Short Article

SirT1 Gain of Function Increases Energy Efficiency and Prevents Diabetes in Mice

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SUMMARY

In yeast, worms, and flies, an extra copy of the gene encoding the Sirtuin *Sir2* increases metabolic efficiency, as does administration of polyphenols like resveratrol, thought to act through Sirtuins. But evidence that Sirtuin gain of function results in increased metabolic efficiency in mammals is limited. We generated transgenic mice with moderate overexpression of SirT1, designed to mimic the Sirtuin gain of function that improves metabolism in *C. elegans*. These mice exhibit normal insulin sensitivity but decreased food intake and locomotor activity, resulting in decreased energy expenditure. However, in various models of insulin resistance and diabetes, SirT1 transgenics display improved glucose tolerance due to decreased hepatic glucose production and increased adiponectin levels, without changes in body weight or composition. We conclude that SirT1 gain of function primes the organism for metabolic adaptation to insulin resistance, increasing hepatic insulin sensitivity and decreasing whole-body energy requirements. These findings have important implications for Sirtuin-based therapies in humans.

INTRODUCTION

The increased prevalence of obesity and diabetes, with the attendant increase in morbidity and mortality, pose a substantial therapeutic challenge (Narayan et al., 2003). Genetic screens in lower organisms provide evidence that gain-of-function of the deacetylase Sir2 results in beneficial metabolic effects and life span extension (Haigis and Guarente, 2006). Sirtuin agonists increase metabolic efficiency in rodents through a mechanism bearing similarity with calorie restriction (Baur et al., 2006; Lagouge et al., 2006; Milne et al., 2007). However, the specificity of these compounds remains undefined.

The function of mammalian Sir2α (also known as SirT1) in metabolism is controversial. Two models of SirT1 gain of function in either pancreatic β cells or adipocytes and brain result in improved insulin secretion and sensitivity, respectively (Bordone

et al., 2007; Moynihan et al., 2005). But increasing SirT1 expression in liver impairs glucose tolerance (Rodgers et al., 2005), and resveratrol increases glucose production by hepatoma cells (Frescas et al., 2005). Thus, it remains unclear whether chronic, ubiquitous SirT1 activation ultimately benefits or impairs metabolic control.

To address this question, we developed transgenic mice designed to mimic the chromosomal duplication leading to the moderate gain of function that improves metabolism in *C. elegans*. In the latter organism, Sir2 function requires the FoxO1 ortholog *daf-16* (Tissenbaum and Guarente, 2001). Thus, in our studies we sought to address whether SirT1 gain of function mediates its effects through FoxO1.

RESULTS

Generation and Analysis of SirT1 Transgenic Mice

To test the hypothesis that SirT1 participates in the regulation of metabolism, we generated BAC transgenic mice overexpressing SirT1 (<u>SirT</u>1 <u>b</u>acterial <u>a</u>rtificial <u>c</u>hromosome <u>o</u>verexpressor, Sir-BACO). SirBACO mice were born in Mendelian ratios and displayed no gross anatomical or reproductive defects. SirT1 levels were 2- to 3-fold higher than control littermates, except in the spleen (~7-fold higher), and distribution patterns were indistinguishable from those of the endogenous protein in all tissues examined (Figures 1A and 1B). SirT1 overexpression was preserved when SirBACO mice were backcrossed onto the *db/db* background (Figure 1C). To probe the transgene's function, we intercrossed SirBACO with *Sirt1*$^{-/-}$ mice (McBurney et al., 2003). The transgene readily rescued developmental defects and postnatal lethality of *Sirt1*$^{-/-}$ mice (data not shown). These findings are consistent the generation of a mouse model of SirT1 gain of function.

Altered Energy Balance in SirBACO Mice

We carried out a metabolic characterization of SirBACO mice on a standard diet. Serum metabolite levels—including insulin and glucose—were normal (Table S1), as was the response to intraperitoneal glucose or insulin challenge tests (Figures 2A and 2B). However, we detected a ~30% increase in plasma adiponectin levels (Table S1).

In *C. elegans* and *D. melanogaster*, increased Sir2 mimics the effects of calorie restriction to extend life span (Haigis and
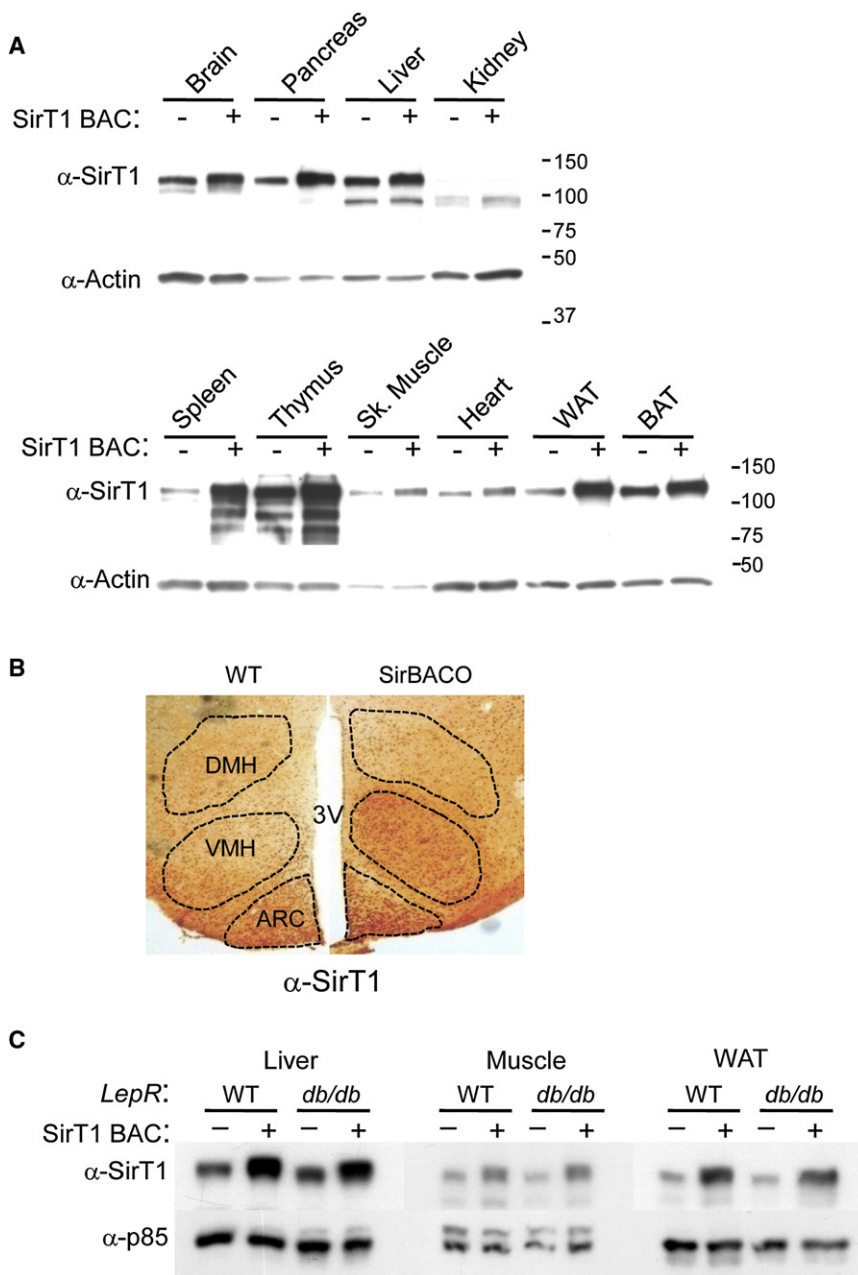
Figure 1. Expression Levels and Tissue Distribution of Sirt1 in SirBACO Mice
(A) Western blot of SirT1 in mouse tissues.
(B) Immunohistochemistry of SirT1 in mouse brain; DMH: dorsomedial hypothalamus; VMH: ventromedial hypothalamus; ARC: arcuate nucleus.
(C) SirT1 expression in SirBACO::*db/db* mice and controls.

(Ravussin et al., 1986). SirBACO mice had decreased resting energy expenditure, but no differences at higher activity levels (Figure S1). Body weight and composition were similar in SirBACO mice and nontransgenic littermates (Figure 2F). Food intake decreased by 15% ± 1.3% in SirBACO mice (Figure 2G), consistent with the preservation of normal body weight, in the face of decreased metabolic rate and activity.

In response to 24 hr food deprivation SirBACO mice showed a normal drop in RQ, reflecting increased fat oxidation (data not shown). These data indicate that, although hypometabolic, SirBACO mice have normal rates of fatty acid oxidation. They also exhibited lower body temperature than control littermates under fed and fasted conditions (Figure 2H), but did not display changes in blood pressure or heart rate (Figure S2), suggesting that the effect on body temperature is secondary to decreased locomotion (Weinert and Waterhouse, 1998).

SirBACO Mice Are Protected from Insulin-Resistant Diabetes

We next asked whether SirT1 overexpression affects the susceptibility to insulin resistance and diabetes in environmental and genetic models of these conditions. When SirBACO mice on an inbred C57BL/6J background were placed on a high-fat diet or backcrossed onto *db/db* mice, they showed lower blood glucose and plasma insulin levels (Figures 3A and 3B; Table S1) and better glucose tolerance (Figure 3C) than nontransgenic controls, despite similar body weight (Figure S3A), weight gain during the diet (Figure 3D), and body fat content (Figure 3E). We provisionally conclude that SirT1 overexpression does not improve glucose tolerance per se, but prevents the adverse effects of obesity on glucose metabolism.

Catalytically Inactive SirT1 Fails to Protect Aging Mice from Glucose Intolerance

To assess the specificity of these findings, we carried out glucose tolerance tests in aging transgenic mice expressing a catalytically inactive SirT1 transgene (H355Y) (Luo et al.,
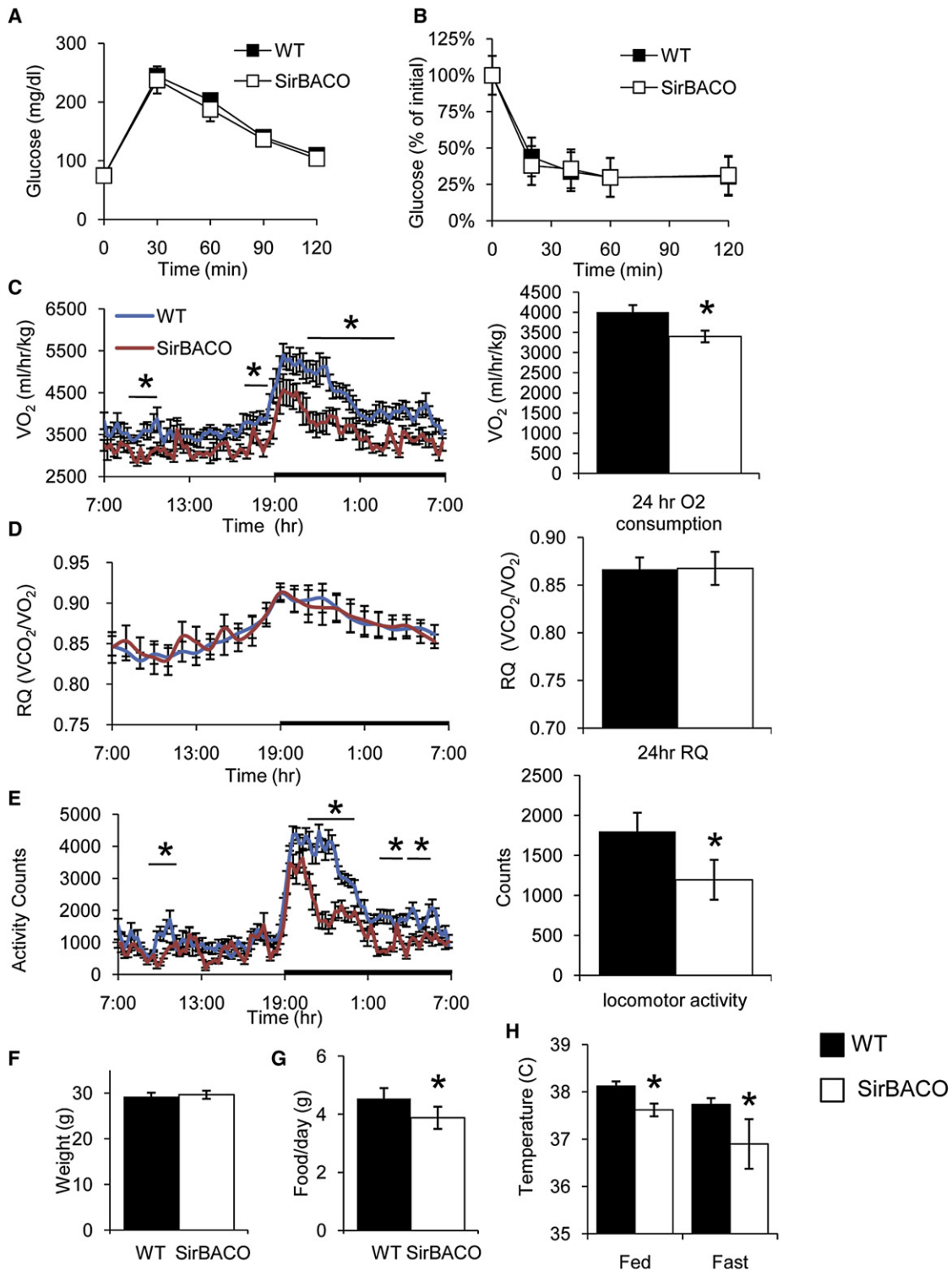
Guarente, 2006). In mammals, prolonged calorie restriction decreases energy expenditure and increases metabolic efficiency by decreasing O_2 consumption (Fontana and Klein, 2007). To determine whether SirT1 overexpression reproduces these subphenotypes, we carried out indirect calorimetry experiments. SirBACO mice demonstrated a coordinate ∼15% decrease in O_2 consumption and CO_2 production (Figure 2C; data not shown). Due to the combined fall in VO_2 and VCO_2, the respiratory quotient (RQ = VCO_2/VO_2) was unchanged (Figure 2D). In addition, spontaneous locomotor activity decreased by 33.6% ± 1.4% (Figure 2E), more markedly during the dark phase.

To investigate differences in basal metabolic rate, we compared the energy expenditure required for locomotor activity

**Figure 2. Metabolic Effects of SirT1 Overexpression**

(A and B) Intraperitoneal glucose tolerance tests and (B) insulin tolerance tests in 4-month-old mice on standard diet (n = 9–11 each).

(C–E) Oxygen consumption (VO_2), (D) respiratory quotient (RQ), and (E) locomotor activity in 8-week-old mice over 24 hr (line chart) and mean 24 hr values (bar graphs) in WT (full bars) and SirBACO mice (empty bars) (n = 7–8 each).

(F) Body weight of chow-fed, 4-month-old mice (n = 10–11 each).

(G) 24 hr food intake (n = 9–11).

(H) Body temperature in fed and 24 hr-fasted mice (n = 9–12 each). * = $p < 0.05$. Error bars represent ± SEM.
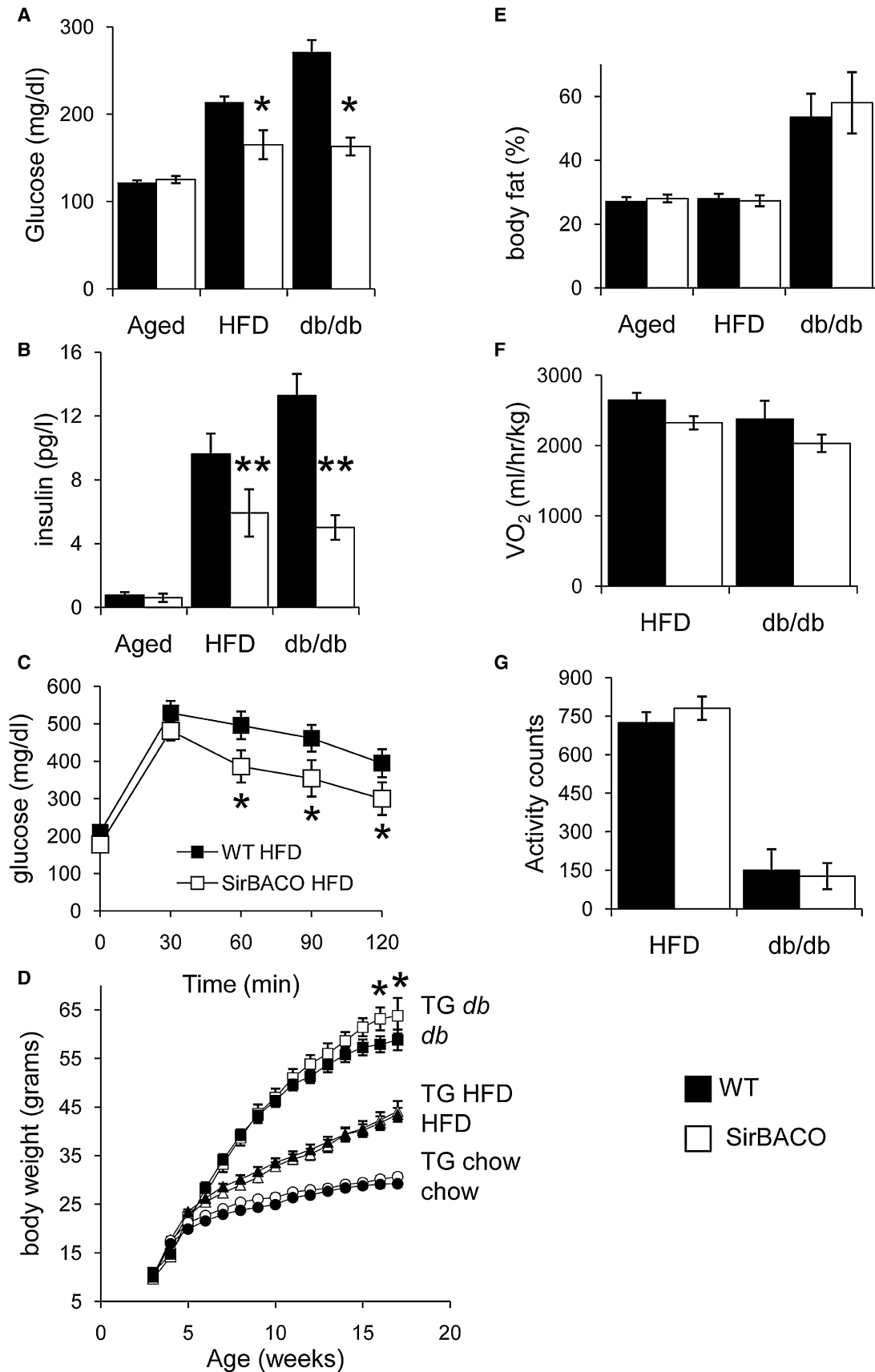
2001). Unlike SirBACO mice, SirT1H355Y transgenics showed no differences in glucose tolerance from control littermates, demonstrating that SirT1 catalytic activity is required for its insulin-sensitizing effects (Figure S3B).

Normal Energy Expenditure in Diabetes-Resistant SirBACO Mice

Based on the decrease of food intake and energy expenditure in SirBACO mice, we wanted to determine whether the transgene's ability to improve glucose homeostasis in diabetes models could be explained by changes in physical activity or metabolic rate. In both the high-fat diet and db/db model of insulin-resistant diabetes, body weight and composition were similar between SirT1 transgenic and nontransgenic mice (Figures 3D and 3E). In contrast to the findings in SirBACO mice on a normal diet (Figure 2), there were no differences in metabolic rate, spontaneous locomotor activity, or food intake in SirBACO mice on a high-fat diet or crossed onto db/db compared to littermate controls (Figures 3F and 3G; data not shown). We conclude that SirT1 promotes a state of increased energy efficiency that protects against insulin resistance and hyperglycemia.

Hyperadiponectinemia and Increased Insulin Sensitivity in SirBACO Mice on High-Fat Diet

To determine the mechanism of improved glucose tolerance in SirBACO mice in a diabetes-predisposing background, we performed hyperinsulinemic euglycemic clamps in high fat-fed and in aging mice (12 months old). In both models, we detected increased insulin-stimulated glucose disposal—as reflected by a higher glucose infusion rate (GIR)—without changes in glucose disappearance rates (Rd), when compared to controls (Figures 4A and S4). Moreover, insulin's ability to suppress hepatic glucose production rose by >30% in high fat-fed SirBACO mice, relative to wild-type littermates (Figures 4B and S4). These findings indicate that the improvement of glucose homeostasis is due to increased hepatic insulin sensitivity (Accili, 2004).

The decrease in hepatic glucose production observed in clamp studies could be due to increased insulin production, improved insulin sensitivity, or both. However, plasma insulin levels were lower in high fat-fed SirBACO and SirBACO::db/db mice (Figure 3B), consistent with improved insulin sensitivity. Islet β cell mass, β cell insulin content, and insulin secretion from isolated pancreatic islets were nearly identical in SirBACO and control mice (Figure S5). Based on prior evidence that adiponectin increases hepatic insulin sensitivity (Combs et al., 2001), we analyzed the role of adiponectin in our transgenic model. Whereas high fat feeding was associated with a modest decrease in plasma adiponectin levels in wild-type mice, in SirBACO mice we observed an ~30%–40% rise (Figure 4C), suggesting that the ability to prevent hyperglycemia is due to increased adipo-

nectin synthesis/secretion, independent of differences in body fat mass. In contrast, we found no differences in resistin, PAI-1, TNF-α, or leptin levels (Table S1). To identify the source of increased adiponectin levels, we measured adiponectin mRNA in white adipose tissue (WAT) from mice fed either standard or high-fat diets and observed a similar increase (Figure 4D). Plasma adiponectin isoform distribution was unchanged, with increases in both the high and medium molecular weight components (Figure 4E).

Adiponectin has been proposed to signal through at least two receptors, AdipoR1 and AdipoR2, which activate AMPK and PPARα, respectively (Yamauchi et al., 2003). Consistent with the observed hyperadiponectinemia, high fat-fed and SirBACO::db/db mice showed elevated phospho-AMPK levels in WAT, liver, and skeletal muscle (Figures 4F and S6). Notably, SirT1 appears to prevent the obesity-driven decrease in phospho-AMPK levels (Martin et al., 2006). Furthermore, in high fat-fed SirBACO mice we also found higher mRNA levels of Pparα and its target genes, Cpt1 and Adipor2, in liver (Figure 4G). These results are consistent with increased adiponectin signaling through AdipoR1 and AdipoR2 and provide a potential mechanism for the increase in hepatic insulin sensitivity.

FoxO1 Deacetylation Phenocopies the Effects of SirT1 Overexpression

Finally, we sought to delineate a mechanism linking SirT1 overexpression to hyperadiponectinemia. Among SirT1 substrates, the transcription factor FoxO1 promotes adiponectin expression (Qiao and Shao, 2006) and FoxO1 haploinsufficiency decreases adiponectin levels (Nakae et al., 2003). SirT1 deacetylates FoxO1 at multiple sites, increasing FoxO1 transcriptional activity (Kitamura et al., 2005). Indeed, acetyl-FoxO1 levels were decreased in hepatocytes from SirBACO mice compared to controls (Figure 4H), as were acetyl-Pgc1α levels in muscle (Figure 4I).

To test the hypothesis that SirT1 and FoxO1 coordinately regulate adiponectin expression, we examined Acrp30 (the gene encoding adiponectin) expression in adipocytes. As constitutive activation of either FoxO1 or SirT1 inhibits adipocyte differentiation (Nakae et al., 2003; Picard et al., 2004), we used 3T3-L1 CARΔ cells, overexpressing the adenoviral receptor, to introduce adenoviruses encoding FoxO1 and SirT1 into differentiated adipocytes (Orlicky et al., 2001). Wild-type FoxO1 increased Acrp30 by ~40%. Similarly, SirT1 increased Acrp30 transcription in a FoxO1-dependent manner (Figure 4J). Conversely, FoxO1 knockdown (Matsumoto et al., 2007) resulted in decreased Acrp30 levels (Figure 4K). Expression of a mutant FoxO1 designed to mimic SirT1-mediated deacetylation (FoxO1-dAc) increased Acrp30 expression, while a mutant FoxO1 mimicking "constitutive acetylation" (FoxO1-Ac) (Kitamura et al., 2005)

Figure 3. SirT1 Increases Insulin Sensitivity in Obese Mice

(A and B) Fasting glucose and (B) insulin levels in SirBACO and WT mice in three conditions: 11-month-old on standard diet (aged), 5-month-old on HFD, or 8-week-old on db/db background.

(C) Glucose tolerance in 4-month-old SirBACO mice on HFD.

(D) Body weight in SirBACO (empty symbols) and WT mice (filled symbols) fed regular chow (n = 13–14 each), HFD (n = 12–16 each), or bred with db/db (n = 9–14 each).

(E–G) Fat mass, (F) indirect calorimetry, and (G) locomotor activity in db/db (full bars), high fat-fed WT (full bars) and SirBACO::db/db or HFD-fed SirBACO mice (empty bars) (n = 5–8 each). * = p < 0.05, ** = p < 0.01. Error bars represent ± SEM.
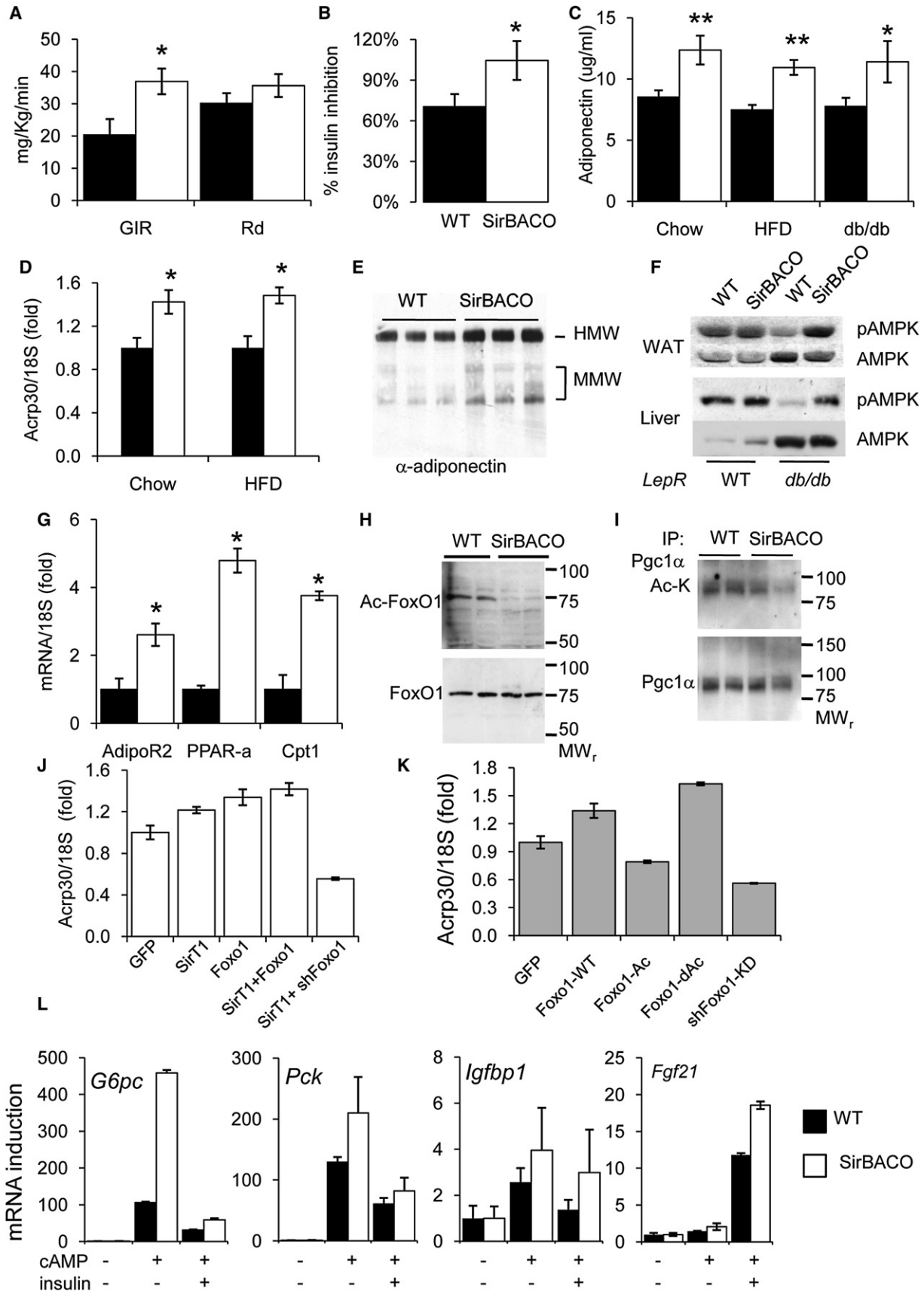
failed to activate *Acrp30* transcription (Figure 4K). These results indicate that FoxO1-dependent *Acrp30* transcription can be regulated by SirT1-mediated deacetylation.

To assess the cell nonautonomous effects of SirT1 on liver gene expression, we isolated primary hepatocytes from WT or SirBACO mice and measured Foxo1/Pgc1α target genes. Consistent with prior observations (Frescas et al., 2005; Rodgers et al., 2005), we observed increased levels of *G6pc*, *Pck1*, and *Igfbp1* in response to cAMP (Figure 4L). The Pparα target *Fgf21* was also elevated in SirBACO hepatocytes but was not regulated by cAMP (Figure 4L). These results indicate that the cell-autonomous and nonautonomous actions of SirT1 on hepatic glucose production are opposite. We speculate that circulating factors account for the latter.

DISCUSSION

The ability of Sirtuin gain of function to extend life span and improve metabolism in simple organisms, together with the insulin-sensitizing properties of Sirtuin agonists in rodents, have led to the exploration of this pathway for therapeutic ends in metabolic disease. But a rigorous genetic test of the hypothesis that Sirtuin gain of function results in positive metabolic effects has not been carried out.

Two models of SirT1 gain of function had previously been reported. SirT1 overexpression in β cells increased insulin secretion (Moynihan et al., 2005). SirBACO mice did not show this phenotype, possibly due to differences in the levels of SirT1 expression. In SirT1 knockin mice (Bordone et al., 2007), the transgene is expressed off the β-actin locus, leading potentially to developmental effects that impair adipocyte differentiation (Picard et al., 2004), and increase insulin sensitivity. Thus, the SirT1 knockin model cannot distinguish between a direct effect of SirT1 and an indirect one due to reduced body fat.

Complex Metabolic Effects of SirT1 Overexpression

Our results demonstrate complex and not easily generalizable effects of SirT1 overexpression. In chow-fed mice, SirT1 gain of function has no discernible effects on insulin sensitivity and glucose utilization, but engenders a behavioral response that partly mimics calorie restriction. While further studies will be required to dissect the site(s) of these Sirtuin effects, they are likely to reflect an involvement of the central nervous system, and thus point to the need for rigorous behavioral/psychometric testing in early-phase clinical trials of Sirtuin agonists. In light of the current prevalence of overweight, the effects of SirT1 to reduce food intake in mice are of some interest vis-à-vis the possibility of developing SirT1 agonists for use in human obesity. In the present study, we cannot discern whether these effects are mediated directly in the central nervous system or by circulating factors.

Resveratrol-treated mice fed a high-fat diet display increased O_2 consumption (Lagouge et al., 2006) and decreased body weight (Baur et al., 2006; Lagouge et al., 2006). SirBACO mice on a high-fat diet do not display these phenotypes, but—similar to resveratrol-treated mice—show decreased locomotor activity (Lagouge et al., 2006). The data suggest that resveratrol acts on additional Sirtuins or on different targets. While both exogenous resveratrol and SirT1 overexpression improve glucose tolerance in high fat-fed and *db/db* mice, the mechanisms appears to differ: our studies point to a mechanistic link with hyperadiponectinemia, while improved ATP utilization seems to protect resveratrol-treated mice from diet-induced diabetes (Baur et al., 2006; Lagouge et al., 2006). The hyperadiponectinemia model allows us to reconcile the decreased hepatic glucose production in SirBACO mice with SirT1's ability to increase it (Rodgers et al., 2005). We propose that, in SirBACO mice, the insulin-sensitizing effect of adiponectin trumps SirT1's cell-nonautonomous activation of the gluconeogenic program.

In Vivo SirT1 Substrates and Metabolic Control

In *C.elegans*, the FoxO1 ortholog *daf-16* is required for life-span extension in response to SirT1 gain of function (Tissenbaum and Guarente, 2001). The widespread role of FoxO1 in mammalian metabolism provides a testable hypothesis on SirT1's mechanism of action. In this regard, we found that, unlike common forms of obesity and type 2 diabetes (Hu et al., 1996), SirBACO mice in diabetic and obese backgrounds display increased adiponectin. We propose that SirT1 acts through FoxO1 to increase adiponectin production. But given the conflicting results on the effect of SirT1 on adiponectin expression (Qiang et al., 2007; Qiao and Shao, 2006), the hyperadiponectinemia of SirBACO mice may have heterogeneous causes, including changes in β-adrenergic signaling or redox potential (Fasshauer et al., 2001; Furukawa et al., 2004). Nonetheless, this phenotype bears similarities with the effect of adiponectin overexpression in *ob/ob* mice (Kim et al., 2007). Moreover, knockin mice bearing constitutively deacetylated FoxO1 alleles mimic the energy balance phenotype of SirBACO mice, supporting the contention that the effects of SirT1 are mediated by FoxO1 (A.B. and D.A., unpublished data).

Conclusions

The ability of SirBACO mice to adapt their basal phenotype and thus prevent insulin-resistant diabetes will rekindle the debate on the evolutionary function of Sirtuins. SirT1 appears to function as a "reverse thrifty gene" that protects against metabolic diseases

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Figure 4. SirT1 Decreases HGP and Regulates Adiponectin

(A and B) Glucose infusion (GIR) and disappearance rates (Rd) and (B) insulin suppression of hepatic glucose production (HGP) in high fat-fed mice (n = 5–11 each).

(C and D) Plasma adiponectin (n = 11–13) and (D) WAT mRNA levels (*Acrp30*) in 16-week-old mice (8-week-old in the SirBACO::*db/db* cross).

(E) Plasma adiponectin isoform distribution.

(F) AMPK phosphorylation in WAT and liver.

(G) mRNA levels of adiponectin target genes (n = 4–5 each).

(H and I) FoxO1 acetylation in hepatocytes and (I) Pgc1α acetylation in gastrocnemius muscle.

(J and K) Regulation of *Acrp30* mRNA levels in 3T3-L1. * = p < 0.05, ** = p < 0.01.

(L) Gene expression in primary mouse hepatocytes (n = 4 each). Error bars represent ± SEM.

by instructing the organism to limit energy consumption and expenditure in the physiologic state. The likely behavioral aspects of SirT1 activation deserve closer scrutiny, if Sirtuin-based approaches are to be introduced in the clinic.

EXPERIMENTAL PROCEDURES

Mice

We used BAC RP23-390D8 to generate SirBACO and SirT H355Y mice. Two lines of WT SirT1 transgenics with similar expression levels (Figure S7) had similar metabolic features (data not shown). db/+ m/+ were from Jackson Laboratory. High-fat diet (60% of calories from fat) (Research Diets) was administered from 4 to 20 weeks of age. All data are from male mice backcrossed at least 10 generations to C57BL/6J.

Antibodies and Cell Culture

Antibodies were from Affinity BioReagents (adiponectin), Cell Signaling Technologies (Acetyl lysine, phospho and total AMPK, and PGC-1α), Santa Cruz Biotechnology (acetyl- and total-FoxO1), Upstate Biotechnology (Sir2, p85), and Calbiochem (actin antibody JLA20). Culture and infection of 3T3-L1 CARΔ cells have been described (Ross et al., 2003).

Metabolic and Glucose Clamp Studies

We performed all assays as described (Lin et al., 2007; Matsumoto et al., 2007).

Energy Balance

Indirect calorimetry was performed using LabMaster (TSE Systems). All mice were acclimatized for 24 hr before measurements every 14 min for 96 hr. Resting metabolic rate was determined by generating average energy expenditure and locomotor activity at each 14 min time point. Energy efficiency plots were obtained as described (Ravussin et al., 1986). Food intake was measured daily for 2 weeks with feeding hoppers. Core body temperature was measured with a probe (YSI Incorporated), blood pressure and heart rate with Hatteras Instrument MC4000, and body composition with either Piximus DEXA scanner (GE Healthcare) or NMR (Bruker Optics).

RNA and Protein Analysis

See Supplemental Data.

Adenovirus Generation

Adenovirus encoding constitutively acetylated (Foxo1-Ac), and deacetylated mutants (Foxo1-dAc), or Foxo1-GFP were generated using previously described cDNAs (Frescas et al., 2005; Kitamura et al., 2005), cloned into AdEasy pShuttle-CMV (Stratagene). Other adenoviruses have been described (Matsumoto et al., 2006).

Statistical Analysis

P values were calculated by unpaired Student's t tests.

SUPPLEMENTAL DATA

Supplemental Data include seven figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found online at http://www.cellmetabolism.org/cgi/content/full/8/4/333/DC1/.

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