Cell

SIRT4 Inhibits Glutamate Dehydrogenase and Opposes the Effects of Calorie Restriction in Pancreatic β Cells

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DOI 10.1016/j.cell.2006.06.057

SUMMARY

Sir2 is an NAD-dependent deacetylase that connects metabolism with longevity in yeast, flies, and worms. Mammals have seven Sir2 homologs (SIRT1–7). We show that SIRT4 is a mitochondrial enzyme that uses NAD to ADP-ribosylate and downregulate glutamate dehydrogenase (GDH) activity. GDH is known to promote the metabolism of glutamate and glutamine, generating ATP, which promotes insulin secretion. Loss of SIRT4 in insulinoma cells activates GDH, thereby upregulating amino acid-stimulated insulin secretion. A similar effect is observed in pancreatic β cells from mice deficient in SIRT4 or on the dietary regimen of calorie restriction (CR). Furthermore, GDH from SIRT4-deficient or CR mice is insensitive to phosphodiesterase, an enzyme that cleaves ADP-ribose, suggesting the absence of ADP-ribosylation. These results indicate that SIRT4 functions in β cell mitochondria to repress the activity of GDH by ADP-ribosylation, thereby downregulating insulin secretion in response to amino acids, effects that are alleviated during CR.

INTRODUCTION

Silent information regulator (Sir2) genes promote longevity in yeast, *C. elegans*, and *Drosophila* (for review, see Blander and Guarente, 2004). An extra copy of the yeast *SIR2* gene extends replicative life span of mother cells compared to wild-type, while a deletion of *SIR2* reduces life span (Kaeberlein et al., 1999). Sir2 proteins are NAD-dependent deacetylases, and the yeast Sir2p silences selected regions of the yeast genome (Imai et al., 2000; Landry et al., 2000). Silencing by Sir2p suppresses the formation of extrachromosomal rDNA circles, which trigger senescence in mother cells (Kaeberlein et al., 1999; Sinclair and Guarente, 1997). In *C. elegans*, the *SIR2* ortholog *sir-2.1* regulates life span by at least two mechanisms. In one, it augments the activity of the forkhead protein DAF-16, a longevity determinant in worms (Berdichevsky et al., 2006). In the other, it regulates the transcription of genes involved in the unfolded-protein stress response (Viswanathan et al., 2005). In yeast and *Drosophila*, *SIR2* and its paralogs are required for the life-span extension brought on by calorie restriction (CR) (Lamming et al., 2005; Lin et al., 2000; Rogina and Helfand, 2004; Wood et al., 2004), raising the question of whether mammalian *SIR2* genes connect nutrient sensing to life span.

Mammals have seven Sir2p homologs (sirtuins, SIRT1–7) (Frye, 2000). While a role for mammalian sirtuins in life-span regulation has not been directly determined, evidence suggests that the ortholog SIRT1 plays roles in numerous physiological pathways, such as control of glucose homeostasis. Pancreatic β cells maintain glucose homeostasis by secreting insulin in response to an increase in ATP due to the metabolism of glucose. SIRT1 deacetylates the forkhead transcription factor FOXO1 (Brunet et al., 2004; Luo et al., 2001; Motta et al., 2004; Vaziri et al., 2001) and thereby promotes resistance to

oxidative stress in β cells (Kitamura et al., 2005). SIRT1 also functions as a positive regulator of insulin secretion in β cells by repressing expression of the mitochondrial uncoupling protein UCP-2 (Moynihan et al., 2005; Bordone et al., 2005). In the liver, SIRT1 interacts with and deacetylates PGC-1α to induce gluconeogenesis in response to fasting (Rodgers et al., 2005).

Functions for the other *SIR2* genes, *SIRT2–7*, in mammalian physiology have only recently begun to emerge. SIRT6 is an ADP-ribosyltransferase (Liszt et al., 2005) and plays an important role in base excision repair of nuclear DNA (Mostoslavsky et al., 2006). *SIRT6* knockout mice show defects in proliferative tissues and a premature aging phenotype (Mostoslavsky et al., 2006). SIRT7 is located in the nucleolus, where, analogous to the yeast Sir2p, it regulates transcription by RNA polymerase I (Ford et al., 2006). However, unlike Sir2p, which silences rDNA by deacetylating histones (Armstrong et al., 2002), SIRT7 appears to bind directly to the RNA pol I complex and exert a positive effect on transcription.

In this paper, we provide the first comprehensive characterization of SIRT4, which was proposed to be a mitochondrial protein because SIRT4-GFP localizes to that cellular compartment (Michishita et al., 2005). We show that, unlike SIRT1–3 (North et al., 2003), SIRT4 does not display NAD-dependent deacetylase activity but instead uses NAD to ADP-ribosylate glutamate dehydrogenase (GDH), an enzyme that converts glutamate to α-ketoglutarate in mitochondria. By ADP-ribosylating GDH, SIRT4 represses the enzymatic activity of GDH and limits the metabolism of glutamate and glutamine to generate ATP. Therefore, SIRT4 represses the ability of β cells to secrete insulin in response to these amino acids, and, as predicted, *SIRT4* knockout mice secrete insulin in response to glutamine. In wild-type mice, CR alleviates the repression of GDH by ADP-ribosylation and thus changes the response of β cells to glutamine and leucine. These findings demonstrate the role of a mammalian sirtuin in regulating an important physiological pathway that responds to CR.

RESULTS

SIRT4 Is Expressed in Mouse Pancreatic β Cells

To identify tissues in which SIRT4 may have a physiologically relevant function, we first investigated the expression pattern of SIRT4 in mouse. SIRT4 was expressed in all tissues examined, with highest levels in the kidney, heart, brain, and liver (Figure 1A). Despite its apparent low level in the pancreas (Figure 1A), an intense SIRT4 signal was detected specifically within pancreatic islets, overlapping the expression of insulin in β cells (Figure 1B, see also close-up in Figure S1A in the Supplemental Data available with this article online), suggesting a possible role in the regulation of insulin secretion (see below). By contrast, SIRT4 showed a diffuse localization pattern in mouse kidney and liver (data not shown).

SIRT4 Is a Mitochondrial ADP-Ribosyltransferase

Mammalian homologs of SIR2 are localized to the nucleus (Liszt et al., 2005; Luo et al., 2001; Vaziri et al., 2001), cytosol (North et al., 2003), and mitochondria (Onyango et al., 2002; Schwer et al., 2002). To determine SIRT4 subcellular localization, we fused GFP to the C terminus of human SIRT4 (SIRT4-GFP). Human liver HepG2 cells expressing phSIRT4GFP displayed colocalization of SIRT4-GFP with a mitochondria-targeted DsRed, but not peroxisome-targeted DsRed (Figure 1C). By contrast, GFP alone showed a diffuse localization pattern. These data are in agreement with a recent report that overexpressed SIRT4-GFP is localized to the mitochondria of human fibroblasts (Michishita et al., 2005).

We verified the mitochondrial localization of endogenous SIRT4 in the mouse insulinoma β cell line MIN6 by subcellular fractionation using a polyclonal antibody generated against the C terminus. SIRT4 was enriched in mitochondrial fractions containing the mitochondrial protein HSP60 (Figure 1D) but not detectable in the cytosolic fraction containing catalase. Endogenous and overexpressed SIRT4 was also detected in the mitochondrial fraction of human 293T cells (data not shown). These results demonstrate that SIRT4 is localized to the mitochondria of human and mouse cells.

We next investigated whether SIRT4 is posttranslationally cleaved during mitochondrial import by sequencing the N terminus of SIRT4-FLAG. The N terminus of many mitochondrial proteins becomes cleaved by signal peptidases during import to the matrix. The first 28 predicted amino acid residues were absent from the N terminus, indicating that SIRT4 had been posttranslationally processed (Figure 1E). Like the putative signal sequence of another mitochondrial sirtuin, SIRT3 (Schwer et al., 2002), that of SIRT4 is enriched in basic residues (Figure 1E). Taken together, our data show that SIRT4 is a mitochondrial protein, is posttranslationally cleaved, and likely resides in the mitochondrial matrix.

While SIRT1–3 have been shown to be NAD-dependent deacetylases, the activity of SIRT4 has not been established. To investigate the enzymatic activity of SIRT4, we overproduced human SIRT4 with a C-terminal FLAG tag in mammalian cells. 293T cells expressing SIRT4-FLAG produced a 34 kDa band that was immunoreactive to both FLAG and SIRT4 antibodies (Figure 2A). We assessed the enzymatic activity of SIRT4-FLAG immunoprecipitated from mammalian cells. Although SIRT4 contains a conserved sirtuin domain (Frye, 2000), we detected no deacetylase activity, in contrast to the known deacetylase SIRT1 (Figure 2B). Instead, we found that SIRT4 transferred a radiolabel indicative of an ADP-ribosyl group from NAD onto histones (Figure 2C), and this transfer was inhibited by 1 mM nicotinamide, which is known to inhibit the activity of sirtuins (Figure 2C). By contrast, SIRT5 did not show ADP-ribosyltransferase activity. Mass spectrometry analysis confirmed that SIRT4 transferred a 540 dalton moiety to histone 2A, corresponding to the molecular weight of ADP-ribose (Figure S1B). These data

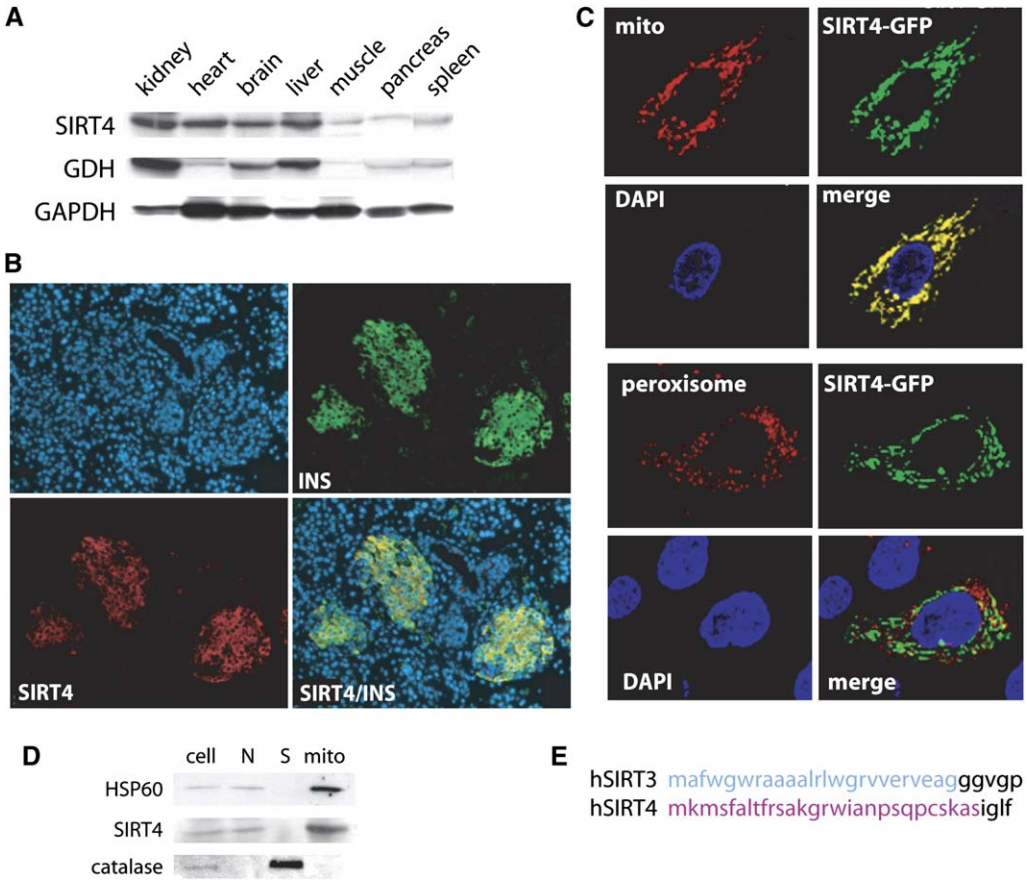

Figure 1. SIRT4 Is a Mitochondrial Protein Expressed in Pancreatic β Cells

(A) Tissue lysates (20 μg) from two mice were combined and analyzed by SDS-PAGE and Western blotting using SIRT4, GDH, and GAPDH antibodies.

(B) Sections of mouse pancreas were analyzed by immunofluorescence using antibodies to SIRT4 (red) and insulin (green). Nuclei were visualized with DAPI (blue, 10× magnification).

(C) HepG2 cells were cotransfected with phSIRT4-EGFP (green), the mitochondrial marker pDs-RED2-Mito (red), or the peroxisomal marker pDs-RED-Peroxi (red).

(D) MIN6 cells were fractionated by sucrose centrifugation. Cell = whole-cell lysate, N = nuclear lysate/unbroken cells, S = light fraction containing cytosol, mito = enriched mitochondria. Blot was probed with antibodies to cytosolic catalase, mitochondrial HSP60, or SIRT4.

(E) N-terminal presequence hSIRT4 as deduced by comparing the N-terminal sequence from protein isolated from mammalian cells to the cDNA sequence. Also shown is the hSIRT3 presequence (Schwer et al., 2002).

show that SIRT4 is an ADP-ribosyltransferase but do not completely rule out a substrate-specific deacetylase activity.

SIRT4 Interacts with GDH

To characterize the in vivo role of SIRT4 as a mitochondrial ADP-ribosyltransferase, we sought to identify proteins that were relevant physiological substrates of this sirtuin. Previous studies suggest that a small number of mitochondrial proteins may be ADP-ribosylated (Jorcke et al., 1998). The best characterized is GDH, a mitochondrial enzyme that converts glutamate into the TCA-cycle intermediate α-ketoglutarate and is inhibited by ADP-ribosylation (Herrero-Yraola et al., 2001). We therefore investigated whether GDH was a bona fide substrate for ADP-ribosylation by SIRT4 within mitochondria.

To probe a functional interaction between these proteins, we first analyzed the expression profile of GDH in mouse tissues. GDH was expressed ubiquitously (Figure 1A) and, like SIRT4, was highly enriched in pancreatic islets localizing to β cells (Figure 2D). We also detected GDH expression in a ring of cells surrounding the insulin-containing cells.

We next investigated whether SIRT4 and GDH physically interact in 293T cells expressing SIRT4-FLAG. Immunoprecipitating SIRT4-FLAG coprecipitated endogenous GDH in cells expressing the tagged construct (Figure 2E). Conversely, immunoprecipitating endogenous GDH pulled down SIRT4-FLAG in these same cells (Figure 2E). Control antibodies against Gal4 did not immunoprecipitate GDH or SIRT4-FLAG. Moreover, we detected GDH in SIRT4 immunoprecipitates from MIN6 cells (Figure 2F),

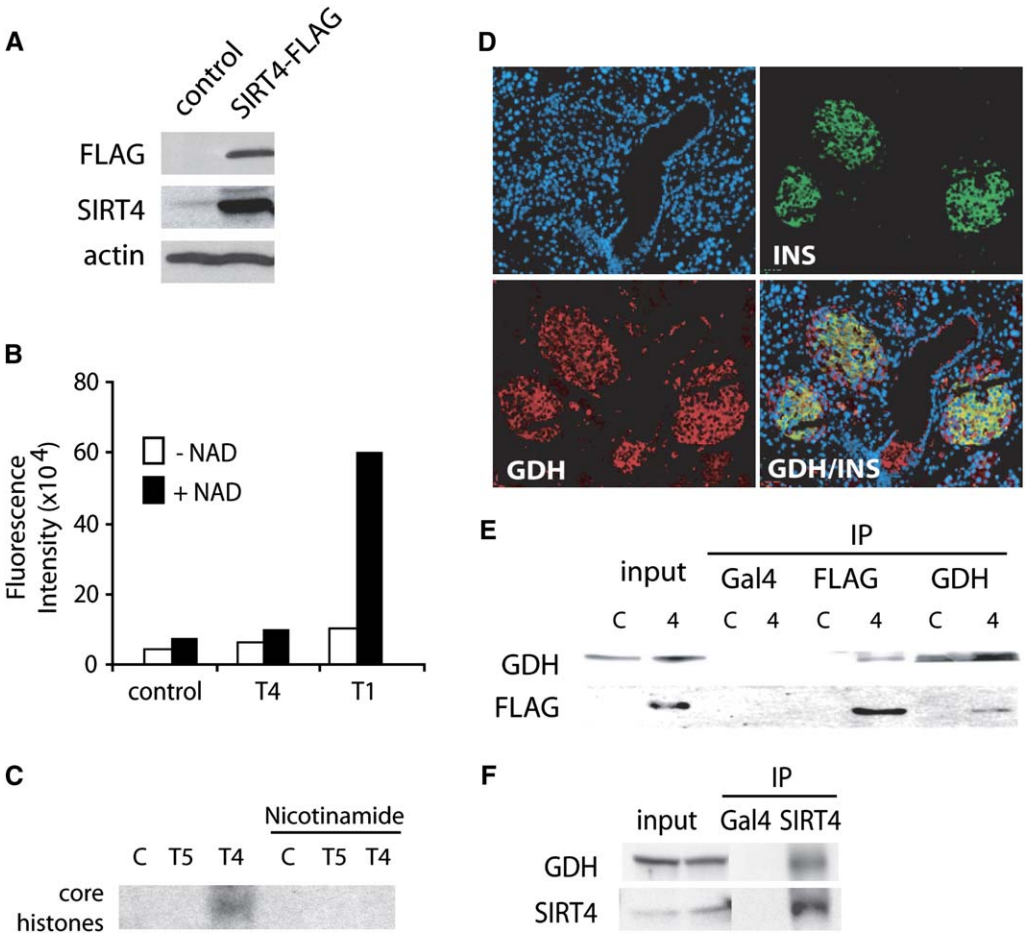

Figure 2. SIRT4 Is a NAD-Dependent ADP-Ribosyltransferase that Interacts with GDH

(A) 293T cells were transiently transfected with pCMV-FLAG vector (control) or phSIRT4-FLAG, and protein expression was verified by Western blotting with FLAG or hSIRT4 antibodies normalized to actin.

(B) Fifty nanograms of immunoprecipitated, dialyzed hSIRT4-FLAG (T4) and hSIRT1-FLAG (T1) were assayed for deacetylase activity using the Fluor de Lys substrate (BIOMOL) in the absence (open bars) or presence (black bars) of 1 mM NAD.

(C) ADP-ribosyltransferase activity of hSIRT4-FLAG (T4), hSIRT5-FLAG (T5), and buffer control (C) was determined by incubating 50 ng of protein with [^{32}P]NAD in the presence of core histone proteins without or with 1 mM nicotinamide.

(D) Sections of mouse pancreas were probed with antibodies to GDH (red) and insulin (green); nuclei were visualized with DAPI (blue, 10× magnification).

(E) 293T cells were transfected with pCMV vector (C) or phSIRT4-FLAG (4), and lysates were immunoprecipitated with Gal4, FLAG, or GDH antibodies and subjected to Western blot using antibodies against FLAG or GDH.

(F) MIN6 cells lysates were immunoprecipitated with Gal4 or SIRT4 antibodies and blotted with antibodies for GDH or SIRT4.

demonstrating that SIRT4 and GDH interact at endogenous levels found in pancreatic β cells.

SIRT4 ADP-Ribosylates and Inhibits GDH

To determine whether SIRT4 could ADP-ribosylate GDH, we incubated SIRT4 immunoprecipitated from isolated mammalian mitochondria with purified GDH for varying times. We monitored ADP-ribosylation of GDH by the transfer of radiolabel from [^{32}P]NAD to GDH. SIRT4 catalyzed the transfer of radiolabel, which was removed by treatment with phosphodiesterase (PDE), an enzyme known to cleave ADP-ribose (Figure 3A) (Cervantes-Laurean et al., 1993; Schwab et al., 2000). ADP-ribosyltransferase activity was inhibited by two structurally distinct inhibitors of sirtuins, nicotinamide and sirtinol (Grozinger et al., 2001) (Figure 3B), further indicating that ADP-ribosylation of GDH was catalyzed by SIRT4.

We next measured the enzymatic activity of recombinant hSIRT4 expressed and purified from *Pichia pastoris*. Like hSIRT4-FLAG, recombinant hSIRT4 exhibited ADP-ribosyltransferase activity on GDH (Figure 3C), and this activity was inhibited by nicotinamide (Figure S1C). In contrast, recombinant SIRT1 purified from bacteria did not ADP-ribosylate GDH to the same extent as SIRT4 (Figure S1C).

Mono-ADP-ribosylated proteins can be generated by two mechanisms: direct enzymatic transfer of ADP-ribose

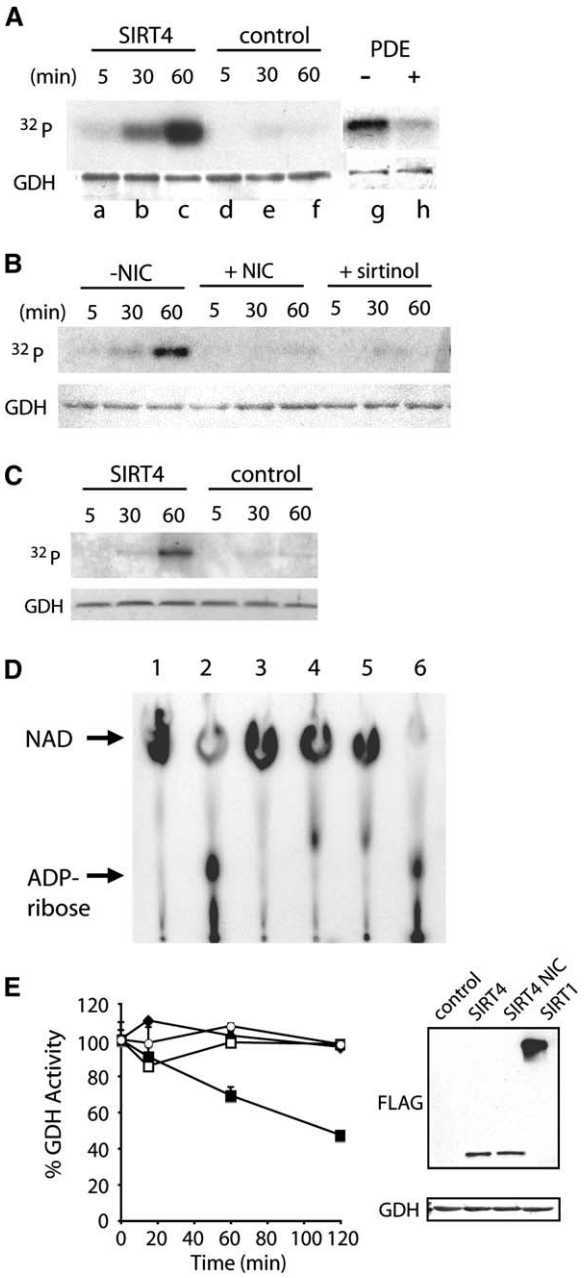

Figure 3. SIRT4 ADP-Ribosylates and Inhibits GDH

(A) ADP-ribosylation of GDH by hSIRT4-FLAG was assessed by incubating 10 μg of GDH with [^{32}P]NAD in the presence (lanes a–c) or absence (lanes d–f) of 10 ng SIRT4 for the indicated times, and the 30 min sample was then treated without (lane g) or with (lane h) phosphodiesterase (PDE; 10 μg/ml). Samples were analyzed by autoradiography, and GDH in each lane from the same gel was detected by Ponceau S.

(B) ADP-ribosylation of GDH by hSIRT4-FLAG was assessed as in (A) without (−NIC) or with nicotinamide (+NIC, 10 mM) or with 40 μM sirtinol for 5, 30, or 60 min at 37°C.

(C) ADP-ribosylation of GDH by recombinant hSIRT4 was assessed by incubating 10 μg of GDH with 0.1 μg of hSIRT4 or control buffer for the indicated times.

(D) Thin-layer chromatography (TLC) was used to analyze [^{32}P]NAD (lane 1) and metabolites generated when [^{32}P]NAD was incubated with NAD glycohydrolase (NADase) (lane 2), GDH (lane 3), hSirT4 (lane 4), hSIRT4 and GDH (lane 5), and Sir2Tm and the acetylated p53 peptide JB12 (lane 6). The reactions were incubated for 1 hr and analyzed by TLC and autoradiography. The arrows indicate the positions where NAD^+ and ADP-ribose migrate.

(E) The enzymatic activity of GDH (50 μg in 200 μl) was measured after a 0, 15, 60, and 120 min incubation at 37°C. Reactions contained 1 mM NAD and 10 ng hSIRT-FLAG without (■) or with (□) 10 mM nicotinamide, 20 ng hSIRT1-FLAG (◆), or mock IP buffer (○). The sirtuins were monitored by blotting with anti-FLAG and GDH by Ponceau S staining. Experiments were repeated in triplicate. In this and all other figures, values are expressed as mean + standard error of the mean (SEM).

from NAD, and noncatalytically by reacting with free ADP-ribose (Jorcke et al., 1998). To explore which mechanism might apply, we tested whether SIRT4 cleaved NAD to generate free ADP-ribose, which could then label GDH nonenzymatically. Thin-layer chromatography (TLC) was used to analyze the NAD metabolites generated when SIRT4 was incubated with NAD in the absence or presence of GDH (Figure 3D). Lane 2 shows the production of free ADP-ribose when NAD was incubated with NAD glycohydrolase, which cleaves NAD to yield ADP-ribose and nicotinamide. As another control, incubation of the bacterial sirtuin Sir2Tm with an acetylated p53 peptide, JB12, produced O-acetyl-ADP-ribose (lane 6), a known product of the deacetylation reaction. In contrast, reactions containing recombinant hSIRT4 and NAD without (lane 4) or with GDH (lane 5) did not produce free ADP-ribose, nor did incubation of NAD with GDH (lane 3). These results suggest that SIRT4 does not function as an NAD glycohydrolase to cleave NAD. Instead, SIRT4 likely ADP-ribosylates GDH by direct transfer of ADP-ribose from NAD.

As ADP-ribosylation is known to inhibit GDH activity (Herrero-Yraola et al., 2001), we determined whether SIRT4 inhibited GDH by incubating SIRT4-FLAG (or SIRT1-FLAG) with bovine GDH in the presence of NAD. Blotting with anti-FLAG approximated the levels of these sirtuins used in the reactions (Figure 3E). After 0–120 min of incubation, an aliquot of the reaction was removed, and GDH activity was measured by monitoring NADH absorbance. SIRT4, but not the buffer control, reduced the enzymatic activity of GDH by at least 50% (Figure 3E and Figure S1D). Moreover, SIRT4 was not inhibitory when NAD was omitted (Figure S1D) or in the presence of nicotinamide (Figure 3E), suggesting that ADP-ribosylation by SIRT4 is required for GDH inhibition. In contrast, SIRT1-FLAG had little effect on GDH activity (Figure 3E).

Generation and Characterization of *SIRT4* Knockout Mice

To test whether SIRT4 regulates GDH activity in vivo, we generated *SIRT4* knockout (KO) mice by replacing exons 1–3 of the *SIRT4* gene with a *LacZ* gene (Figure 4A). The lack of SIRT4 protein was verified by Southern blot analysis of genomic DNA (Figure 4B) and by Western blot

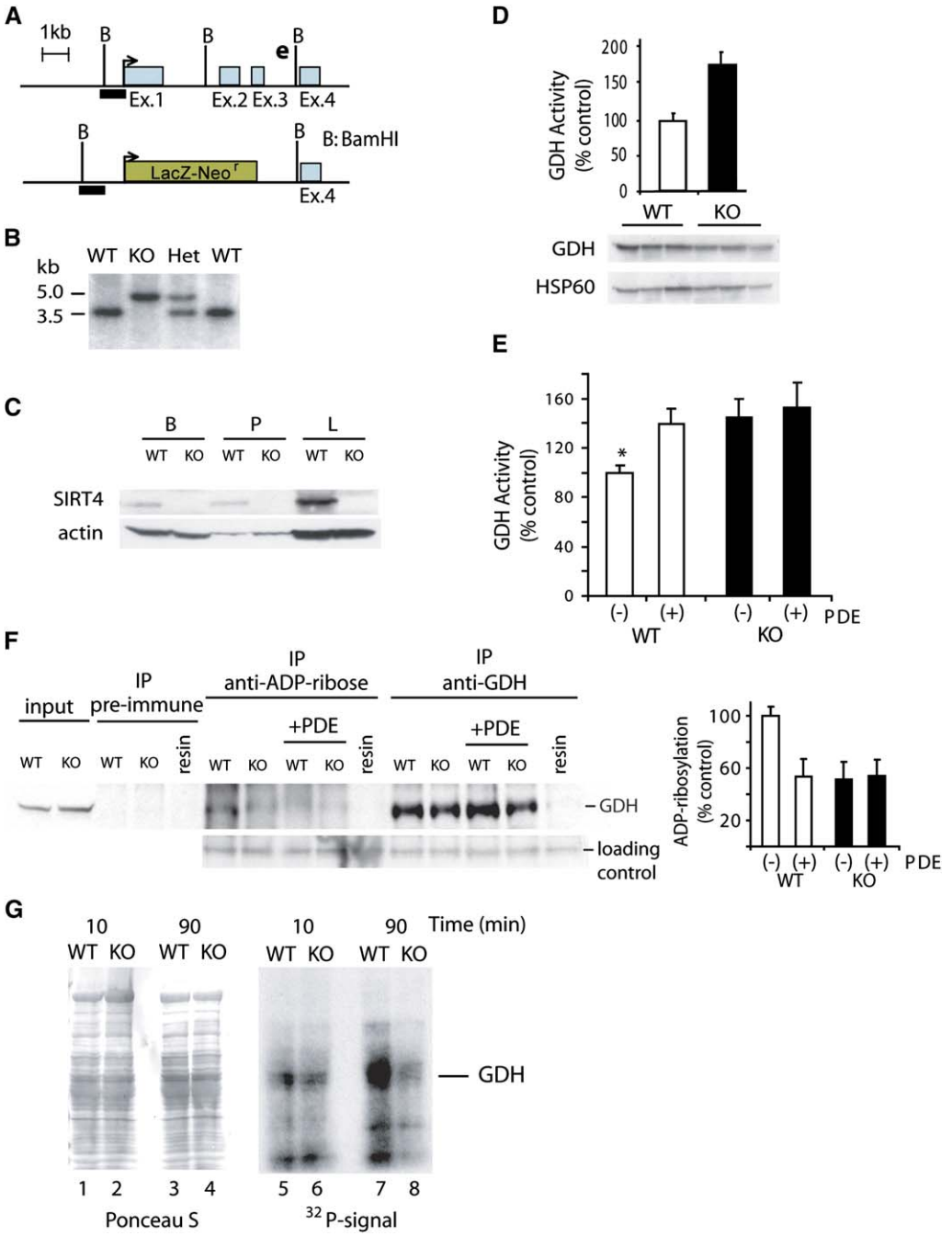

Figure 4. SIRT4 Mediates GDH ADP-Ribosylation In Vivo

(A) A *LacZ* cassette was inserted into the mouse *SIRT4* gene in-frame with the first part of exon 1, deleting exons 1–3.

(B) Genotypes were determined by Southern blotting of BamHI-digested genomic DNA with the probe indicated by the black bar in (A).

(C) Lysates from wild-type and *SIRT4* KO brain (B), pancreas (P), and liver (L) were blotted with the indicated antibodies.

(D) GDH activity was measured in pancreatic mitochondrial lysates (20 μg) from *SIRT4* wild-type (open bar) or KO mice (black bar) (n = 4). GDH protein levels were measured in three of these samples by immunoblotting for GDH and HSP60.

(E) GDH activity was measured from 100 wild-type (open bar) or KO (black bar) islets lysed in NP-40 buffer with or without PDE treatment (10 μg/ml) for 15–20 min at 37°C.

(F) Left: Liver mitochondrial lysates were immunoprecipitated with resin conjugated with preimmune serum, anti-mono-ADP-ribose, or anti-GDH in the absence or presence of PDE for 4 hr at 4°C. Immunoprecipitates were run out adjacent to antibody-conjugated resin that had not been incubated with lysate and were then blotted with GDH antibodies conjugated to horseradish peroxidase. The blot was reprobed with anti-rabbit secondary antibodies; the heavy chain is shown as a loading control. Right: Nine separate experiments of the anti-ADP-ribose immunoprecipitation were quantitated by ImageJ (NIH), as shown.

analysis of brain, pancreas, and liver lysates (Figure 4C). *SIRT4* KO mice were viable, fertile, and did not display gross phenotypic abnormalities when compared with wild-type littermates. All subsequent measurements were performed on isogenic 129 strain littermates of wild-type and KO mice.

Pancreatic mitochondrial lysates from KO mice exhibited higher GDH activity than wild-type controls without an increase in GDH protein levels (Figure 4D; $p < 0.01$). As β cells comprise less than 1% of total pancreas, we next measured GDH activity from isolated pancreatic islets and found that activity was significantly higher in *SIRT4* KO mice compared with littermate controls (Figure 4E). To understand why GDH activity was higher in *SIRT4* KO mice, we investigated whether GDH activity in islet lysates was increased by PDE treatment. PDE can serve as a probe for whether ADP-ribosylation downregulates enzymes because it cleaves the ADP-ribose moiety and can thereby relieve inhibition (Witters and McDermott, 1986). Incubation with PDE increased the activity of wild-type lysates to the level of KO samples (Figure 4E). By contrast, *SIRT4* KO lysates were not affected by PDE treatment (Figure 4E). These results strongly suggest that GDH activity is inhibited by SIRT4-mediated ADP-ribosylation in vivo.

We further examined the ADP-ribosylation status of GDH in wild-type and KO mitochondrial lysates by a physical assay. Mono-ADP-ribose antibodies pulled down more GDH from wild-type liver lysates than from KO (Figure 4F). This difference was minimized when lysates were preincubated with PDE. Data from nine such experiments were compiled and are represented in the bar graph in Figure 4F. Taken together, the above data suggest that SIRT4 represses GDH activity by ADP-ribosylating it in vivo.

We tested whether SIRT4 affects mitochondrial ADP-ribosylation by another assay, in which we incubated comparable amounts of wild-type and KO liver mitochondrial lysates (Figure 4G, lanes 1–4) with [^{32}P]NAD. We observed higher ADP-ribosylation in wild-type mitochondria compared with KO samples in the longer, 1.5 hr time point (Figure 4G, lanes 7 and 8), indicating that KO mitochondria have decreased ADP-ribosyltransferase activity. Specifically, we observed a significant difference in the ADP-ribosylation of a 56 kDa band, which was previously determined to be GDH (Herrero-Yraola et al., 2001), a conclusion we verified by immunoprecipitation (data not shown). The labeling observed in the short time point (10 min, lanes 5 and 6) or long time point of the KO sample (lane 8) could represent a background due to nonenzymatic labeling. Alternatively, there may be another mitochondrial ADP-ribosyltransferase or nonmitochondrial contaminant in our preparations.

SIRT4 Represses Insulin Secretion in Response to Glucose and Amino Acids

We next investigated a biological role for the regulation of GDH by SIRT4. Insulin release from pancreatic β cells can be induced by amino acids, a process termed amino acid-stimulated insulin secretion (AASIS) (Cline et al., 2004; Kelly and Stanley, 2001; Sener and Malaisse, 1980). For example, leucine is a potent insulin secretagogue because its conversion into TCA-cycle intermediates provides energy (for review, see MacDonald et al., 2005). In addition, leucine directly stimulates GDH, thereby allowing glutamate to fuel the TCA cycle and induce insulin secretion (Sener and Malaisse, 1980). Glucose represses AASIS, in part, by triggering a surge in GTP, which binds to and inhibits GDH (Li et al., 2003). Indeed, humans with mutations in GDH that render it insensitive to inhibition by GTP display hyperinsulinism/hyperammonemia (HI/HA) syndrome (Stanley et al., 1998).

To test whether SIRT4 regulates insulin secretion by repressing GDH, we first examined how this sirtuin affects insulin secretion in MIN6 cells. Using two separate RNAi sequences, we constructed stable MIN6 pools with decreased levels of SIRT4 compared to control cells (Figure 5A). SIRT4 RNAi cells secreted more insulin compared to control cells at noninducing and inducing concentrations of glucose (Figure 5B; $p < 0.05$) and also displayed increased levels of ATP and rates of oxygen consumption (Figures S1E and S1F). SIRT4 RNAi cells had growth rates similar to control cells (Figure 5C).

To investigate whether knockdown of *SIRT4* potentiates AASIS, cells were challenged with glutamine, which is converted to glutamate in cells. Glutamine must normally be added with leucine to induce insulin, as discussed above (Li et al., 2003; Sener and Malaisse, 1980). Strikingly, in the SIRT4 RNAi cells, glutamine alone stimulated insulin secretion, while control MIN6 cells were unresponsive (Figure 5D; $p < 0.01$). SIRT4 RNAi also increased GDH activity (Figure S1G). We tested whether GDH is required for the increases in glucose and amino acid insulin secretion and AASIS in *SIRT4* knockdown cells by constructing stable MIN6 lines expressing RNAi for both SIRT4 and GDH (Figure 5A). Double-knockdown cells displayed reduced levels of both SIRT4 and GDH. Moreover, these cells did not secrete more insulin in response to glucose and did not respond to glutamine (Figure 5D), indicating that the high insulin secretion in response to glucose and the AASIS seen in the SIRT4 deficiency were GDH dependent.

We next determined whether insulin secretion was regulated by SIRT4 in vivo by studying *SIRT4* KO mice. In the absence of SIRT4, mice had a 30% increase in circulating insulin levels during ad libitum feeding (Figure 6A; $p < 0.001$). When subjected to an overnight fast, KO animals had significantly higher insulin levels compared with

(G) Mitochondria from wild-type and KO livers were incubated with [^{32}P]NAD for 10 or 90 min, run on SDS-PAGE, and transferred to nitrocellulose. Proteins were visualized by Ponceau S staining (lanes 1–4), and ADP-ribosylation was monitored by autoradiography (lanes 5–8).

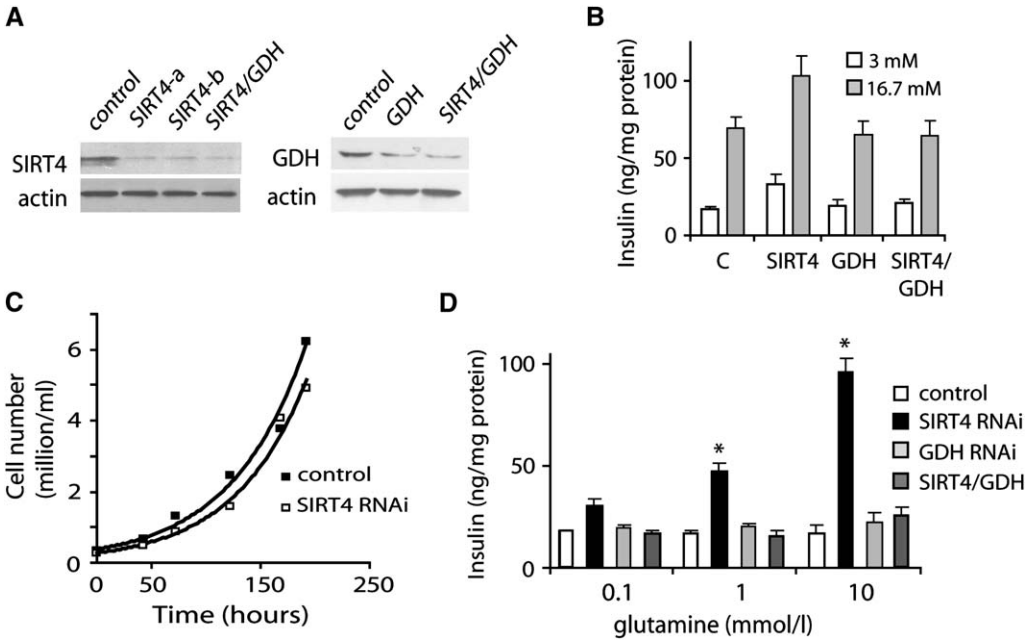

Figure 5. SIRT4 Regulates Insulin Secretion in MIN6 Insulinoma Cells via GDH

(A) Immunoblot showing that levels of SIRT4, GDH, or both (SIRT4/GDH) were decreased by RNAi compared to control MIN6 cells. Cells were stably infected with viruses that contained two SIRT4 (SIRT4-a and SIRT4-b) RNAi sequences, GDH RNAi, both SIRT4/GDH RNAi, or pSUPER-retro (control). SIRT4a and SIRT4b lines gave similar results for secretion assays; only SIRT4a is shown in further experiments.

(B) Insulin secretion was measured from control or the indicated RNAi cells in buffer containing 3 mM (open bars) or 16.7 mM glucose (filled bars).

(C) Representative growth curves from control (■) and SIRT4 RNAi (□) cells.

(D) Insulin secretion was measured in control cells (open bars) or cells expressing RNAi for SIRT4 (black bars), GDH (light gray bars), or both SIRT4/GDH (dark gray bars) incubated in buffer containing 0.1, 1, or 10 mmol/l glutamine for 2 hr at 37°C. Assays in (B) and (D) were performed at least three times using cell lines from two to four separate transfections.

wild-type littermates (Figure 6A; $p < 0.001$). Fed glucose levels were similar between wild-type and KO mice, while male KO mice showed slight but significant fasting hypoglycemia compared to littermate controls (Figure 6B; $p < 0.01$). Females did not show a statistical difference in glucose levels (data not shown). The increase in insulin levels in KO mice was not due to glucose intolerance; if anything, the KO mice were slightly more tolerant (Figure 6C). We next measured whether an IP injection of glutamine stimulated insulin secretion in fasted KO mice. Strikingly, glutamine increased plasma insulin substantially in KO mice (Figure 6D). In contrast, wild-type animals did not respond to glutamine (Figure 6D).

To test whether β cells from the *SIRT4* KO mice display elevated insulin secretion, we isolated islets and measured insulin secretion in response to glucose. We found significantly higher insulin secretion from KO islets under glucose-induced conditions (Figure 6E). Moreover, KO islets showed a dosage-dependent increase in glutamine-stimulated insulin secretion, while wild-type islets were unresponsive (Figure 6F). KO islets also showed higher leucine-stimulated insulin secretion (Figure 6F) but resembled wild-type islets with respect to total insulin content and morphology (Figure 6G). These data show that SIRT4 functions as a negative regulator of insulin secretion and AASIS in mouse β cells in vivo and ex vivo.

CR Regulates GDH Activity and AASIS

Sir2 is involved in physiological changes during CR in yeast and *Drosophila*. More recently, SIRT1 has been shown to be required for at least one phenotype induced by CR in mice, the increase in physical activity (Chen et al., 2005). We hypothesized that SIRT4 may also play a role in physiological changes during CR in mammals. A substantial body of data suggests that protein turnover is increased by CR and that amino acids are used as carbon and energy sources to drive gluconeogenesis in the liver (Dhahbi et al., 2001; Hagopian et al., 2003a, 2005). Thus, it seemed reasonable that β cells of CR mice might respond to amino acids as insulin secretagogues.

To address whether CR potentiates AASIS, we first measured changes in SIRT4 and GDH protein levels in pancreatic islets from CR mice. While GDH protein levels were unaffected by CR, there was significantly higher GDH activity in CR islet lysates compared with non-CR control lysates (Figures 7A and 7B). Moreover, treatment of the non-CR islet lysates with PDE increased GDH activity to the level of the CR lysates but did not further increase GDH activity in the CR lysates (Figure 7B), suggesting that GDH is ADP-ribosylated and downregulated in islets under non-CR conditions, but not during CR. The effects of CR on GDH activity parallel what we observed in *SIRT4*

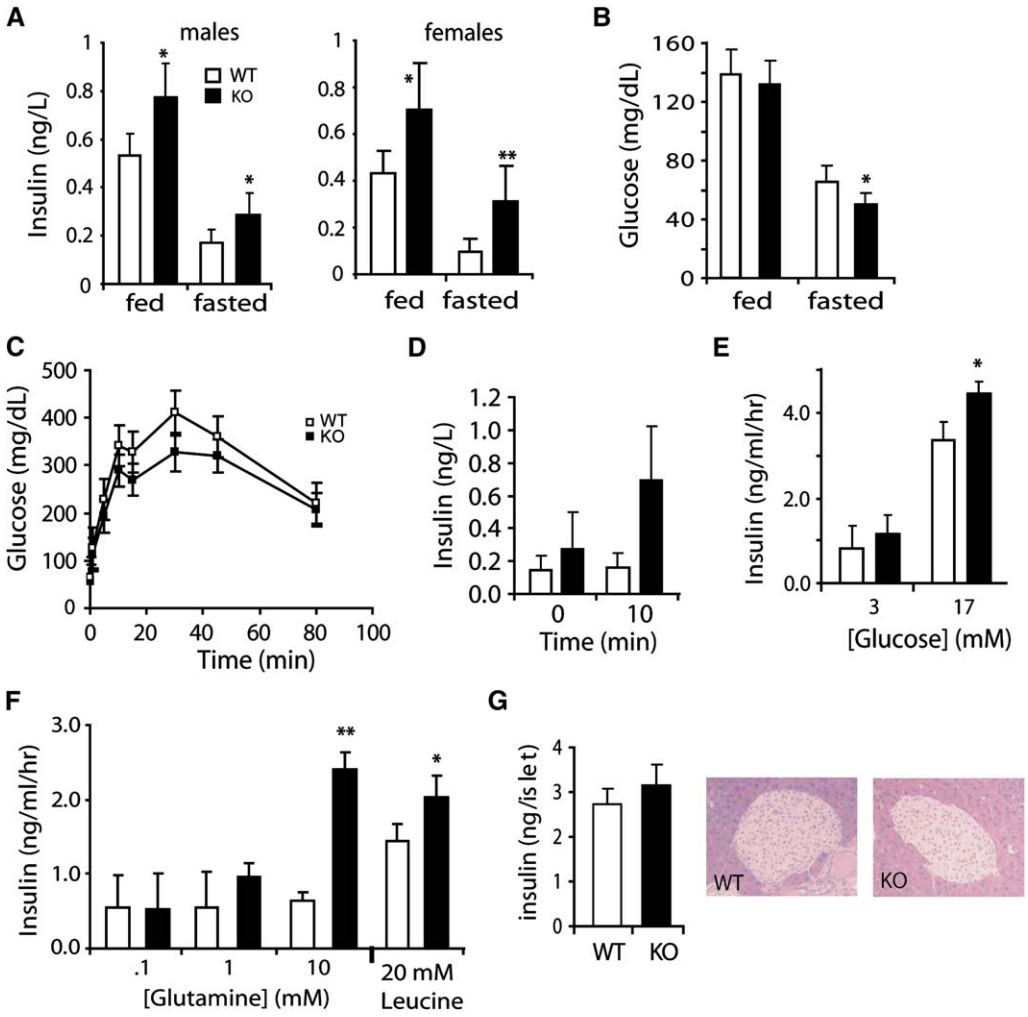

Figure 6. SIRT4 Regulates Insulin Secretion In Vivo

(A) Plasma insulin was measured in *SIRT4* wild-type (open bars throughout figure) or *SIRT4* KO (black bars throughout figure) mice fed ad libitum or after an overnight (14 hr) fast. (n = 15–25).

(B) Blood glucose was measured in *SIRT4* wild-type or *SIRT4* KO mice fed ad libitum or after an overnight (14 hr) fast (n = 15–25).

(C) Blood glucose was measured after an IP injection in fasted animals (1 g/kg body weight from a 10% glucose [w/v] solution) (n = 6).

(D) Glutamine-stimulated insulin secretion in *SIRT4* wild-type or *SIRT4* KO mice. Glutamine (0.2 g/kg body weight of a 2% solution) was injected IP in fasted mice, and insulin was measured at 0 min (before injection) and 10 min after injection (n = 6).

(E and F) Insulin secretion was measured in isolated islets (eight islets per concentration in duplicate) from *SIRT4* wild-type or *SIRT4* KO mice stimulated with 3 mM or 17 mM glucose (E) or 0.1, 1, or 10 mM glutamine or 20 mM leucine (F).

(G) Insulin content of pancreatic islets was measured in batches of eight islets (n = 15 batches in three separate experiments). Representative islets from *SIRT4* wild-type or *SIRT4* KO mice are shown. *p < 0.01, **p < 0.001 by Wilcoxon rank-sum test.

KO pancreas, thereby suggesting that SIRT4 activity is downregulated by CR.

To test directly whether CR potentiates AASIS, we isolated islets from control and CR pancreas for ex vivo analysis. We found that glucose-stimulated insulin secretion was not affected by CR compared with control islets (Figure 7C). However, there was a striking increase in glutamine- and leucine-stimulated insulin secretion in isolated CR islets compared to controls (Figures 7D and 7E). These results demonstrate a switch to AASIS during CR, similar to what was observed in *SIRT4* KO mice, further suggesting that SIRT4 activity is downregulated by CR to mediate this switch.

Because GDH activity has been reported to be upregulated in liver during CR (Hagopian et al., 2003a), we tested whether changes in ADP-ribosylation were also responsible for this regulation. As in islets, CR did not affect the levels of GDH in liver lysates (Figure 7F) but gave rise to higher GDH activity compared to controls (Figure 7G). Also, repeated analyses of SIRT4 did not reveal significant differences in protein levels in control versus CR. Moreover, the GDH activity

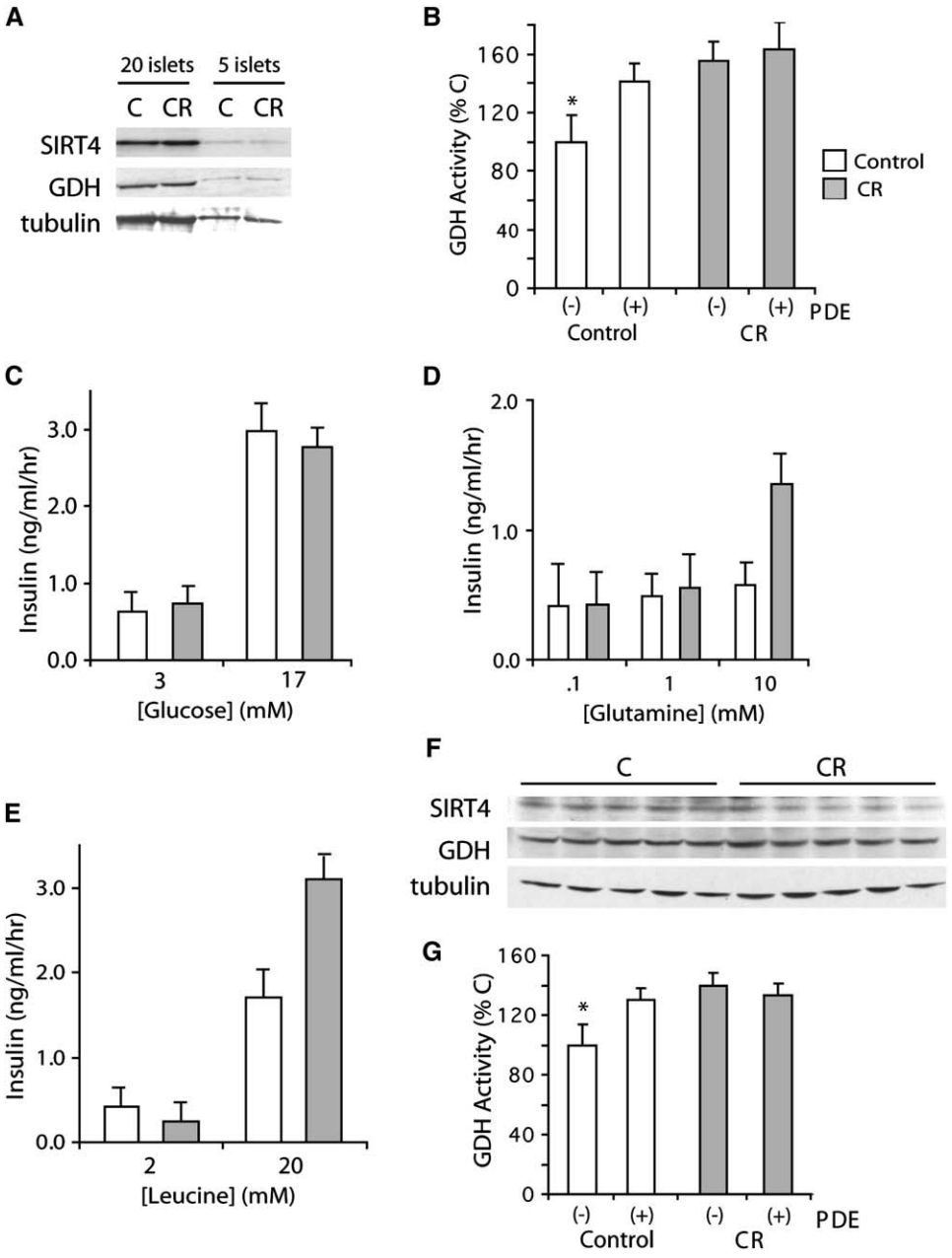

Figure 7. Calorie Restriction Potentiates AASIS

(A) Lysates from two sets of islets from control (C) or calorie-restricted (CR) mice were analyzed by SDS-PAGE and Western blotting using antibodies to SIRT4, GDH, and tubulin.

(B) GDH activity was measured in 100 control (open bars) or CR (gray bars) islets without or with PDE treatment, as described for Figure 4E.

(C–E) Insulin secretion was measured in isolated islets (duplicate assays using five islets for each measurement) from control (open bars) or CR (gray bars) mice stimulated with glucose (C), glutamine (D), or leucine (E). Islets from six control and six CR mice were used.

(F) Liver lysates from five control and five CR mice were analyzed by Western blotting using antibodies to SIRT4, GDH, and tubulin.

(G) GDH activity was measured in liver lysates from five control (open bars) and five CR (gray bars) mice without or with PDE treatment, as described for Figure 4E.

in control lysates was increased by PDE treatment to levels observed in CR livers. PDE did not affect GDH activity in the CR liver lysates. These findings suggest that, as in islets, GDH is ADP-ribosylated and downregulated in the liver under non-CR conditions, but not during CR.

To address whether GDH activity is altered in tissues other than pancreas and liver during CR, we also

measured GDH activity and sensitivity to PDE in control and CR brain (cerebellum) lysates. SIRT4 and GDH protein levels were not significantly altered by CR (Figure S2A). However, as in pancreas and liver, we found that GDH activity was increased in CR compared with control lysates (Figure S2B). Again, the lower level of activity in control lysates was stimulated by PDE, but the higher activity in CR lysates was not, indicating that regulation occurred by ADP-ribosylation of GDH.

DISCUSSION

We show that the mammalian SIR2 homolog SIRT4 regulates insulin secretion in pancreatic β cells. Several lines of evidence support a model in which SIRT4 regulates AASIS in β cells in response to diet by ADP-ribosylating and repressing mitochondrial GDH. First, SIRT4 and GDH are expressed (Figure 1B and Figure 2D) and interact in mitochondria of β cells (Figure 2F). Second, both immunoprecipitated and recombinant SIRT4 ADP-ribosylate GDH in vitro (Figure 3). Third, SIRT4 inhibits GDH activity in vitro (Figure 3E). Fourth, GDH is activated in wild-type lysates by PDE, indicating that it is normally repressed by ADP-ribosylation (Figure 4E). Fifth, GDH activity is higher in *SIRT4* KO mice and can not be further activated by PDE treatment (Figures 4D and 4E). Sixth, *SIRT4* KO mice display increased insulin secretion in response to glutamine and leucine (Figure 6F). These KO mice do not show a change in levels of GDH (Figure 4D) or other markers of mitochondrial function (Figure S2C). Seventh, GDH activity is elevated during CR and is not further stimulated by PDE (Figure 7B). Eighth, CR islets secrete more insulin in response to glutamine and leucine compared to controls (Figures 7D and 7E).

We conclude that SIRT4 ADP-ribosylates and downregulates GDH in β cells during calorie-sufficient conditions, thereby preventing glutamine from serving as an insulin secretagogue. However, during CR, there is an increase in GDH activity, which upregulates insulin secretion in response to glutamine and leucine. Unlike GDH from control mice, GDH from CR mice is insensitive to PDE treatment, suggesting that it is less ADP-ribosylated. We thus surmise that the SIRT4 ADP-ribosyltransferase is downregulated by CR, a change that goes against the expectation that sirtuin functions increase during CR. However, it is consistent with the following molecular and physiological considerations. First, we did not observe an induction in SIRT4 protein levels in β cells of CR mice (Figure 7A). Second, it has been reported that fatty-acid oxidation in mitochondria is upregulated by CR to provide energy (Tsuchiya et al., 2004), which should result in a substantial conversion of NAD to NADH in that cellular compartment. Indeed, a reduction in the NAD/NADH ratio has been reported in liver mitochondria during CR (Hagopian et al., 2003b) and in pancreas during overnight starvation (Bordone et al., 2005). A change in the NAD/NADH ratio regulates yeast Sir2p during CR (Lin et al., 2004). A lower NAD/NADH ratio during CR may therefore downregulate SIRT4 activity and thus reduce ADP-ribosylation of GDH.

Coordination between β Cells and Liver during CR

Because amino acids serve as carbon and energy sources during CR (Dhahbi et al., 2001; Hagopian et al., 2003a; Tsuchiya et al., 2004), they have the potential to fuel glucose synthesis in the liver. Our finding that CR induces AASIS in β cells (Figures 7D and 7E) fits the idea that this regimen shifts the burden of energy provision from carbohydrates toward proteins derived from the diet. A previous study found elevated liver GDH activity during CR (Hagopian et al., 2003a), and our data suggest that this regulation occurs by a reduction in ADP-ribosylation of the liver enzyme (Figure 7G). By this logic, ADP-ribosylation of GDH may coordinate amino acid metabolism in different tissues with changes in diet—i.e., during CR, a reduction in ADP-ribosylation upregulates GDH in the liver to potentiate gluconeogenesis and in β cells to potentiate AASIS. In contrast to the altered response of CR islets to amino acids, their response to glucose was unchanged from calorie-sufficient mice (Figure 7C), consistent with previous findings (Reaven et al., 1983).

Opposing Roles of SIRT4 and SIRT1 in Insulin Secretion

SIRT1 functions as a positive regulator of glucose-stimulated insulin secretion (GSIS) in β cells (Moynihan et al., 2005) by repressing the mitochondrial uncoupling protein UCP-2 (Bordone et al., 2005). In contrast, SIRT4 appears to downregulate GSIS and AASIS. It is not certain whether the observed effect of SIRT4 on GSIS, like its effect on AASIS, is due to downregulation of GDH. While the in vitro study in MIN6 insulinoma cells suggests that the increase in GSIS in *SIRT4* knockdown cells requires GDH, more experiments will be required to resolve the question of whether SIRT4 affects other mitochondrial proteins that can influence GSIS.

It is striking that at least two of the seven sirtuins appear to regulate insulin secretion, apparently in opposing directions. It is possible that regulation by these sirtuins is invoked under subtly different dietary conditions. SIRT1-mediated repression of UCP-2 is alleviated by acute food deprivation (Bordone et al., 2005), indicating that this mechanism may function to dampen insulin secretion during starvation. In contrast, SIRT4 repression of GDH was alleviated during long-term CR, resulting in activation of AASIS in β cells and potentially gluconeogenesis in liver. Thus, SIRT1 and SIRT4 may serve to regulate insulin secretion under conditions that span the range of food availability, from acute starvation to chronic food limitation.

Mitochondrial Sirtuins

SIRT4 is a mitochondrial ADP-ribosyltransferase that modifies GDH to affect insulin secretion and amino acid metabolism in pancreas, liver, and brain. It will be interesting to determine whether SIRT4 regulates additional pathways by ADP-ribosylating other mitochondrial proteins.

Indeed, our study of ADP-ribosylation of mitochondrial proteins in vitro (Figure 4G) indicated other potential targets of SIRT4. The other mitochondrial sirtuin that has been studied in detail is SIRT3. Unlike SIRT4, SIRT3 displays NAD-dependent deacetylase activity (Onyango et al., 2002; Schwer et al., 2002). SIRT3 has been reported to be upregulated by CR and cold exposure in brown fat, where it may alter the metabolic activity of this tissue (Shi et al., 2005). Because the enzymatic activity of both sirtuins is influenced by mitochondrial NAD/NADH ratios and the level of nicotinamide, it will be interesting to learn how coordination of acetylation and ADP-ribosylation by these sirtuins regulates mitochondria.

Summary and Perspective

SIRT4 regulates insulin secretion in β cells by ADP-ribosylating and downregulating GDH in mitochondria. ADP-ribosylation of GDH, as judged by sensitivity to PDE, is reduced by long-term CR in islets and liver, suggesting that CR downregulates SIRT4 activity, perhaps by lowering the NAD/NADH ratio. The increase in GDH activity during CR potentiates amino acids as insulin secretagogues. It will be important to determine whether other mammalian sirtuins also help coordinate the multiple effects of CR on health and life span.

EXPERIMENTAL PROCEDURES

Animal Experimentation

Littermate 129/Sv (8–12 weeks old) mice were used for the present studies. The *SIRT4* KO targeting vector was constructed by replacing exons 1–3 with a *LacZ* gene inserted in-frame after the first 21 bp of exon 1 using the recombineering method (Valenzuela et al., 2003). Mice were housed under controlled temperature (25°C) and light, fed normal chow, and euthanized by CO_2. Organs were harvested and homogenized and mitochondria from cells and tissues were isolated as described (Schwer et al., 2002). For CR studies, C57BL/6 mice were subjected to CR for 12 months as described (Pugh et al., 1999).

Insulin Assays

Plasma insulin levels were determined by ELISA (Alpco Diagnostics, Windham, NH, USA). Glucose levels were measured using a one-step glucometer. For islet insulin secretion assays, pancreata were digested by incubation with collagenase (0.4 mg/ml) in HBSS, 1 $MgCl_2$, and 3 mM glucose at 37°C while shaking. Islets were separated from tissue by a series of centrifugation and wash steps. Handpicked groups of 5–8 islets were cultured for 2 hr or overnight in RPMI media (10 mM glucose, 10% FBS). To stimulate insulin secretion, islets (or MIN6 cells, passage 29–40) were incubated in Krebs-Ringer-HEPES buffer (KRB; pH 7.4 128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4, 20 mM HEPES) containing 3 mM glucose for 1 hr. Then, islets were incubated in KRB containing glucose, glutamine, or leucine at 37°C for 1–2 hr. Supernatants containing insulin were stored at −20°C until analysis. Total islet insulin was measured in islets treated with ethanol overnight at −20°C. Retroviral infection was performed as described (Picard et al., 2004) using MIN6 cells. Four independent stable lines were created for each RNAi, and experiments were repeated two or three times in each line.

Immunoprecipitation/Microscopy

For immunoprecipitation, cells were lysed for 30 min at 4°C in ice-cold NP40 buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP40) containing protease inhibitors (EDTA-free Complete; Roche Molecular Biochemicals, Indianapolis, IN, USA) and 1 mM DTT (Sigma, St. Louis). The lysate was clarified by centrifugation for 15 min at 4°C at 14,000 rpm in a tabletop centrifuge, immunoprecipitated at 4°C for 2 hr, washed, and immunoblotted; signal was detected using ECL (Amersham Biosciences, Piscataway, NJ, USA). One percent to five percent of the lysate was used as the input control.

HepG2 cells were grown on coverslips and transfected with phSIRT4GFP, pDsRed2-Mito, or pDsRed2-Peroxi (BD Biosciences, Palo Alto, CA, USA). After 48 hr, cells were fixed with paraformaldehyde and analyzed using a confocal microscope (Zeiss LSM 510; Zeiss, Thornwood, NY, USA).

Immunohistochemistry in mouse pancreas was performed using a microwave citrate unmasking protocol. An antibody for mouse SIRT4 was created in rabbit against the peptide LEMNFPLSSAAQDP. Antibodies to insulin and GDH were from Zymed Laboratories (San Francisco) and USBiological, respectively. Antibodies to FLAG and Gal4 were from Sigma. Mono-ADP-ribose antibody was a generous gift from H. Hilz (Meyer and Hilz, 1986). Secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA).

Recombinant SIRT4

The EasySelect *Pichia* expression kit (Invitrogen, Carlsbad, CA, USA) was used to prepare recombinant *P. pastoris* clones expressing hSIRT4 (residues 25–314) for intracellular expression. Recombinant hSIRT4 was dialyzed in 50 mM Tris (pH 8.0), 10 mM 2-mercaptoethanol, 100 mM NaCl; protein was applied to a MonoQ anion exchange column (Amersham Biosciences); and hSIRT4 was eluted with a 0.1–1 M NaCl gradient.

Enzyme Assays

FLAG-tagged SIRT1, SIRT4, and SIRT5 proteins were purified by immunoprecipitation and eluted in FLAG buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM DTT, 0.1 mg/ml FLAG peptide). Deacetylase activity of dialyzed protein (50 ng) was assessed with or without 1 mM NAD by the Fluor de Lys kit (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA). ADP-ribosylation activity was assessed as described (Tanny et al., 1999) using FLAG proteins (50 ng) and core histone (1 μg) in the presence of 1 mM NAD and with or without 1 mM nicotinamide in 50 mM Tris buffer (pH 8.0), 10 mM DTT, 150 mM NaCl. For recombinant hSIRT4, assays were performed by incubating 5 μCi [^{32}P]NAD with GDH (10 μg) at 37°C for 1 hr with or without hSIRT4 (1 μg), hSIRT4 alone, 2 μg *Neurospora crassa* NAD glycohydrolase (Sigma), TmSIR2 (2 μg), or 1 μM acetylated peptide JB12 (Sauve et al., 2001).

GDH activity was assessed as described (Herrero-Yraola et al., 2001). Briefly, GDH (10 μg, Sigma) activity was measured after incubation in the presence or absence of SIRT4 (10 ng) or 1 mM NAD^+ at 37°C for the indicated times.

Thin-layer chromatography was used to analyze NAD metabolites produced by sirtuin activity. Two microliters from each reaction was spotted on PEI-cellulose TLC plates (Macherey-Nagel, Easton, PA, USA) and developed with 300 mM LiCl. After drying the TLC plate, it was exposed to film for 15 min.

Mitochondrial labeling experiments were performed as described (Jorcke et al., 1998). In brief, wild-type or KO liver mitochondria were incubated with 1 mM NAD (containing 20 μCi [^{32}P]NAD) for 10 or 90 min at 37°C. Labeled proteins were visualized by autoradiography.

Statistical Analysis

Analysis was performed using an unpaired Student's t test, and significant differences are indicated by an asterisk when $p < 0.05$.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and can be found with this article online at http://www.cell.com/cgi/content/full/126/5/941/DC1/.

ACKNOWLEDGMENTS

We thank P. Bradshaw and J. Huang for help with the CR experiments. We also thank D. Lombard, L. Bordone, R. Olivieira, X. Li, and N. Bishop for comments on the paper and helpful discussion and S. Imai for discussion and cells. We also thank C. Stanley for helpful discussions. This work was supported by grants from the NIH to L.G., R.W., and M.C.H.; a grant from Estee Lauder to G.B.; an Ellison Foundation Senior Scholar Award to F.W.A.; and grants from the Human Frontier Science Program and The Leukemia & Lymphoma Society to R.M. K.M.H. is a Robert Black Fellow of the Damon Runyon Cancer Research Foundation. F.W.A. and C.W. are investigators of the Howard Hughes Medical Institute.

A.J.M., D.M.V., G.D.Y., and M.K. are employees and shareholders of Regeneron Pharmaceuticals, Inc. L.G. is a founder and board member of Elixir Pharmaceuticals. R.W. and T.A.P. are founders and board members of LifeGen Technologies, LLC.

Received: January 20, 2006
Revised: April 26, 2006
Accepted: June 26, 2006
Published: September 7, 2006

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