demonstration of a positive action of SIRT1 on lipolysis (Picard et al., 2004), the RNA levels of the hormone-sensitive lipase were enhanced in SRT1720-treated mice. The enhanced expression of oxidative markers, such as the PGC-1 coactivators and the nuclear receptor PPAR β/δ , suggests that SRT1720 could promote energy expenditure in WAT. Nevertheless, the minor contribution of WAT to whole-body energy consumption suggests that this regulation is not the primary cause of the lean phenotype, which most likely results from the reduced availability of lipids for storage.

Since the absence of fat accumulation in SRT1720-treated mice suggested that energy expenditure could be stimulated, we analyzed the metabolic capacities of energy dissipating organs. Skeletal muscle function was examined utilizing endurance and locomotor tests (Figure 4A). Mice treated with SRT1720 on HF diet ran approximately twice the distance as control animals in an endurance exercise test. To rule out an indirect effect of weight difference on running capacities, we exercised chow-fed mice treated for 6 weeks with SRT1720 and

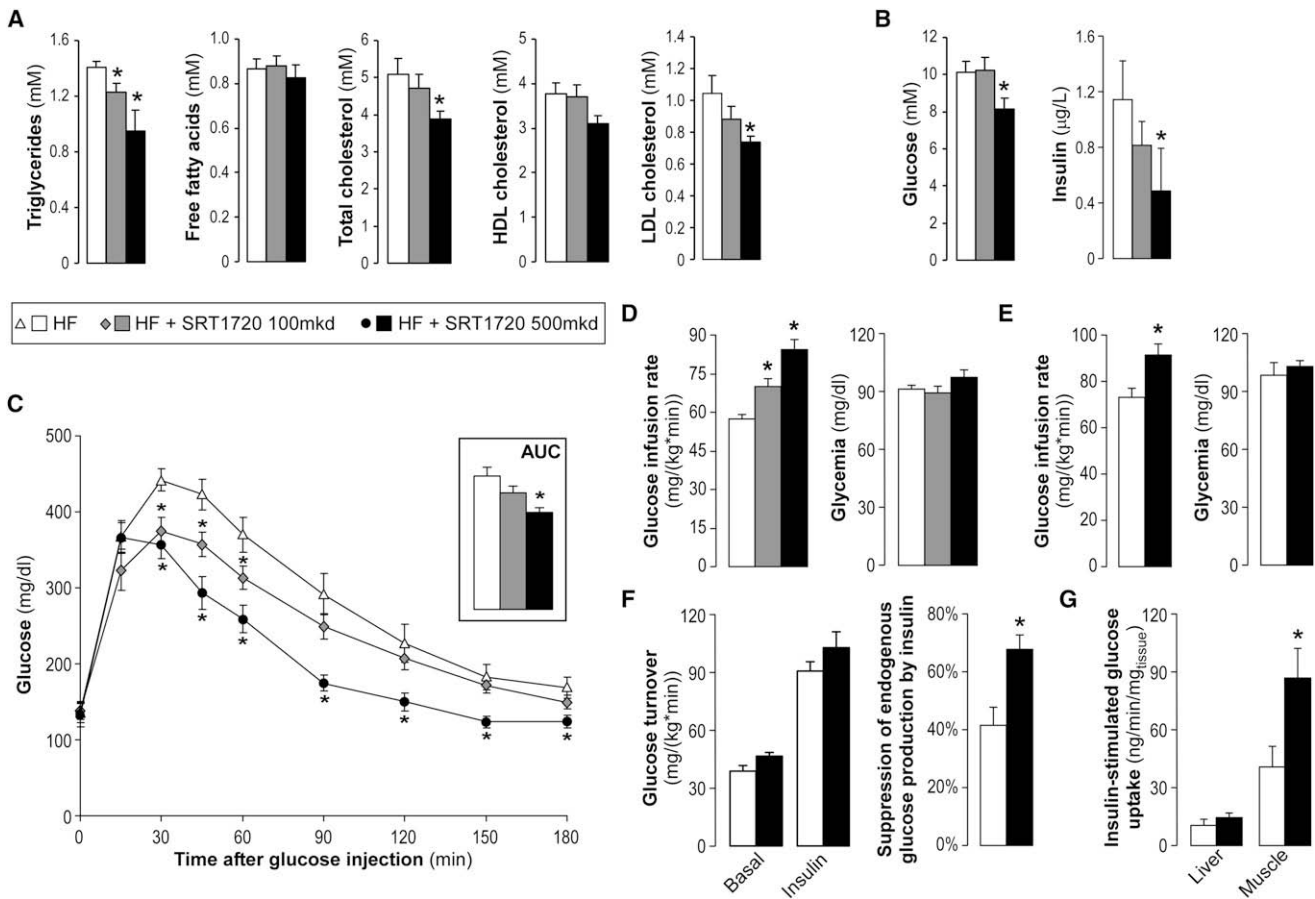


Figure 3. SRT1720 Improves Glucose and Cholesterol Homeostasis

(A and B) Lipid (A) and glucose and insulin (B) levels were measured after 16 hr of fasting in the plasma of mice treated with HF diet alone or supplemented with SRT1720 at 100 or 500 mkd for 6 weeks ($n = 8$).

(C) Intraperitoneal glucose tolerance test on mice treated with SRT1720 as in (A) for 12 weeks and injected with 2 g glucose/kg after 12 hr of fasting. The adjacent bar graph represents the average area under the curve ($n = 10$).

(D) Insulin sensitivity evaluated through the average glucose infusion rate at equilibrium in a hyperinsulinemic-euglycemic clamp (18 mU insulin/min/kg) in mice treated with SRT1720 as in (A) for 20 weeks. The equilibrium at clamp is reflected by the euglycemia shown in the adjacent graph ($n = 5$).

(E–G) A hyperinsulinemic-euglycemic clamp (18 mU insulin/min/kg) was performed using radioactive tracers in mice treated with SRT1720 as in (A) for 3 weeks. The evaluation of whole-body glucose turnover (F) and glucose uptake in tissues (G) was performed using ^3H -glucose and ^{14}C -2-deoxy-glucose tracers, respectively.

Error bars represent SEM, and significant differences compared to untreated controls (p value < 0.05) are indicated by an asterisk.

demonstrated that the animals on SRT1720 also became much better endurance runners (Figure S3D). Increased muscle strength in the grip strength test, as well as better locomotor behavior, as concluded from the increased latency to fall in a rotarod test and the reduced time to hind paw equilibration in a string test, all provide additional support to the improved muscle function upon SRT1720 administration. Since improved endurance can result from variations in the proportion of glycolytic and oxidative muscle fibers, we evaluated whether SRT1720 can promote fiber type switching through a succinate dehydrogenase (SDH) staining of muscle fibers (Figure 4B). The proportion of blue-stained oxidative fibers was higher in the gastrocnemius of SRT1720-treated mice, whereas it could not be further enhanced by the treatment in the purely oxidative soleus muscle. The shift toward more oxidative fibers occurred without changes in mitochondrial density (Figures S5B and S5C). In addition,

citrate synthase (Figure S5D) and cytochrome C oxidase (Figure S5E) activities were not altered in SRT1720-treated mice, suggesting that SRT1720 enhances oxidative metabolism through other mitochondrial mechanisms.

Therefore, to understand the molecular basis of the improved endurance and fiber type switch, we analyzed gene expression from gastrocnemius skeletal muscle (Figure 4D). Despite an increased mRNA level of cytochrome C oxidase (Cox IV), the expression of most of the genes controlling mitochondrial function and oxidative phosphorylation (OxPhos) was only minimally affected by SRT1720 treatment. We observed, however, a switch in the contractile phenotype of the muscle fibers, as SRT1720-treated mice expressed higher mRNA levels of both troponin I slow (TropoI_{slow}) and type I and IIa myosin heavy chains (MyHC), three markers of slow-twitch oxidative fibers, but had reduced expression of the mRNA encoding the fast-twitch

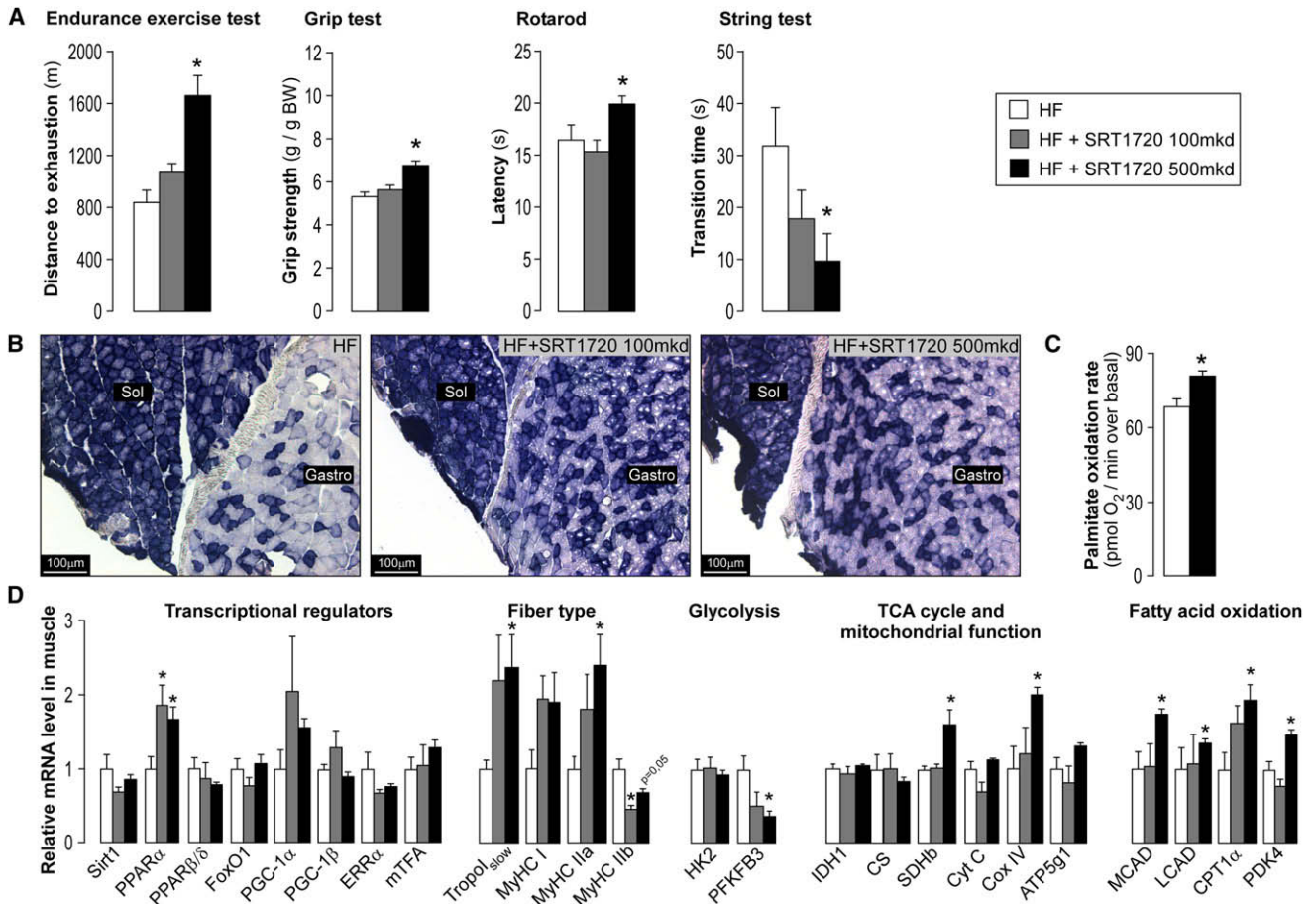


Figure 4. SRT1720 Improves Endurance and Locomotor Functions

(A) Endurance, evaluated by the average distance run until exhaustion on a treadmill, and locomotor functions, evaluated through the string, the grip strength, and the rotarod tests, were performed in mice treated for 15 weeks with HF diet alone or supplemented with either 100 or 500 mkd SRT1720 (n = 10).

(B) Representative succinate dehydrogenase staining on gastrocnemius (gastro) and soleus (sol) muscle sections.

(C) Fatty acid oxidation rates were measured in C2C12 cells treated for 24 hr with vehicle (white bars) or 10 nM SRT1720 (black bars) using oxygen consumption as a readout (n = 10).

(D) Gene expression in gastrocnemius skeletal muscle expressed relative to β2-microglobulin in the same mice as in (C). Abbreviations not used previously: HK, hexokinase; IDH, isocitrate dehydrogenase; Cyt C, cytochrome C; ATP5g1, ATP synthase subunit 5g1; CPT-1, carnitine palmitoyl transferase 1.

Error bars represent SEM, and significant differences compared to untreated controls (p < 0.05) are indicated by an asterisk.

glycolytic marker MyHC type IIb. Interestingly, this switch correlated with a lower expression of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) and an increased expression of the pyruvate dehydrogenase kinase 4 (PDK4) mRNA, which favors the utilization of fatty acids over glucose by lowering the utilization of pyruvate through inhibition of the pyruvate dehydrogenase complex. Moreover, SRT1720 also stimulated the expression of genes promoting FAO, such as PPARα and the medium- and long-chain acyl-CoA dehydrogenases (MCAD and LCAD), to concomitantly increase the flux of fatty acid utilization in the muscle. In line with these observations, the genes upregulated by SRT1720 at the genome-wide level were enriched in genes from the mitochondrial β-oxidation pathway (Figure S5A). Moreover, these gene expression changes correlated functionally with enhanced FAO as SRT1720 increased palmitate oxidation rates in muscle cells (Figure 4C). Therefore, SRT1720 induces a switch toward more oxidative

muscle fiber types by primarily stimulating FAO rather than by improving mitochondrial function per se.

Given the important contribution of hepatic FAO to whole-body energy expenditure, we evaluated whether the beneficial effects of SRT1720 on muscle oxidative functions could also be observed in the liver. Reduced staining with oil red O confirmed that hepatic fat storage in lipid droplets was significantly reduced in SRT1720-treated mice (Figure 5A), predominantly because the accumulation of triglycerides was inhibited (Figure 5B). In contrast, hepatic free fatty acid levels were not altered. We then tested the different metabolic pathways that SRT1720 could regulate to promote energy expenditure. The expression of genes controlling glycolysis was not altered in SRT1720-treated mice (Figure 5C), and gluconeogenesis was not consistently changed, as the expression of the phosphoenolpyruvate carboxykinase (PEPCK) was modestly enhanced, while that of glucose-6-phosphatase (G6Pase) remained unchanged. SRT1720, however, had

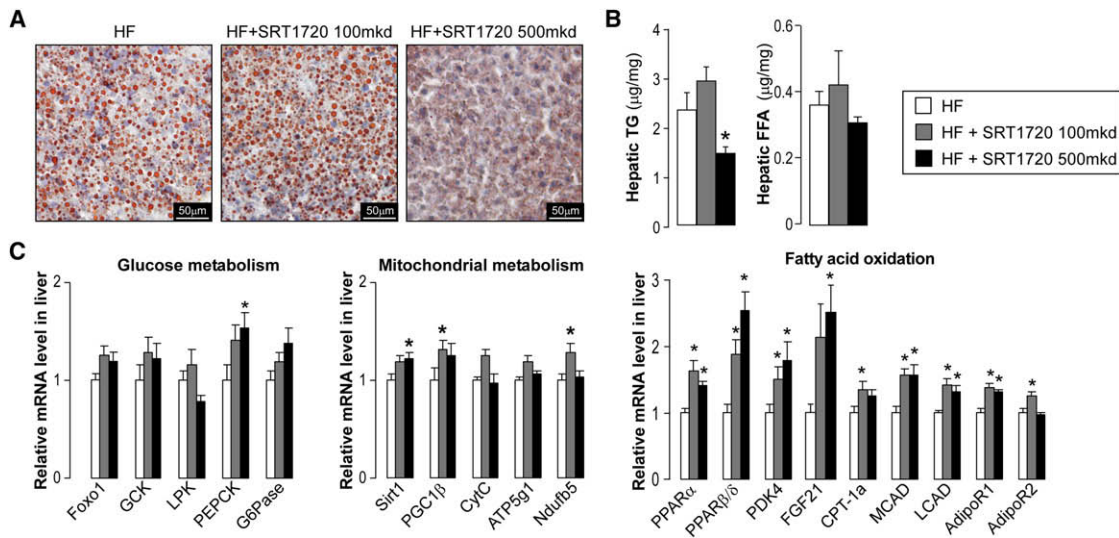


Figure 5. SIRT1720 Promotes Hepatic Oxidative Functions

(A) Representative oil-red-O staining of liver sections from mice fed for 20 weeks with HF diet alone or supplemented with SIRT1720 at 100 or 500 mkd.

(B) Hepatic lipid content was measured following Folch's extraction (n = 10).

(C) Gene expression in liver expressed relative to β -actin and 36B4 (n = 10). Abbreviations not used previously: GCK, glucocarcboxykinase; LPK, L-type pyruvate kinase; Ndufb5, NADH dehydrogenase 1 β 5; AdipoR, adiponectin receptors.

Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

a strong impact on the expression of enzymes and regulators controlling FAO (Figures 5C and S5A). The expression of the two nuclear receptors PPAR α and β/δ was robustly induced in the liver of SIRT1720-treated mice. The functional relevance of this induction was validated by an increased expression of direct PPAR target genes. Among these, SIRT1720 exerted particularly prominent actions on the mRNA expression of PDK4 and the fibroblast growth factor 21 (FGF21), an antiobesity signaling molecule secreted by the liver in response to PPAR α activation (Kharitonov and Shanafelt, 2008). Consistent with what was observed in the muscle, the actions of SIRT1720 on hepatic oxidative metabolism occurred with rather discrete changes in mitochondrial function and OxPhos (Figure 5C), suggesting that this SIRT1 activator stimulates hepatic energy expenditure by primarily acting on the degradation of fatty acids.

BAT is another major tissue where energy, coming predominantly from fat, is dissipated to maintain body temperature. Consistent with the lean phenotype induced by the treatment, mice on SIRT1720 had smaller brown adipocytes, in which the size of lipid droplets was reduced (Figures 6A and 6B). Similar to what was observed in skeletal muscle, the number of mitochondria did not seem to be affected by SIRT1720 despite a lower density caused by reduced lipid droplet size (Figure 6B). Low fat accumulation in BAT correlated with the global activation of a network of genes controlling energy expenditure (Figure 6C). The expression of several transcription factors, including PPAR α and γ , thyroid hormone receptors (TR) α and β , and PGC-1 α and β , was strongly activated in the BAT of SIRT1720-treated animals. In addition, the upregulation of type 2 deiodinase (DIO2), an enzyme that converts inactive thyroid hormone T4 into T3, most likely synergizes with the elevated TR levels to promote thyroid hormone signaling. Several direct targets of PGC-1, such as the estrogen-related receptor α (ERR α), the mitochon-

drial transcription factor A (mTFA), and the superoxide dismutases (SOD) 1 and 2, were also significantly induced. Surprisingly, the expression of the uncoupling protein (UCP) 1, which uncouples mitochondrial electron transport from ATP synthesis to dissipate energy as heat, or that of its homolog UCP2 were not altered. In contrast, the expression of UCP3 was robustly increased by SIRT1720, contributing potentially to energy dissipation by short-circuiting OxPhos. SIRT1720 also potently stimulated the expression of genes controlling FAO, whereas its action on components of the mitochondrial electron transport chain was fairly limited. In SIRT1720-treated mice, the excess of calories from the diet is most likely transformed to NADH and FADH2 by enhanced fat oxidation and generates a proton gradient in mitochondria, which can subsequently be eliminated through UCP3-mediated uncoupling. In line with these observations, SIRT1720 stimulated oxygen consumption in primary brown adipocytes (Figure 6D). SIRT1720 treatment did not change the body temperature at night. The physiological circadian temperature drop during daytime, because of inactivity and spontaneous fasting as normally observed in control mice, was, however, amplified by the treatment, which lowered body temperature by $\sim 1^\circ\text{C}$ (Figures 6E and 6F). Since this temperature phenotype could not be recapitulated in a short-term experiment where SIRT1720 was administered by daily gavage, it is likely that the temperature phenotype from Figure 6F results from low fat accumulation in SIRT1720-treated mice, which does not allow sufficient insulation and lipolysis to generate heat during resting periods.

SIRT1720 Administration Mechanistically Mimics Low Energy Levels

SIRT1 has emerged as a pleiotropic modulator of transcription factor and coregulator activity, which it regulates through

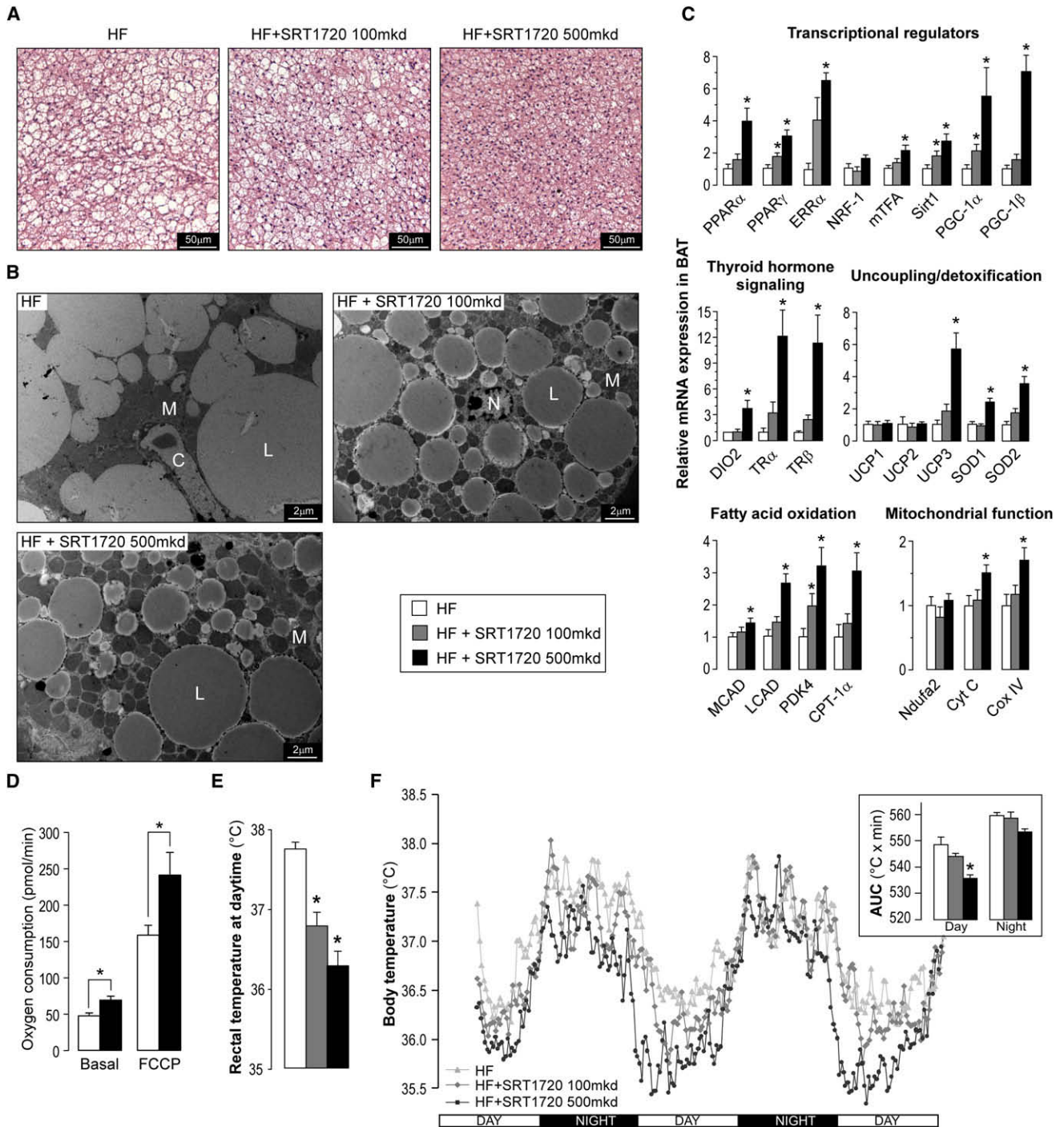


Figure 6. SRT1720 Stimulates Energy Expenditure from the Brown Adipose Tissue

(A and B) Representative hematoxylin and eosin staining (A) and electronic micrographs (B) from BAT sections of mice fed for 20 weeks with HF diet alone or supplemented with SRT1720 at 100 or 500 mkd ($n = 10$). Abbreviations not used previously: L, lipid droplet; M, mitochondrium; N, nucleus; C, capillary.

(C) Gene expression in BAT expressed relative $\beta 2$ -microglobulin ($n = 10$). Abbreviation not used previously: NRF1, nuclear respiratory factor 1.

(D) Oxygen consumption was measured in primary brown adipocytes treated for 24 hr with vehicle (white bars) or 10 nM SRT1720 (black bars) before or after a 1 hr treatment with the uncoupler FCCP at 500 nM.

(E) Rectal temperature measured at 11 a.m. ($n = 10$).

(F) Body temperature monitored by telemetry over 72 hr. The adjacent bar graph represents the average area under the curve over the diurnal and nocturnal phases, respectively ($n = 5$).

Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

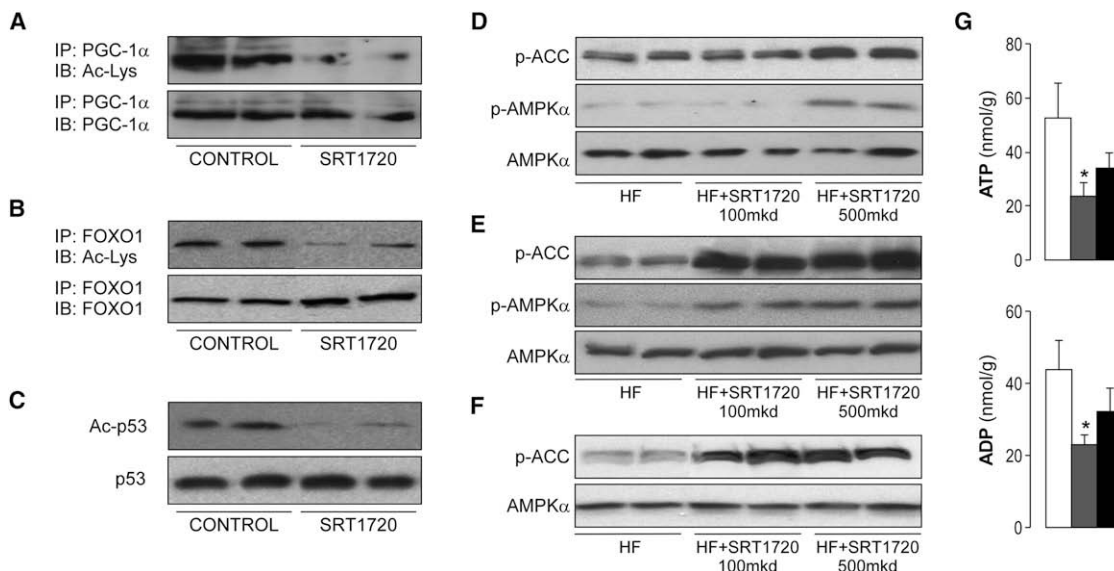


Figure 7. SRT1720 Promotes the Deacetylation of SIRT1 Targets and Indirectly Activates AMPK

(A–C) Acetylation levels of SIRT1 targets in response to chronic SRT1720 administration over 20 weeks. The acetylation of PGC-1 α (A) and FOXO1 (B) was analyzed by immunoprecipitation from nuclear extracts from the gastrocnemius muscle of control mice or mice treated with SRT1720 at 500 mg per kg of body weight per day (mkd), followed by an immunoblot against acetylated lysine residues (top panel) or PGC-1 α /FOXO1 (lower panel). P53 acetylation levels (C) were determined by direct immunoblotting against acetylated (top panel) or total (lower panel) p53 from hepatic nuclear extracts of the same animals as in (A) and (B). (D–F) AMPK activation in mice treated for over 20 weeks with SRT1720 was evaluated in the gastrocnemius muscle (D), the liver (E), and BAT (F) by analyzing AMPK α and ACC phosphorylation levels by western blot. (G) ATP and ADP concentrations were measured using a luciferase-based assay from acid-extracted BAT samples from mice described in (D)–(F) ($n = 8$). Error bars represent SEM, and significant differences compared to untreated controls ($p < 0.05$) are indicated by an asterisk.

deacetylation (Feige and Auwerx, 2008). Since our gene profiling experiments suggested that the metabolic effects of SRT1720 rely at least in part on transcriptional mechanisms, we analyzed the acetylation levels of metabolically relevant transcriptional targets of SIRT1 in response to SRT1720. One of the best characterized metabolic targets of SIRT1 is PGC-1 α , which is activated by SIRT1-mediated deacetylation (Gerhart-Hines et al., 2007; Lagouge et al., 2006; Rodgers et al., 2005, 2008). Endogenous PGC-1 α immunoprecipitated from the gastrocnemius muscle of mice treated with SRT1720 was robustly deacetylated (Figure 7A). In addition, SRT1720 also promoted the deacetylation of the Forkhead transcription factor family O1 (FOXO1) in skeletal muscle and of p53 in liver (Figures 7B and 7C), two other SIRT1 targets implicated in the coordination of metabolic homeostasis (Bensaad and Vousden, 2007; Feige and Auwerx, 2008; Frescas et al., 2005; Gross et al., 2008; Luo et al., 2001; Qiao and Shao, 2006; Vaziri et al., 2001). The ability of SRT1720 to induce the deacetylation of three established SIRT1 targets in different tissues, therefore, unequivocally demonstrates that it is a potent activator of SIRT1 in vivo.

SIRT1 is a well-recognized effector of the beneficial effects of calorie restriction on homeostasis (Guarente, 2006), and it has been speculated that treatment with SIRT1 activators might trigger metabolic pathways activated by low energetic levels (Chen and Guarente, 2007). Given that the systemic response to caloric deprivation is a metabolic switch toward the oxidation of lipids released from adipose tissue stores, SRT1720 seems to induce, at least in part, metabolic pathways activated by low energetic levels. Since AMPK is another key activator of fatty acid catabo-

lism that senses low energetic levels, we tested whether prolonged SRT1720 administration could modulate AMPK activity. Although AMPK was not activated by SRT1720 treatment in cellular models or upon acute exposure in vivo (Figures 1F and 1G), the phosphorylation of AMPK α and its downstream target ACC was increased in the muscle, liver, and BAT of mice treated chronically with 500 mkd SRT1720 (Figures 7D, 7E, and 7F). Given that ATP and ADP levels were reduced in BAT of SRT1720-treated mice (Figure 7G), it is likely that SRT1720 targets AMPK indirectly through the metabolic status.

Altogether, these observations, therefore, support the concept that the SIRT1 activator SRT1720 acts as a calorie restriction mimetic that favors fat utilization by promoting the direct deacetylation of multiple SIRT1 targets and by inducing chronic metabolic adaptations that involve the indirect activation of AMPK.

DISCUSSION

Pharmacologically targeting transcriptional networks to regulate global gene expression programs favoring energy expenditure represents an attractive concept to combat metabolic diseases. In this context, SIRT1 has emerged as an interesting target with the demonstration that the naturally occurring SIRT1 activator resveratrol protects from diet-induced metabolic disorders (Baur et al., 2006; Lagouge et al., 2006). At present, it is, however, difficult to distinguish how many of the effects of resveratrol are specifically mediated by SIRT1 versus other resveratrol targets such as AMPK (Baur et al., 2006; Dasgupta and Milbrandt,

2007). A new generation of selective synthetic SIRT1 activators structurally unrelated to resveratrol and with improved potency, efficacy, and specificity have recently been developed (Milne et al., 2007). In the present study, we have analyzed the global metabolic actions of SRT1720, a member of this new generation of agonists that is more potent and efficacious than resveratrol and exhibits higher specificity with respect to off-target activation of other sirtuin homologs or AMPK (Milne et al., 2007; Figure 1). When incorporated in the diet at a dose of 100 mkd, SRT1720 improves glucose homeostasis by enhancing insulin sensitivity in mice where insulin resistance was induced by HF feeding, as also reported in other rodent models of type 2 diabetes using daily oral administration (Milne et al., 2007). These antidiabetic actions are even more pronounced at a dose of 500 mkd, which also protects from the weight gain induced by HF feeding. Importantly, the antidiabetic actions of SRT1720 are not the indirect consequences of reduced fat accretion, as they can be recapitulated after short-term treatment or under chow diet, where weight differences between groups are minimal. By selectively activating SIRT1, SRT1720 treatment, therefore, mimics the phenotype that is induced by resveratrol administration, as oxidative metabolism and energy expenditure are enhanced in mice treated with both compounds, leading to protection from diet-induced obesity and to improved muscle performance. Although it can be surprising to observe that high doses of SRT1720 are required to induce physiological effects *in vivo* despite a high affinity for SIRT1, this apparent discrepancy relates to the pharmacokinetics of the compound. The plasma concentrations of SRT1720 only reach nanomolar concentrations at both doses administered, presumably because of rapid metabolism and/or distribution, and further chemical refinement of the structure of SRT1720 will be required to enhance bioavailability. In addition, the observation that SRT1720 transiently affects core body temperature and spontaneous locomotor activity could constitute potential side effects for the treatment of metabolic disorders but could also be explored for other therapeutic interventions.

Despite a high correlation of the phenotypic outputs induced by resveratrol and SRT1720, which involve in both cases enhanced energy expenditure in muscle, liver, and BAT, the physiological mechanisms through which these compounds exert their actions only partially overlap. The most prominent difference relates to the way through which both compounds induce oxidative metabolism. Resveratrol acts primarily on mitochondrial biogenesis and function by activating PGC-1 α and, subsequently, inducing the expression of regulators of mitochondrial metabolism such as NRF1, ERR α , and mTFA (Lagouge et al., 2006). In contrast, SRT1720 seems to have a more limited activity on mitochondrial density. It is, therefore, plausible that off-target effects, such as the direct activation of AMPK, are required for the actions of resveratrol on mitochondrial biogenesis. It seems that the activation of SIRT1 by SRT1720 acts upstream on oxidative metabolism by activating the pathways controlling the oxidation of fatty acids. Consistently, SRT1720 promotes palmitate oxidation in cellular models, and the nuclear receptors PPAR α and PPAR β/δ , two major regulators of FAO (Evans et al., 2004; Feige et al., 2006), are upregulated by SRT1720 in several tissues with high rates of fatty acid utilization. Moreover, many oxidative PPAR target genes are also induced, suggesting that

SIRT1 activation by SRT1720 drives a global oxidative program by stimulating fatty acid utilization. In this context, the strong enhancement of endurance and running capacities observed in SRT1720-treated mice most likely results both from a switch in the contractile phenotype of muscle fibers and from fast-twitch glycolytic fibers acquiring more resistance to fatigue by switching their substrate preference toward fatty acids. At the molecular level, the deacetylation of PGC-1 α and FOXO1 by SRT1720 most probably plays a prominent role in this oxidative switch of skeletal muscle fibers, as the metabolic actions of these transcriptional regulators are stimulated by SIRT1-mediated deacetylation, leading to the promotion of oxidative muscle function (Frescas et al., 2005; Gross et al., 2008; Handschin et al., 2007; Lin et al., 2002; Rodgers et al., 2008). The SRT1720-mediated induction of PPAR α expression could be linked to the coactivation of the PPAR α promoter by PGC-1 α (Huss et al., 2004). In addition, activation of PGC-1 α signaling most likely synergizes with increased PPAR expression, as PGC-1 α is a coactivator of PPAR α - and PPAR β/δ -mediated FAO (Vega et al., 2000; Wang et al., 2003). Despite the induction of PGC-1 α expression in various metabolic tissues of SRT1720-treated mice and the ability of SRT1720 to promote PGC-1 α deacetylation, it is interesting to observe that the activation of PGC-1 α signaling by SRT1720 seems limited to its action on FAO but, unlike resveratrol, does not extend to other PGC-1 α -dependent actions. Several hypotheses could explain this more restricted activity. The bioavailability of SRT1720 in metabolic tissues could be distinct from that of resveratrol and dictate tissue-specific regulations. It is also possible that the interplay with other metabolically relevant SIRT1 targets such as FOXO1 or p53 (Bensaad and Vousden, 2007; Feige and Auwerx, 2008; Frescas et al., 2005; Gross et al., 2008; Luo et al., 2001; Qiao and Shao, 2006; Vaziri et al., 2001), which are deacetylated by SRT1720, also modulates the systemic metabolic response. Alternately, the observation that specific SIRT1 agonists modulate only a subset of PGC-1 α target promoters in cellular models (Figure S6) suggests that SRT1720 can drive a selective activation of PGC-1 α signaling.

It is well established that SIRT1 is activated by low energetic levels such as those occurring during fasting or calorie restriction (Michan and Sinclair, 2007; Rodgers et al., 2005), and it has been suggested that SIRT1 activators act, at least in part, by stimulating physiological pathways activated by low energetic levels (Barger et al., 2008; Pearson et al., 2008). Consistently, SRT1720 activates a global network enhancing fat oxidation, a process also stimulated upon fasting in mammals when fatty acids coming from adipose tissue lipolysis become the prominent energetic substrate. In this context, it is highly interesting to observe that prolonged SIRT1 activation by SRT1720 can activate AMPK, a sensor of low energetic levels, through indirect mechanisms. It is possible that the recently described SIRT1-dependent regulation of LKB1 plays a role in this regulation (Hou et al., 2008). However, since SRT1720 does not directly activate AMPK in cellular models or *in vivo* and energetic levels are reduced in certain tissues of SRT1720-treated mice, we believe that SRT1720 could act as a calorie restriction mimetic, which would induce a global metabolic adaptation similar to what occurs under low energetic levels. Given the important role of AMPK in inducing oxidative metabolism in response to low energetic levels, the indirect activation of this kinase and of its downstream effectors by SRT1720

most likely allows the amplification of increased fat oxidation. Since it has been recently suggested that the response to calorie restriction varies greatly between tissues (Chen et al., 2008), it will, therefore, be of importance to precisely characterize how the tissue-specific response to SIRT1 activators relates to energetic levels of individual organs.

Altogether, our results further validate SIRT1 as a bona fide target to combat metabolic disorders and establish SIRT1720 as a prime candidate to explore the potential of SIRT1 as a therapeutic target.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

5-aminoimidazole-4-carboxamide riboside (AICAR; Toronto Research Chemicals) was dissolved in 0.9% NaCl, and metformin (Sigma), resveratrol (Orchid Chemicals), SIRT1720, and SIRT2183 (Sirtris) were dissolved in DMSO. Viral constructs for control and SIRT1 shRNAs, as well as PGC-1 α WT and R13, were previously described (Rodgers et al., 2005). CytC and PDK4 reporter constructs were kind gifts of P. Puigserver and D. Kelly, respectively.

Animal Experiments

Male 7-week-old C57BL/6J mice (Charles River) were housed with a 12 hr light-dark cycle and fed a standard or HF diet containing 60% energy as fat (D12492; Research diet), supplemented or not with SIRT1720, as described in Feige et al. (2008). Phenotyping tests and histology were performed according to EMPRESS standardized protocols as described in Argmann et al. (2006), Heikkinen et al. (2007), and Lagouge et al. (2006). For acute experiments, SIRT1720 and AICAR were injected intraperitoneally at 500 mg/kg 4 hr before the sacrifice. Radiolabeled hyperinsulinemic-euglycemic clamps were performed as described in the Supplemental Experimental Procedures, using ³H-glucose and ¹⁴C-2-deoxy-glucose to evaluate glucose turnover and organ-specific glucose uptake, respectively.

Biochemistry and Immunoblotting

Plasmatic parameters and hepatic and fecal lipid content were measured as previously described (Mataki et al., 2007). Cytochrome C oxidase activity was evaluated by following the oxidation of fully reduced cytochrome C (Sigma) at 550 nm, and citrate synthase activity was measured as previously described (Lagouge et al., 2006). ATP and ADP concentrations were measured using a luciferase-based assay (Biovision) after acid extraction in 5% perchloric acid followed by neutralization with potassium carbonate. Western blots were performed on 0.5–2 mg of total proteins using antibodies directed against the total or phosphorylated subunit α of AMPK, phosphorylated ACC, p53, and acetylated p53 and diluted at 1/1000. PGC-1 α and FOXO1 acetylation was evaluated by immunoprecipitating 1000 μ g of total proteins from skeletal muscle with PGC-1 α or FOXO1 antibodies, followed by immunoblotting against acetylated lysine or against PGC-1 α or FOXO1. All antibodies were from Cell Signaling, and detection was performed using ultrasensitive horseradish peroxidase chemiluminescence (Pierce).

Gene Expression Profiling

SYBR-green qPCR and Affymetrix expression arrays were performed as described in the Supplemental Experimental Procedures on cDNAs from trizol-extracted (Invitrogen) RNA samples. Microarrays were analyzed using gene set enrichment analysis as previously described (Lagouge et al., 2006).

Oxygen Consumption Measurements

Cellular oxygen consumption and palmitate oxidation was measured using a Seahorse bioscience XF24 analyzer with ten biological replicates per condition, as described in the Supplemental Experimental Procedures.

Statistics

Statistical analyses were performed with a Student's t test for independent samples. Data are expressed as mean \pm SEM, and p values smaller than 0.05 were considered as statistically significant.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at [http://www.cellmetabolism.org/supplemental/S1550-4131\(08\)00284-2](http://www.cellmetabolism.org/supplemental/S1550-4131(08)00284-2).

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