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Mammalian SIRT1 Represses Forkhead Transcription Factors

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Summary

The NAD-dependent deacetylase SIR2 and the forkhead transcription factor DAF-16 regulate lifespan in model organisms, such as yeast and *C. elegans*. Here we show that the mammalian SIR2 ortholog SIRT1 deacetylates and represses the activity of the forkhead transcription factor Foxo3a and other mammalian forkhead factors. This regulation appears to be in the opposite direction from the genetic interaction of SIR2 with forkhead in *C. elegans*. By restraining mammalian forkhead proteins, SIRT1 also reduces forkhead-dependent apoptosis. The inhibition of forkhead activity by SIRT1 parallels the effect of this deacetylase on the tumor suppressor p53. We speculate how downregulating these two classes of damage-responsive mammalian factors may favor long lifespan under certain environmental conditions, such as calorie restriction.

Introduction

Over the past several years, numerous genes have been identified that alter the lifespan of model organisms. One of these is *SIR2*, which encodes an NAD-dependent protein deacetylase (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). In yeast, the deacetylation of histones by Sir2p results in silencing of mating type genes, telomeres, and ribosomal DNA (Shore, 2000). Silencing in the ribosomal DNA promotes longevity (Kennedy et al., 1997; Sinclair and Guarente, 1997), and increased dosage of the yeast *SIR2* gene extends the lifespan of mother cells beyond the wild-type (Kaeberlein et al., 1999). In *C. elegans*, increased dosage of the worm *sir-2.1* gene also extends the lifespan of worms beyond the wild-type (Tissenbaum and Guarente, 2001). This conserved function predicts that mammalian SIR2 genes will also regulate lifespan.

Yeast Sir2p is upregulated by changes in metabolic activity due to calorie restriction (Lin et al., 2000). In particular, reducing glucose levels in the media shifts the metabolic strategy of yeast cells for energy production from fermentation to respiration, and this shift is required for long life (Lin et al., 2002). This metabolic shift upregulates the Sir2p deacetylase by increasing the NAD/NADH ratio (Guarente, 2000; Lin et al., 2004). Moreover, *sir2* mutants do not live longer when calorie restricted, implying that calorie restriction is a regulated process with the Sir2p deacetylase playing an essential regulatory role.

A major genetic pathway that regulates the lifespan of *C. elegans* is the insulin-like signaling pathway. Mutations in the insulin receptor, DAF-2, slow signaling in this pathway and extend the lifespan (Kenyon et al., 1993; Kimura et al., 1997). DAF-2 is coupled to a series of kinases, including AKT kinase, which phosphorylate the forkhead family transcription factor DAF-16 causing its retention in the cytoplasm (Ogg et al., 1997; Lin et al., 1997). Thus, mutations in *daf-2* lead to the translocation of DAF-16 to the nucleus, where it is active (Henderson and Johnson, 2001; Lin et al., 2001; Lee et al., 2001). DAF-16 activity is required in *daf-2* mutants for the entry into dauer in larvae and for the extended lifespan in adults (Kenyon et al., 1993; Kimura et al., 1997).

Genetic data in *C. elegans* suggest that the long lifespan conferred by extra copies of *sir-2.1* will extend the lifespan in wild-type but not in *daf-16* mutants, indicating that in worms, SIR-2 requires DAF-16 for longevity (Tissenbaum and Guarente, 2001). However, the mechanism by which SIR-2.1 may regulate insulin signaling to activate DAF-16 is not known.

In mammals, both *SIR2* and forkhead genes are conserved and play important roles in cellular and organismal biology. *SIR2* is represented by seven homologs, *SIRT1-7* (Frye, 1999). The ortholog, *SIRT1*, is an NAD-dependent deacetylase (Imai et al., 2000) that deacetylates and downregulates the p53 tumor suppressor (Luo et al., 2001; Vaziri et al., 2001). Accordingly, increased SIRT1 activity in cultured cells reduces p53-mediated apoptosis in response to radiation or oxidative stress. SIRT1 has also been shown to repress the terminal differentiation in dividing myocytes by binding to the myoD cofactor, pCAF (Fulco et al., 2003). In these cases, SIRT1 favors cell survival and division in the face of stress or signals for terminal differentiation.

SIRT1 knockout mice have a severe phenotype, with a high degree of embryonic and postnatal lethality (Mc-

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Burney et al., 2003; Cheng et al., 2003). In outcrossed backgrounds, rare homozygous knockout mice survive to adulthood and are invariably smaller than wild-type and sterile. Consistent with the possibility that SIRT1 regulates p53 in vivo, these knockout adults display hyperacetylated p53 and show increased apoptosis, at least in thymocytes (Cheng et al., 2003) and spermatogonia (McBurney et al., 2003).

The forkhead (or winged helix) proteins are structurally related transcriptional activators of which the mammalian Foxo members Foxo1, Foxo3a, and Foxo4 represent a subfamily that are regulated by growth factors through the activation of the PtdIns-3-kinase pathway (Tran et al., 2003). Growth factor-induced activation of PtdIns-3-kinase leads to an increase in the activity of the ser/threonine kinase AKT/PKB (Anderson et al., 1998; Stephens et al., 1998), which in turn leads to phosphorylation and inactivation of Foxo proteins by retention in the cytoplasm (Rena et al., 2001; Cahill et al., 2001; Brunet et al., 2002; Tzivion et al., 2001).

Under conditions that activate Foxo proteins, such as serum withdrawal, Foxo proteins move from the cytoplasm to the nucleus, where they activate or repress suites of genes (Ramaswamy et al., 2002; Murphy et al., 2003). Depending on the activation signal, Foxo can regulate apoptosis (Brunet et al., 1999; Nakamura et al., 2000; Tang et al., 2002), cell cycle arrest (Dijkers et al., 2000; Kops et al., 2002b; Medema et al., 2000), differentiation (Hribal et al., 2003; Bois and Grosveld, 2003; Nakae et al., 2003), or the activation of genes involved in DNA repair (Tran et al., 2002; Furukawa-Hibi et al., 2002) and oxidative stress resistance (Murphy et al., 2003; Kops et al., 2002a; Nemoto and Finkel, 2002).

Other genes activated by Foxo in mammals, such as PEPCK, control the gluconeogenic pathway that drives the production of glucose in the liver upon starvation (Daitoku et al., 2003; Barthel et al., 2001; Nakae et al., 2001). Indeed, the reduced level of Foxo1 in heterozygous mice suppresses a diabetic phenotype induced in mice heterozygous for the insulin receptor gene (Nakae et al., 2002).

In this study, we examine a possible connection between SIRT1 and Foxo3a in mammalian cells. Our findings indicate that SIRT1 deacetylates and downregulates forkhead proteins. The implications of these findings for the biology of aging in mammals will be discussed.

Results

Binding of SIRT1 to Foxo3a and to p300

Human embryonic kidney cells (HEK293T) were transfected with Ha-tagged Foxo3a, Flag-tagged SIRT1, or both. Immunoprecipitation with anti-Flag and blotting with anti-Flag (Figure 1A) showed SIRT1 in lanes 2 and 3, as expected. Likewise, immunoprecipitation with anti-HA and blotting with anti-HA revealed Foxo3a in lanes 1 and 3, as expected. In the experimental case of immunoprecipitation with anti-Flag and blotting with anti-HA, Foxo3a was evident when SIRT1 was cotransfected (lane 3), but not when Foxo3a was transfected alone (lane 1). This experiment shows that SIRT1 can interact with Foxo3a.

In addition, we carried out coimmunoprecipitation of transfected SIRT1 (tagged with Myc) and p300 (tagged with HA) (Figure 1B). The dominant-negative mutant of SIRT1 (dn) changing an active site His to Ala (Imai et al., 2000; Luo et al., 2001) was also used in this assay. Transfection with SIRT1 (lanes 1 and 4) or the SIRT1dn (lanes 2 and 5) gave rise to elevated levels of SIRT1, visualized by blotting with an anti-Myc antibody (bottom panel). Extracts were immunoprecipitated with anti-HA and blotted with anti-Myc antibody. Transfection of SIRT1 (lane 1, top), SIRT1dn (lane 2, top), or HA-p300 in the absence of SIRT1 (lane 3, top) did not result in a signal of SIRT1 in the precipitate. In contrast, cotransfection of HA-p300 and SIRT1 (lane 4, top) or SIRT1dn (lane 5, top) gave rise to a strong SIRT1 signal in the precipitate. In a reciprocal experiment, cells were transfected with HA-p300 (lane 6) or HA-p300 and myc-SIRT1dn (lane 7). Immunoprecipitation with anti-myc followed by blotting with anti-HA showed that p300 was pulled down only when cotransfected with SIRT1. These experiments indicate that p300 can interact with SIRT1 or the dn mutant.

SIRT1 Represses the Ability of Foxo3a to Activate Transcription

In a first attempt to determine whether SIRT1 could alter the activity of Foxo3a, HeLa cells were transfected with a luciferase construct driven by the promoter of the forkhead target gene, *bim*, a proapoptotic factor (Dijkers et al., 2000). Expression vectors for Foxo3a, or Foxo3a plus SIRT1 or SIRT1dn, were also transfected, as indicated (Figure 2A). The Foxo3a expression vector used in this experiment actually expresses a mutant Foxo3a, Foxo3aA3, in which the three phosphorylation sites for AKT/PKB are mutated to alanine, thereby rendering nuclear localization as constitutive.

Transfection with the Foxo3a expression vector (lane 2) gave rise to transactivation of the luciferase construct compared to transfection with a vector control (lane 1). Activation by Foxo3aA3 was largely repressed by cotransfection of a SIRT1 expression vector (lane 5) but only partially repressed by the SIRT1dn (lane 6). The SIRT1 or SIRT1dn constructs did not affect the activity of luciferase in cells that were not transfected with Foxo3aA3 (lanes 3 and 4). These findings suggest that SIRT1 can repress Foxo3a and that the deacetylase activity contributes to repression. To further test whether the deacetylase activity plays a role in repression, we added 10 mM nicotinamide, known to block the enzymatic activity of SIRT1. Nicotinamide prevented repression by SIRT1 (lane 5) but did not affect the mild repression by SIRT1dn (lane 6).

In a second experiment, HeLa cells were transfected with a luciferase reporter construct driven by synthetic Foxo binding sites (DBE) plus expression vectors for Foxo3a (all lanes), as well as SIRT1 or PTEN, a 3′-phosphoinositide phosphatase (Figure 2B). PTEN expression is expected to downregulate insulin signaling through the PtdIns-3-kinase pathway and enhance the activity of Foxo3a. Transfection of the SIRT1 vector repressed luciferase activity (lane 2) compared to a vector control (lane 1). This repression was more manifest in cells treated with wortmannin (lanes 4 and 5), an inhibitor

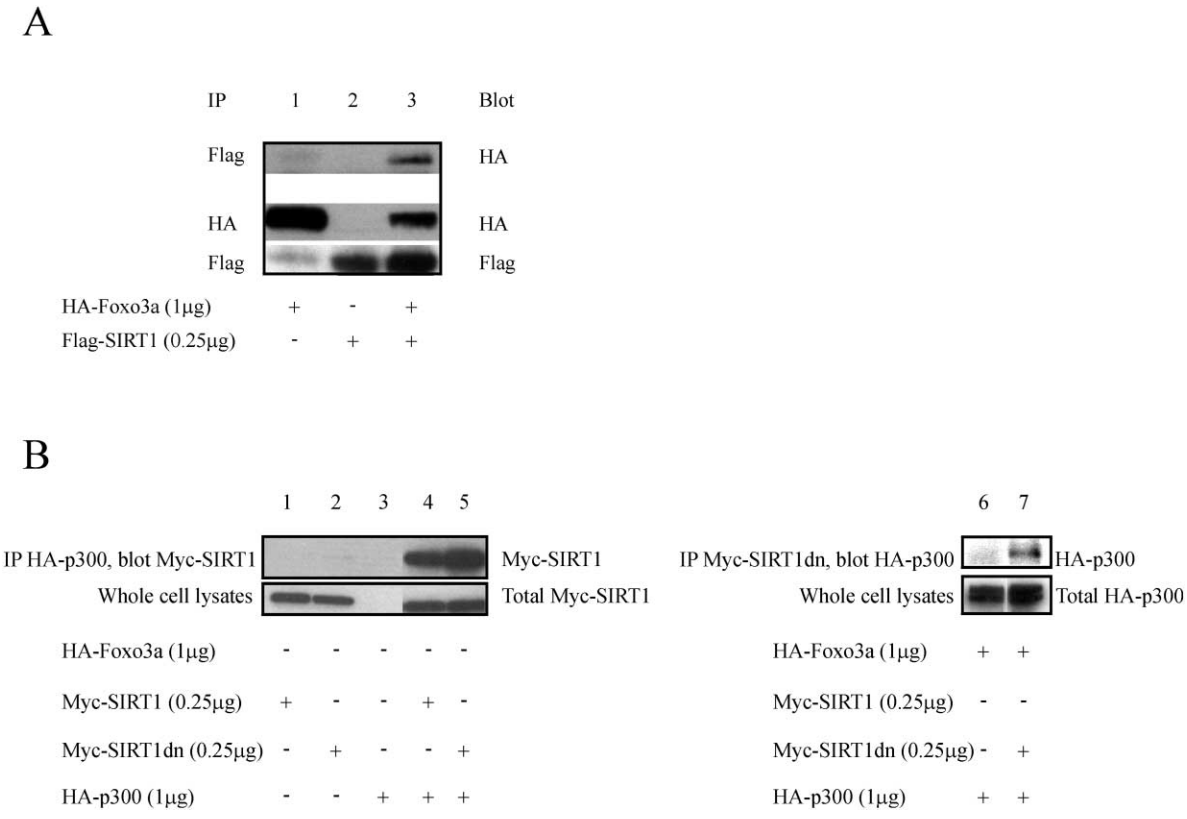


Figure 1. SIRT1 Interacts with Foxo3a and p300

(A) SIRT1 interacts with Foxo3a. HEK293T cells were transfected with HA-Foxo3a (lane 1), Flag-SIRT1 (lane 2), or HA-Foxo3a together with Flag-SIRT1 (lane 3). Lysates were immunoprecipitated as indicated (left) and Western blotted with the indicated antibodies (right). HA-Foxo3a is only immunoprecipitated by the Flag antibody when Flag-SIRT1 is present.

(B) SIRT1 interacts with p300. HEK293T cells were transfected with the indicated plasmids lysed and immunoprecipitated with either an anti-HA antibody (lane 1–5) or an anti-Myc antibody (lanes 6 and 7). Immunoprecipitates were Western blotted and probed with anti-Myc antibody (lanes 1–5) or with an anti-HA antibody (lanes 6 and 7). The top panel of lanes 1–5 demonstrate that SIRT1 (both wt or the dominant-negative version dn) is only immunoprecipitated by the HA antibody when HA-p300 is expressed. The lower panel (lanes 1–5) demonstrates that equal amounts of SIRT1 were expressed in the total cell lysates. Lanes 6 and 7 demonstrate that HA-p300 was only immunoprecipitated by the Myc antibody when both Ha p300 and SIRT1dn were expressed. Lanes 6 and 7 (bottom panel) demonstrate that the levels of p300 in the total lysates were comparable.

of the PI3 kinase, which, like PTEN, will downregulate PtdIns-3-kinase signaling and activate Foxo3a. In both control (lane 3) and wortmannin-treated cells (lane 6), PTEN expression hyperactivated Foxo3a, demonstrating the effectiveness of this reporter assay in reflecting levels of signaling in the insulin pathway.

In a third experiment, we analyzed the ability of SIRT1 to repress Foxo3a or Foxo3aA3 on promoters of known target genes, bim and the cell cycle regulator, p27 (Figure 2C). HeLa cells were transfected with the DBE construct (DBE, lanes 1–5), the p27 promoter (p27, lanes 6–10), or the *BIM* promoter (Bim, lanes 11–15). The level of transcription that was driven by transfection of the Foxo3a expression construct alone was arbitrarily set to 100. Repression of Foxo3a (lanes 2 and 3) or Foxo3aA3 (lanes 4 and 5) by SIRT1 was observed on the DBE construct. Importantly, much stronger repression was observed on the p27 or *BIM* promoters in similar experiments (lanes 7–10 and 12–15). In order to be certain that the repression by SIRT1 or differences between promoters were not due to differences in Foxo3a expression, Western blots were performed and showed comparable levels of the protein in each case (Figure 2D). The above experiments show that SIRT1 can repress Foxo3a activity, and the catalytically inactive SIRT1 is less effective.

In an analogous study, the activity of Foxo1 (Foxo1A3, mutated in the three PKB phosphorylation sites) was also repressed by SIRT1 (Figure 3A). In this case, we examined expression of a luciferase reporter driven by the bim promoter. This reporter was activated by cotransfection of the Foxo1A3 expression construct (lane 2) versus a vector control (lane 1). SIRT1 repressed this activation (lane 3), and the repression was largely abolished by nicotinamide (lane 6). Nicotinamide did not affect the basal activity of the reporter (lane 4) or activation by Foxo1A3 in the absence of SIRT1 (lane 5).

Finally, luciferase assays were carried out using an expression vector for a third forkhead factor, Foxo4 (Foxo4A3, mutated in the three PKB phosphorylation sites), in U2OS cells. In this case, marked activation of the reporter by forkhead was observed; this activation was greatly repressed by cotransfection with the SIRT1 expression vector (Figure 3B). In addition, Foxo4A3 also

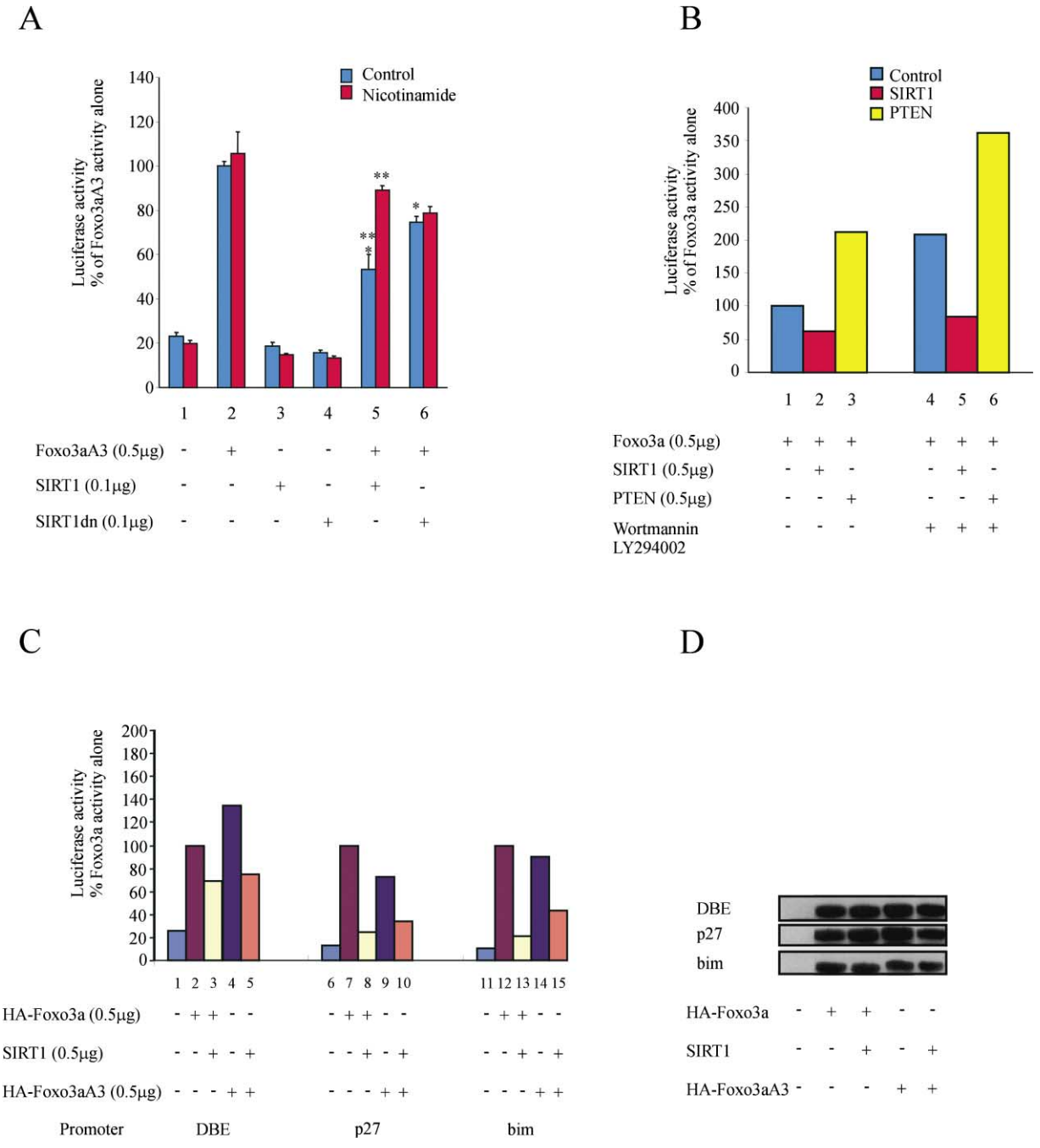


Figure 2. SIRT1 Represses Foxo3a Transcriptional Activity

(A) HeLa cells were transfected as indicated together with the bim-luciferase and β-galactosidase reporter construct. Sixteen hours after transfection, the cells were either maintained as controls (control) or were treated with nicotinamide (10 mM) for a further 16 hr. The data are normalized with respect to β-galactosidase activity and are expressed as a percent of the activity of Foxo3aA3 alone. The data are shown as the average + the standard deviation of triplicate samples. The data demonstrate that SIRT1 represses the transcriptional activity of Foxo3aA3 (compare lane 2 and lanes 5 and 6, control. *denotes a p value < 0.02). Repression by SIRT1 is significantly reversed by incubation with nicotinamide (compare lanes 5 ± nicotinamide. **denotes a p value < 0.01).

(B) HeLa cells were transfected with indicated constructs together with the DBE-luciferase and β-galactosidase reporter constructs. The data are normalized with respect to the β-galactosidase and are expressed as a % of the activity of Foxo3a alone (control). Transfected cells were either maintained as controls or were treated with wortmannin and LY294002 as indicated. PTEN is a 3′-phosphoinositide phosphatase, expected to act as a positive regulator of Foxo3a, and demonstrates that the assay procedure is able to discriminate between potential activators (PTEN) or inhibitors (SIRT1) of Foxo3a activity.

(C) SIRT1 represses Foxo3a-mediated transcription from various promoters. HeLa cells were transfected with the indicated plasmids and reporter constructs and the β-galactosidase reporter. In the cases where the transcriptional activity of Foxo3a was assessed, wortmannin and LY294002 were added for the final 6 hr. The data are shown as the % of the activity obtained in the Foxo3a sample and are single points representative of three experiments. The data demonstrate that SIRT1 is more efficient at repressing Foxo3a activity at natural promoters (p27 and bim).

(D) Repression by SIRT1 cannot be accounted for by changes in the total levels of Foxo3a. Proteins from the samples used for the luciferase activity (Figure 2C) were separated by SDS-PAGE and probed for HA to detect Foxo3a and Foxo3aA3.

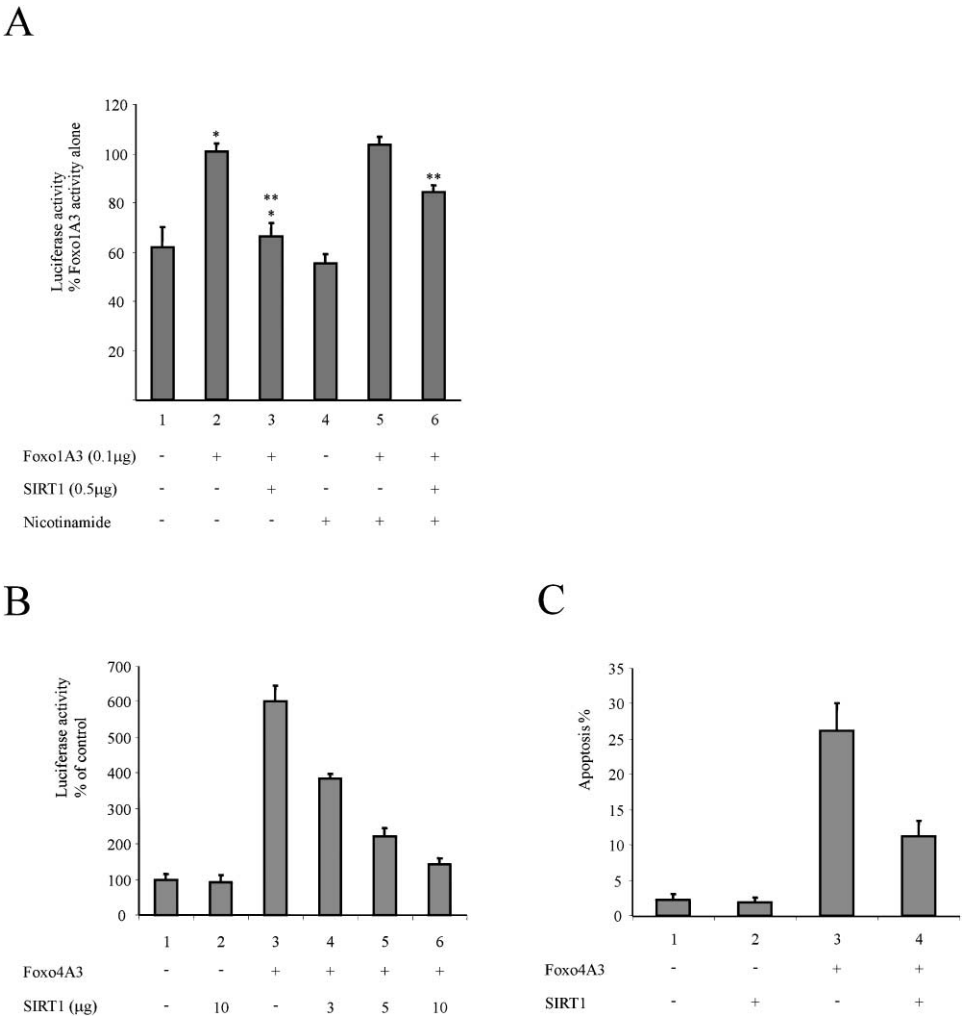


Figure 3. SIRT1 Represses Foxo1 and Foxo4

(A) SIRT1 represses Foxo1 activity in a nicotinamide-dependent manner. HeLa cells were transfected with the indicated constructs together with the bim-luciferase and β-galactosidase reporter constructs. Sixteen hours after transfection, the cells were either maintained as controls or were treated with 10 mM nicotinamide (as indicated) for a further 16 hr. The Foxo1 construct is a constitutively active form in which the PKB phosphorylation sites were mutated (Foxo1A3). The data are normalized to β-galactosidase and presented as a percent of the activity obtained in Foxo1A3 alone. The data are shown as the average + the standard deviation. The data (compare lanes 2 and 3) demonstrate that SIRT1 represses Foxo1A3 activity (* denotes a p value < 0.001) and that this repression is blocked by incubation with 10 mM nicotinamide (compare lanes 3 and 6. **denotes a p value < 0.002).

(B) SIRT1 negatively regulates transcription induced by Foxo4. U2OS cells were transfected as indicated together with the pGL2-3XIRS-luc and β-galacosidase reporter constructs. The Foxo4 construct is the mutant form in which the PKB phosphorylation sites T28, S193, and S258 were converted to alanines (Foxo4A3). The data are normalized for β-galacosidase and shown as the average + the standard deviation.

(C) SIRT1 inhibits apoptosis induced by Foxo4. H1299 cells were transfected with Foxo4A3 and/or SIRT1 as indicated together with GFP as a sorting control. Forty-eight hours after transfection, the cells were fixed, stained with propidium iodide, and analyzed for apoptotic cells (sub-G1) by flow cytometry. The data are presented as the % of green cells + the standard deviation.

caused efficient apoptosis of H1299 cells (Figure 3C). This also was strongly repressed by SIRT1. These findings indicate that the repression of Foxo3a by SIRT1 applies to other forkhead family members.

SIRT1 Deacetylates Foxo3a and p300 and Represses p300-Mediated Activation of Foxo3a

The above experiments suggested that SIRT1 represses the activity of forkhead proteins. To test whether SIRT1 deacetylated forkhead, we transfected HEK293T cells with HA-Foxo3a (Figure 4A, all lanes), p300 (lane 2), and, in some cases, p300 and SIRT1 (lanes 3–5). Total cell extracts were equilibrated for roughly comparable levels of p300 and Foxo3a (lanes 1–5) and the extracts were processed further. Foxo3a (and p300) was immunoprecipitated with anti-HA and subsequently analyzed. Similar amounts of total HA-Foxo3a were brought down in all cases, as determined by blotting the precipitates with anti-HA (lanes 1′–5′, middle). The precipitates were also blotted with anti-acetyl-lysine (lanes 1′–5′, top). Acetylated Foxo3a was observed in cells cotransfected with Foxo3a and p300 (lane 2′, top) but not in cells transfected with Foxo3a alone (lane 1′, top). SIRT1 expression virtually abolished the appearance of acetylated Foxo3a (lanes 3′–5′, top), indicating that Foxo3a can be

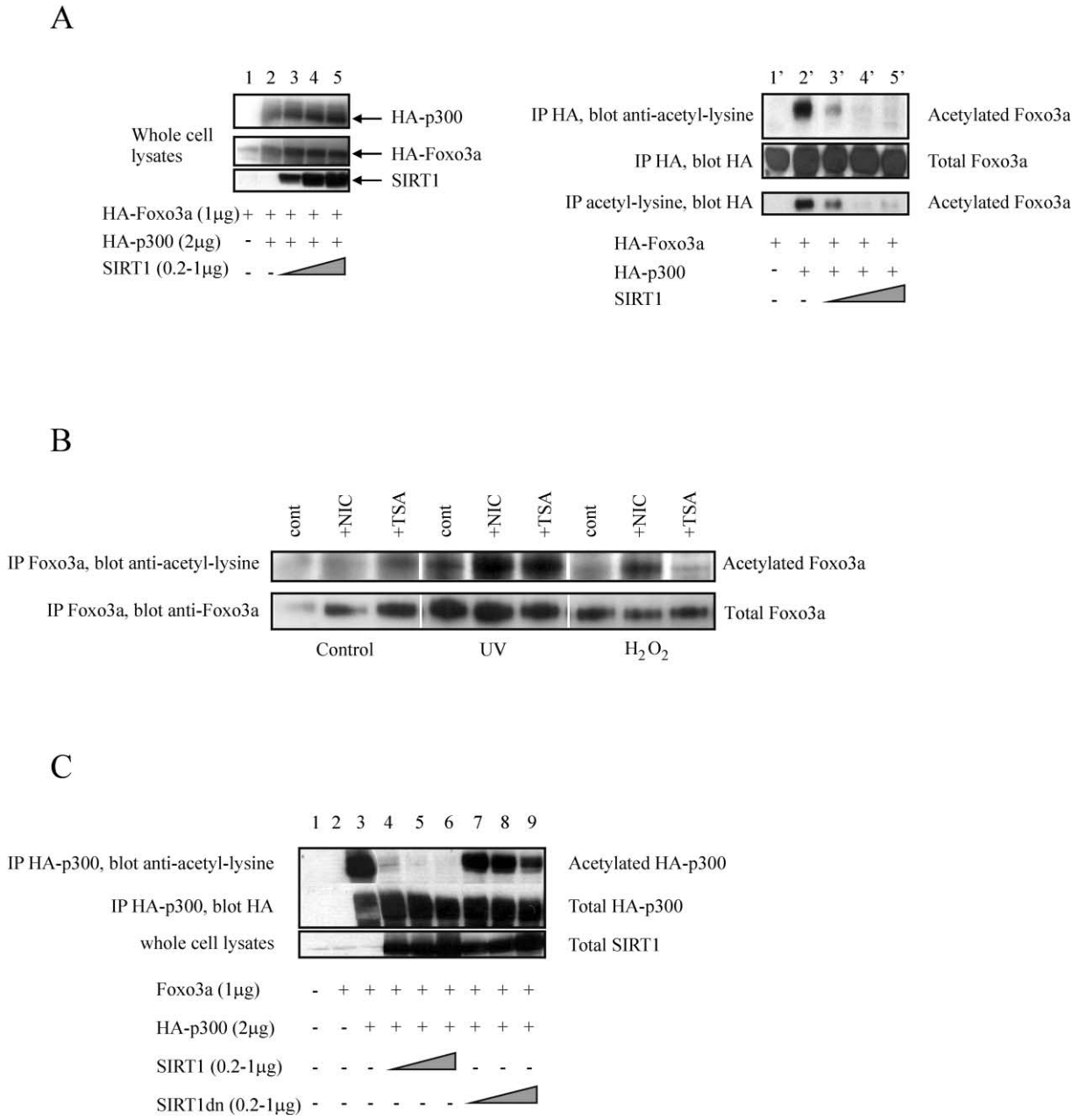


Figure 4. SIRT1 Deacetylates Foxo3a and p300

(A) HEK293T were transfected with the indicated constructs and lysed 36 hr later. Total cell lysates were Western blotted for the presence of HA-p300, HA-Foxo3a, and SIRT1 (lanes 1–5). Comparable levels of p300 (lanes 2–5) and Foxo3a (lanes 1–5) were immunoprecipitated using antibodies either directed against acetylated-lysine (lanes 1′–5′, bottom panel) or the HA epitope (lanes 1′–5′, top panels) and Western blotted for the presence of HA epitope (in the case of immunoprecipitation with anti-acetyl-lysine antibodies, bottom panel) or for acetylated-lysine residues (in the case of immunoprecipitation with the HA antibody, top panel). The data demonstrate that cotransfection with p300 leads to Foxo3a acetylation (compare lanes 1′ and 2′), which is reversed by SIRT1 expression (compare lanes 3′–5′ with lane 2′). The blot of the HA immunoprecipitate was reprobed using the anti-HA antibody to demonstrate approximately equal presence of HA-Foxo3a (1′–5′, middle panel).

(B) Endogenous Foxo3a is acetylated in response to UV irradiation and oxidative stress. HeLa cells were maintained either as controls, irradiated with 200 μjoules/cm^2 UVC (UV), or treated with H_2O_2 (500 μm). Growth medium was replaced and cells were incubated for 2 hr after which the indicated deacetlylase inhibitors were added for a further hour. The endogenous Foxo3a was immunoprecipitated and Western blotted using antibodies directed against acetylated-lysine residues (top panel). The blot was reprobed for the total amount of Foxo3a (bottom panel). Acetylation was enhanced by nicotinamide (NIC) or TSA.

(C) SIRT1 deacetylates p300. HEK293T cells were transfected as indicated, lysed, immunoprecipitated with an anti-Ha antibody, and Western blotted using an anti-acetyl-lysine antibody (top panel). The data demonstrate that p300 is acetylated when expressed in HEK293T cells (compare lanes 1 and 3) and that this acetylation is a target for SIRT1 deacetylase activity (compare lane 3 with lanes 4–6). The HA-IP blot was reprobed for the HA epitope (middle panel) to demonstrate that approximately equal amounts of p300 were present in the immunoprecipitates. Lysates were immunoblotted to demonstrate equal amounts of SIRT1 or SIRT1dn (bottom panel).

deacetylated by SIRT1. In a reciprocal experiment, we immunoprecipitated the extracts with anti-acetyl-lysine and blotted with anti-HA (lanes 1′–5′, bottom). Again, this experiment showed that Foxo3a was acetylated by p300 and deacetylated by SIRT1.

We next addressed whether endogenous Foxo3a was acetylated upon UV or oxidative stress conditions, which should activate the protein to turn on target genes for stress management. The endogenous Foxo3a was immunoprecipitated from HeLa cells and blotted with the anti-acetyl-lysine or anti-Foxo3a antibodies. As shown in Figure 4B, both UV and H2O2 treatment trigger acetylation of Foxo3a. The levels of Foxo3a protein also appear higher in UV-treated cells (our preliminary results indicate that acetylated Foxo3a is more stable than unacetylated Foxo3a [not shown]). As a test of SIRT1 involvement in deacetylation of the endogenous Foxo3a, we treated these cells with the SIRT1 inhibitor, nicotinamide (NIC), and found a further enhancement of acetylation for both stressors. Treatment with the inhibitor of non-sirtuin histone deacetylases TSA also elicited greater acetylation of Foxo3a upon UV stress. These results parallel findings with stress-activated p53 using these inhibitors (Luo et al., 2001; Vaziri et al., 2001).

We also assessed whether SIRT1 would deacetylate p300. HEK293T cells were transfected with Foxo3a (Figure 4C, lanes 2–9), HA-p300 (lanes 3–9), SIRT1 (lanes 4–6), and SIRT1dn (lanes 7–9). Extracts were immunoprecipitated with anti-HA and blotted for HA (middle) or anti-acetylated-lysine (top). Increasing amounts of SIRT1 protein (lanes 4–6) or SIRT1dn (lanes 7–9) were present in extracts from SIRT1-transfected cells (bottom). Also, comparable amounts of p300 were precipitated from transfected cells (middle, lanes 3–9). p300 was clearly acetylated in p300 transfected cells (lane 3, top) and, strikingly, was deacetylated by SIRT1 (lanes 4–6) but to a much lesser degree by SIRT1dn (lanes 7–9). Thus, p300, like Foxo3a, is deacetylated by SIRT1.

We next determined whether the p300 acetylase and SIRT1 deacetylase influenced the ability of Foxo3a to activate transcription. HEK293T cells were transfected with the Foxo3a expression vector (Figure 5A, lanes 2–9) or a vector control (lane 1). Increasing amounts of the p300 expression vector were cotransfected without SIRT1 (lanes 2–5) or in addition to the SIRT1 vector (lanes 6–9). In extracts, SIRT1 expression was constant (lanes 6–9, middle), while slight increases in Foxo3a levels were observed with increasing levels of p300 (bottom panel). Cells used in lanes 1–9 above had also been cotransfected with a luciferase vector driven by the bim promoter, along with a β-galactosidase vector driven by a constitutively active SV40 promoter. Expression of luciferase and β-galactosidase were determined in all cases (Figure 5B). Compared to the β-galactosidase control, luciferase levels were elevated with increasing levels of p300 (Figure 5B, lanes 1–5). This finding indicates that p300 increases the activity of Foxo3a. Cotransfection with the SIRT1 vector damped this activation (Figure 5B, lanes 6–9), which shows that SIRT1 represses the ability of p300 to activate Foxo3a.

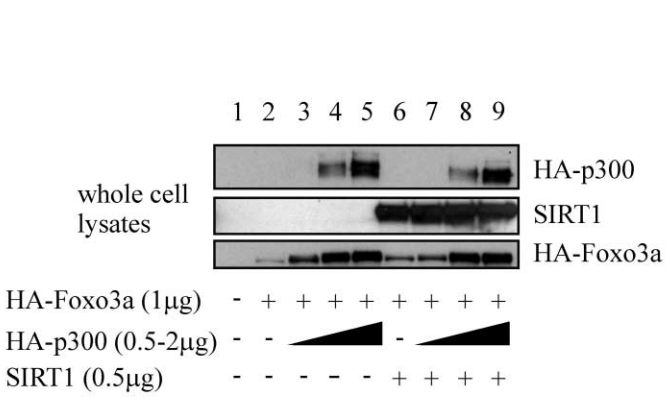
In a second test of the effect of SIRT1 and p300 on Foxo3a activity, we transfected HEK293T cells with Foxo3a (Figure 6, lanes 2–7) without (lane 2) or with (lanes 3–7) p300. We also cotransfected SIRT1 (lanes 4–5) or SIRT1dn (lanes 6–7). In this experiment, we monitored expression of the endogenous p27 gene by a Western blot for p27 protein (results were also confirmed by RT-PCR, not shown). The Foxo3a vector activated p27 levels (lane 2) compared to a vector control (lane 1). Cotransfection of p300 further stimulated p27 (lane 3). This activation by Foxo3a and p300 was completely eliminated by cotransfection of SIRT1 (lanes 4 and 5). In contrast, activation was not prevented by cotransfection of SIRT1dn (lane 6). These findings bolster the claim that p300 activates Foxo3a and SIRT1 prevents this. Note that the ability of p300 and SIRT1 to activate and repress Foxo3a correlates very well with their ability to acetylate and deacetylate the protein (Figure 4A). The activation of p27 appears partially blunted at the higher levels of SIRT1dn (lane 7). These findings may be an indication of residual deacetylase activity of the SIRTdn mutation. Alternatively, they may suggest a deacetylation-independent activity of SIRT1.

Expression of Foxo Target Genes in SIRT1 Knockout Mice

We next examined the effects of the SIRT1 knockout mutation on Foxo activity in embryonic stem (ES) cells and KO mice. ES cells were transfected with a luciferase reporter driven by Foxo DNA binding sites (Figure 7A). SIRT1$^{-/-}$ ES cells supported a higher level of luciferase activity than wild-type ES cells (lanes 1 and 2). To obtain further evidence that it was Foxo activity that we were measuring in these cells, we transfected them with Foxo3a and repeated the assays. Foxo3a gave higher levels of reporter activity in wild-type cells (lane 3), and this activity was again derepressed in SIRT1$^{-/-}$ cells (lane 4). Reintroduction of SIRT1 into the SIRT1$^{-/-}$ ES cells restored repression of the reporter (not shown). Thus, SIRT1 represses Foxo activity in ES cells.

In Figure 7B, we show two pairs of littermates of genotypes SIRT1$^{+/+}$ (lanes 1, 1′), +/− (lanes 3, 3′), and −/− (lanes 2, 2′ and 4, 4′). Liver and kidney tissues were harvested, RNA extracted, and probed for the forkhead target gene *PEPCK* by Northern blot. Expression was elevated in the liver (left panel) and kidney (right panel) of the −/− mice compared to the wild-type or +/− controls. In Figure 7C, a similar analysis was carried out for IGFBP1 mRNA, another known forkhead target, on livers from two pairs of littermates (AB and CD) with the indicated genotypes. Again, expression was higher in the −/− mice than in the wild-type littermates.

Finally, to demonstrate that SIRT1 is present at Foxo DNA sites in vivo, livers of +/+ and −/− mice were harvested and subject to chromatin immunoprecipitation, using SIRT1 or several control antibodies (Figure 7D). Primers bracketing a Foxo DNA site in the *IGFBP1* promoter were used to probe by PCR the DNA in the immunoprecipitates. p300 (lanes 2 and 7), Foxo1 (lanes 3 and 8), and Foxo3a (lanes 4 and 9) were bound to the DNA site in SIRT1$^{+/+}$ and $^{-/-}$ tissues. SIRT1 was bound at the Foxo sites in +/+ mice (lane 5) and, as expected, was absent in the knockout control (lane 10). The above studies in ES cells and knockout mice validate that SIRT1 negatively regulates forkhead-responsive genes in vivo.



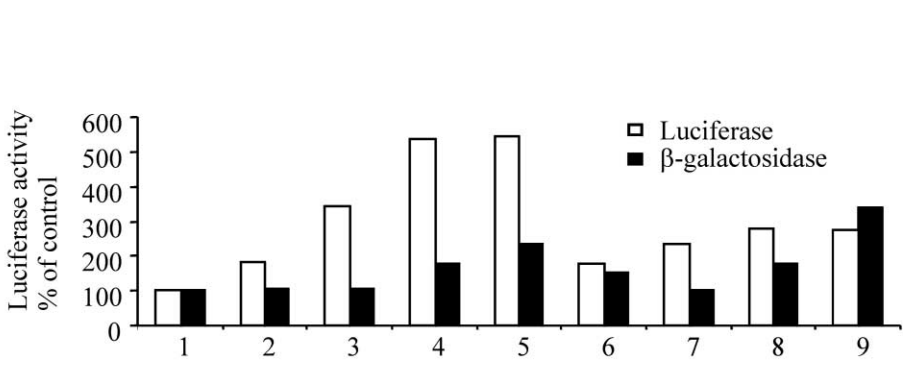


Figure 5. p300 Enhances Transcriptional Activity of Foxo3a while SIRT1 Represses the p300-Mediated Activation of Foxo3a

(A) HEK293T cells were transfected as indicated together with bim-driven luciferase and control β-galactosidase vectors. Cells were treated with wortmannin and LY294002 for the final 6 hr before being lysed in luciferase assay buffer (Promega). Cell lysates were immunoprobed using an anti-HA antibody (p300), anti-SIRT1, and anti-Foxo3a.

(B) Samples (1–9) from above were assayed for luciferase and β-galactosidase activities. The data clearly demonstrate that Foxo3a-dependent luciferase activity was induced by p300 compared to the β-galactosidase control and this induction was dramatically reduced in the presence of the deacetylase SIRT1.

Discussion

In this report, we investigate a possible connection between the mammalian SIR2 ortholog, SIRT1, and the forkhead transcription factor, Foxo3a. Several findings indicate that SIRT1 is a repressor of Foxo3a activity on target genes that are normally activated by this factor. First, SIRT1 binds to Foxo3a in mammalian cells. Second, SIRT1 represses the ability of Foxo3a to activate reporter constructs, as well as the endogenous target gene, p27. Third, SIRT1 deacetylates Foxo3a. Fourth, SIRT1 counteracts p300-mediated activation of Foxo3a. Fifth, ES cells in which SIRT1 is knocked out support a higher level of transactivation of a luciferase reporter driven by Foxo DNA binding sites. Sixth, SIRT1 KO mice display an increase in transcription of Foxo target genes, *IGFBP1* and *PEPCK*. Seventh, SIRT1 is present at the Foxo binding site within the promoter of at least one

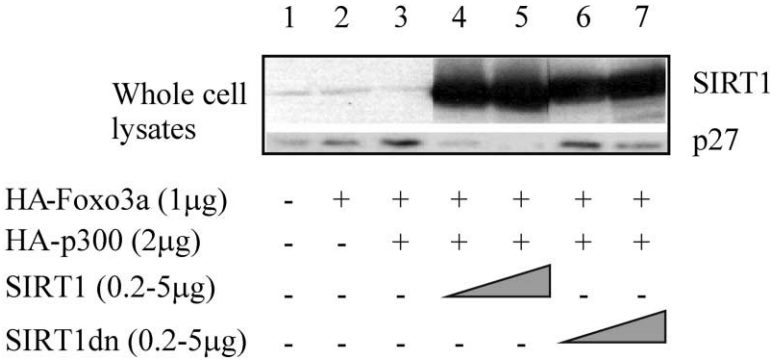


Figure 6. SIRT1 Inhibits Foxo3a Activation of p27

HEK293T cells were transfected as indicated and lysed 36 hr later. Total cell lysates were immunoprobed for the presence of SIRT1 (top panel) or for the Foxo3a target p27. The data show that Foxo3a induces endogenous p27, coexpression of p300 enhances p27 upregulation, and that this enhancement is inhibited by the expression of SIRT1. The inhibition by SIRT1 is at least partially dependent on its deacetylase activity as demonstrated by the lower efficiency of inhibition of p27 upregulation by the SIRT1dn.

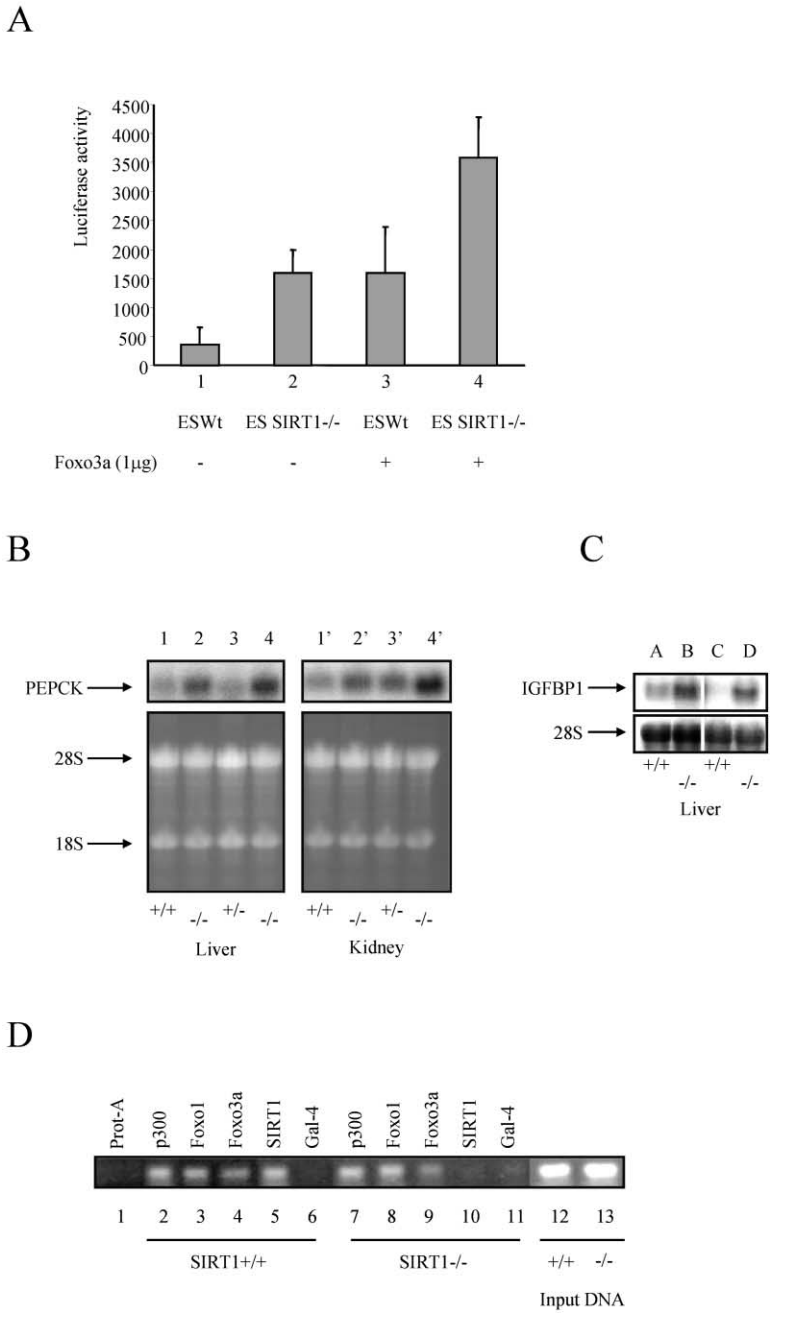


Figure 7. Foxo Activity Is Upregulated in SIRT1 Knockout ES cells and Mice

(A) Wild-type (ESWt) or SIRT1$^{-/-}$ (ES SIRT1$^{-/-}$) embryonic stem cells were transfected with 3 μg of pGL3-FHRE-luc reporter construct either without (−) or with (+) 1 μg of Foxo3a. Luciferase activity was determined using the dual luciferase kit (Promega), and the data were normalized to the activity of a cotransfected CMV renilla plasmid. The error bars represent the standard deviation of triplicate assays.

(B–D) Foxo target genes bind SIRT1 and are upregulated in SIRT1 knockout mice. (B) Northern blots of RNA from liver (lanes 1–4) and kidney (lanes 1′–4′) of mice of indicated genotypes were probed for PEPCK. Mice 1 and 2 were littermates, as were mice 3 and 4. The RNA-stained gel is shown as a loading control. (C) Northern blot of liver RNA from mice of indicated genotypes was probed for IGFBP1. Mice A and B were littermates, as were mice C and D. A negative image of 28S RNA is shown as a control. (D) Chromatin immunoprecipitates using the indicated antibodies were probed with primers flanking a Foxo DNA site in the *IGFBP1* promoter. Liver samples were derived either from SIRT1 wild-type (SIRT1$^{+/+}$. lanes 2–6) or SIRT1$^{-/-}$ (lanes 7–11) mice. In lane 1, protein A beads alone were used as a control. As can be seen, p300, Foxo1, Foxo3a antibodies precipitate the DNA site from both the wild-type and SIRT1$^{-/-}$ mouse, while SIRT1 antibodies precipitate the DNA in the wild-type but not the SIRT1$^{-/-}$ mouse (compare lanes 5 and 10). Gal-4 antibody (lanes 6 and 11) is used as a negative control. Lanes 12 and 13 represent the input DNA from SIRT1$^{+/+}$ mice and SIRT1$^{-/-}$ mice.

Foxo target gene in mouse tissue. We also show that activation of Foxo3a by UV or oxidative stress increases its acetylation status. SIRT1 may downregulate activated forkhead by destabilizing the protein, decreasing its DNA binding activity, or changing protein/protein interactions, as suggested for other transcription factors (Brooks and Gu, 2003). SIRT1 may also mediate repression at Foxo target genes by deacetylating histones at those loci.

While these studies have focused on the forkhead factor Foxo3a, our results also indicate that SIRT1 can repress Foxo1 and Foxo4, in the latter case mitigating Foxo-driven apoptosis. SIRT1 may thus generally downregulate genes that are normally activated by forkhead proteins. Previous data have suggested that p300 acts as a transcriptional coactivator for all three mammalian Foxo proteins and for *C. elegans* DAF-16 (Nasrin et al., 2000). Furthermore the interaction between p300 and Foxo and the level of Foxo acetylation decreased in response to growth factor activation (Mahmud et al., 2002). Since we show that SIRT1 also binds to and deacetylates p300, our data strengthen the claim that p300 is a coactivator of Foxo proteins.

Our findings link SIRT1 and forkhead, two of the regulators shown to control lifespan in lower organisms. Surprisingly, the regulation of Foxo3a by SIR2 in mammalian cells is of opposite sign from the apparent regulation in *C. elegans*. Why does the mammalian SIRT1 function to

repress forkhead? In many ways, Foxo seems to parallel p53 in its functions. Both may play a role in damage surveillance and oxidative stress responses. In the case of Foxo, activation triggers translocation from the cytoplasm to the nucleus (Rena et al., 2001; Cahill et al., 2001; Brunet et al., 2002; Tzivion et al., 2001; Henderson and Johnson, 2001; Lin et al., 2001; Lee et al., 2001). Both p53 and Foxo appear to interact with the coactivator p300 and both are acetylated in the course of their activation (Avantaggiati et al., 1997; Nasrin et al., 2000; Mahmud et al., 2002). Both can cause apoptosis, p53 in response to damage and Foxo in response to withdrawal of growth factors and when overexpressed (Brunet et al., 1999; Nakamura et al., 2000; Tang et al., 2002).

The repression of Foxo3a by SIRT1 appears to parallel the repression of p53. It is possible that downregulation of both of these factors raises the threshold for apoptosis and cell senescence (Luo et al., 2001; Langley et al., 2002) and mitigates the progressive erosion of organ systems with age. It is of interest that Akt/PKB$^{-/-}$ mice, which have elevated levels of activated forkhead proteins, are more sensitive to damage-induced apoptosis and genotoxic stress (Chen et al., 2001). This finding is consistent with the suggestion that restraint of forkhead activity may be important in the context of long-term survival.

SIR2 mediates the ability of a low calorie diet to increase the lifespan in the yeast model system (Lin et al., 2000, 2002). If this is also true in mammals, the regulation of p53 and Foxo proteins by SIRT1 may set the appropriate levels of these factors to achieve longevity under low calorie conditions (Koubova and Guarente, 2003). However, it would seem essential that repression of p53 and Foxo by SIRT1 be integrated with other activities of this sirtuin, if cancer is to be avoided and longevity is to be engendered. Calorie-restricted animals display a reduction in several hormones, including insulin and certain growth factors (Weindruch and Walford, 1988), changes that are expected to drive Foxo proteins to the nucleus. This effect may be compensated for by the downregulation of Foxo by SIRT1 in nuclei of hormone-responsive tissues as a mechanism to avoid excessive apoptosis. Such a reduction in hormonal levels and a compensatory desensitization of apoptosis may not result in any net increase in cancer and, in fact, may engender overall stress resistance.

Finally, the regulation of Foxo by SIRT1 gives this sirtuin a window into many forkhead-mediated metabolic changes in mammals (Tran et al., 2003; Kamei et al., 2004; Nadal et al., 2002; Nakae et al., 2002, 2003). SIRT1 may therefore play an important role in altering metabolism in response to diet, for example impinging on gluconeogenesis, insulin secretion/action, lipid usage, and ketogenesis, during calorie restriction. Tuning the metabolism and stress resistance to the diet may help engender the long life observed on a low calorie regimen.

Experimental Procedures

Plasmids and Antibodies

pECHA-Foxo3a and Foxo3aA3 (mutated in the three PKB phosphorylation sites T32, S253, and S315 all converted to alanines), DBE-luciferase (a luciferase reporter construct driven by synthetic Foxo DNA binding sites), pGL2-p27 ^{kip1}(luciferase reporter driven by the p27 promoter), bim-luc (luciferase reporter driven by the *BIM* promoter), and the plasmid encoding Foxo1A3 are gifts from Dr. R. Medema (NKI), Ha-p300 is a gift from R. Bernards (NKI); Fla- tagged SIRT1 (Luo et al.,2001); pGL2-3XIRS-luc and pGL3-FHRE-luc (gifts from M. Greenberg, Harvard). Antibodies used: anti-Flag antibody (Sigma M2, 1:5000 for Western blotting and 2 μl for immunoprecipitation), anti-Ha (clone 12CA5, 1:250 for Western blotting and 50 μl for immunoprecipitation), anti-acetyl-lysine (Cell Signaling, polyclonal and monoclonal antibodies, 1:1000 for Western blotting and 2 μl each for immunoprecipitation), anti-Foxo3a (Upstate 06-951, 1:1000), anti-SIRT1 (Upstate 07-131, 1:5000), anti-p27^{kip} (Transduction laboratories, 1:1000), anti-p300 (Santa Cruz, sc-585), anti-FKHR (Santa Cruz H-128), anti-GAL4 (Santa Cruz sc-729).

Cell Culture and Reporter Assays

HEK293T, HeLa, and U2OS cells were maintained in DMEM 10% fetal calf serum and routinely passaged at 60% confluence. HEK293T (100000/well) or HeLa cells (30000) were plated in 12 well plates and the indicated plasmids together with a Foxo3A-dependent reporter construct (0.1 μg in HEK293T cells and 0.5 μg in HeLa cells) were transfected using calcium phosphate (2.5 μg total DNA). To monitor transcriptional activation induced by wild-type Foxo3a, wortmannin (200 nM; Sigma) and LY294002 (10 μM; Sigma) were added for the final 6 hr to inhibit endogenous PKB activity. Ten millimolar nicotinamide (Sigma), to inhibit SIRT1 activity, was added for 12 hr before lysis. Luciferase activity measurements (Promega luciferase assay kit) were carried out using an automated Wallach 96 well microplate reader. Data was normalized by assessing β-galactosidase activity derived from cotransfection with a plasmid encoding β-galactosidase (0.1 μg in HEK293T cells and 0.5 μg in HeLa cells) driven by an SV40 promoter.

Assessment of Foxo4 activity was determined by transfection of the indicated constructs together with a reporter construct (pGL2-3XIRS-Luc) into U20S cells. Forty-eight hours later, the cells were lysed and luciferase activity was assessed.

Transfections into mouse embryonic stem cell were performed using GeneJuice (Novagen). Briefly, SIRT1$^{+/+}$ or SIRT12$^{-/-}$ mouse embryonic stem cells were seeded in 60 mm plates at a density of 500000/dish. Cells were cotransfected with 3 μg of the indicated luciferase reporter gene, 2 μg of a GFP expressing construct, and either 1 μg of WT Foxo3a or 1 μg of empty vector. Forty-eight hours posttransfection, cells were harvested in lysis buffer and luciferase activity was assayed according to the Promega protocol. GFP fluorescence was measured on a fluorometer and used to control for differences in transfection efficiency.

Immunoprecipitations and Western Blotting

HEK293T cells (300000) were plated in six well plates, transfected using calcium phosphate, and 24 hr later were lysed in 0.5 ml of lysis buffer (50 mM Tris pH 8.0, 50 mM KCl, 10 mM EDTA, 1%NP40) containing deacetylase inhibitors (10 mM Nicotinamide and 1 μM TSA), protease inhibitors (Roche EDTA free cocktail), and phosphatase inhibitors (20 mM NaF and 1 mM orthovanadate). Lysates were immunoprecipitated (12 hr), washed three times with 50 mM Tris (pH 7.5) (5 mM EDTA, 150 mM NaCl 0.1% tween 20), and were probed by Western blotting.

Apoptosis Analysis

H1229 cells were transfected as indicated together with GFP to mark cells that were transfected. After a further incubation of 48 hr, the cells were harvested, fixed, stained with propidium idodide, and analyzed by flow cytometry. The sub-G1 peak was used to assess the % of apoptotic cells.

Stress Treatments

Confluent HeLa cells were either maintained as controls, UVC irradiated (200 μJoules/cm^2), or treated with H_2O_2 (500 μm 2 hr later Nicotinamide (10 mM) or TSA (1 μM) was added for 1 hr and lysed using IP lysis buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 50 mM KCl, 0.2%NP40, 10% glycerol) containing protease inhibitors (Roche complete protease tablets), 10 mM nicotinamide, 1 μM TSA, 1 mM orthovanadate, and 20 mM NaF. Endogenous Foxo3a was immuno-

precipitated and Western blotted using anti-acetyl-lysine antibodies (Upstate, polyclonal and monoclonal, 1:1000 diluted in PBS tween 0.2%, 5% BSA).

Northern Blot Analysis

Total RNA was extracted from mice of the indicated SIRT1 genotypes (129/Sv CD1 mixed background) using guanidium thiocyanate, separated on 1.2% agarose, transferred to nitrocellulose, and hybridized according to standard procedures with ^{32}P-labeled probes specific for murine phosphoenolpyruvate carboxylase (*PEPCK*) or insulin-like growth factor binding protein 1 (*IGFBP1*).

Chromatin Immunoprecipitation

Assay was performed on 4-month-old females (129/Sv CD1 mixed background) as previously described (Wells et al., 2000; Weinmann et al. 2001) using a protocol for mouse tissues (http://mcardle.oncology.wisc.edu/farnham/protocols/tissues.html) with the following modifications: (1) formaldehyde crosslinking was for 1 hr; (2) protein A Sepharose was used; (3) preclear was for 1 hr; (4) centrifugation step (14000 rpm for 20 min) was included after incubation with primary antibody; (5) immunoprecipitates were washed two times with IP dilution buffer, two times with 0.1% SDS, 1% Triton X100, 2 mM EDTA, 20 mM TrisHCl, pH 8, 500 mM NaCl, two times with IP wash buffer, two times with TE 1X; (6) DNA complexes were eluted using 50 mM TrisHCl, pH 8, 1% SDS 10 mM EDTA; (7) after reversion of formaldehyde crosslink, DNA was recovered by diluting the samples with milli Q water to 0.5% SDS, 5 mM EDTA, 25 mM TrisHCl, 100 mM NaCl, treating with proteinase K (1 mg/ml for 2 hr at 37°C), extracting with phenol, chloroform followed by ethanol precipitation in the presence of 5 μg glycogen and Na acetate (300 mM). DNA was washed with 70% Ethanol, dried, resuspended in TE, and analyzed by PCR. Immunoprecipitations were performed with 1 μg of the indicated antibodies. IGFBP1 primers (available upon request) span the Foxo binding site (Tomizawa et al., 2000; Hall et al., 2000).

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References

Anderson, K.E., Coadwell, J., Stephens, L.R., and Hawkins, P.T. (1998). Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. Curr. Biol. *8*, 684–691.

Avantaggiati, M.L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A.S., and Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. Cell *89*, 1175–1184.

Barthel, A., Schmoll, D., Kruger, K.D., Bahrenberg, G., Walther, R., Roth, R.A., and Joost, H.G. (2001). Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells. Biochem. Biophys. Res. Commun. *285*, 897–902.

Bois, P.R., and Grosveld, G.C. (2003). FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. EMBO J. *22*, 1147–1157.

Brooks, C.L., and Gu, W. (2003). Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr. Opin. Cell Biol. *15*, 164–171.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J.V., Dalal, S.N., DeCaprio, J.A., Greenberg, M.E., and Yaffe, M.B. (2002). 14–3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. J. Cell Biol. *156*, 817–828.

Cahill, C.M., Tzivion, G., Nasrin, N., Ogg, S., Dore, J., Ruvkun, G., and Alexander-Bridges, M. (2001). Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14–3-3-dependent and 14–3-3-independent pathways. J. Biol. Chem. *276*, 13402–13410.

Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., et al. (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. *15*, 2203–2208.

Cheng, H.L., Mostoslavsky, R., Saito, S., Manis, J.P., Gu, Y., Patel, P., Bronson, R., Appella, E., Alt, F.W., and Chua, K.F. (2003). Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc. Natl. Acad. Sci. USA *100*, 10794–10799.

Daitoku, H., Yamagata, K., Matsuzaki, H., Hatta, M., and Fukamizu, A. (2003). Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. Diabetes *52*, 642–649.

Dijkers, P.F., Medema, R.H., Pals, C., Banerji, L., Thomas, N.S., Lam, E.W., Burgering, B.M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., and Coffer, P.J. (2000). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol. Cell. Biol. *20*, 9138–9148.

Frye, R.A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem. Biophys. Res. Commun. *260*, 273–279.

Fulco, M., Schiltz, R.L., Iezzi, S., King, M.T., Zhao, P., Kashiwaya, Y., Hoffman, E., Veech, R.L., and Sartorelli, V. (2003). Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. Mol. Cell *12*, 51–62.

Furukawa-Hibi, Y., Yoshida-Araki, K., Ohta, T., Ikeda, K., and Motoyama, N. (2002). FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. J. Biol. Chem. *277*, 26729–26732.

Guarente, L. (2000). Sir2 links chromatin silencing, metabolism, and aging. Genes Dev. *14*, 1021–1026.

Hall, R.K., Yamasaki, T., Kucera, T., Waltner-Law, M., O'Brien, R., and Granner, D.K. (2000). Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin. The role of winged helix/forkhead proteins. J. Biol. Chem. *275*, 30169–30175.

Henderson, S.T., and Johnson, T.E. (2001). daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr. Biol. *11*, 1975–1980.

Hribal, M.L., Nakae, J., Kitamura, T., Shutter, J.R., and Accili, D. (2003). Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. J. Cell Biol. *162*, 535–541.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature *403*, 795–800.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. *13*, 2570–2580.

Kamei, Y., Mizukami, J., Miura, S., Suzuki, M., Takahashi, N., Kawada, T., Taniguchi, T., and Ezaki, O. (2004). A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. J. Lipid Res. *1*, 81–88.

Kennedy, B.K., Gotta, M., Sinclair, D.A., Mills, K., McNabb, D.S., Murthy, M., Pak, S.M., Laroche, T., Gasser, S.M., and Guarente, L. (1997). Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in S. cerevisiae. Cell *89*, 381–391.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R.

(1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461–464.

Kimura, K.D., Tissenbaum, H.A., Liu, Y., and Ruvkun, G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science *277*, 942–946.

Kops, G.J., Dansen, T.B., Polderman, P.E., Saarloos, I., Wirtz, K.W., Coffer, P.J., Huang, T.T., Bos, J.L., Medema, R.H., and Burgering, B.M. (2002a). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature *419*, 316–321.

Kops, G.J., Medema, R.H., Glassford, J., Essers, M.A., Dijkers, P.F., Coffer, P.J., Lam, E.W., and Burgering, B.M. (2002b). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell. Biol. *22*, 2025–2036.

Koubova, J., and Guarente, L. (2003). How does calorie restriction work? Genes Dev. *17*, 313–321.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. USA *97*, 5807–5811.

Langley, E., Pearson, M., Faretta, M., Bauer, U.M., Frye, R.A., Minucci, S., Pelicci, P.G., and Kouzarides, T. (2002). Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. EMBO J. *21*, 2383–2396.

Lee, R.Y., Hench, J., and Ruvkun, G. (2001). Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. Curr. Biol. *11*, 1950–1957.

Lin, K., Dorman, J.B., Rodan, A., and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science *278*, 1319–1322.

Lin, S.J., Defossez, P.A., and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science *289*, 2126–2128.

Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. *28*, 139–145.

Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturtz, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R., and Guarente, L. (2002). Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature *418*, 344–348.

Lin, S.J., Ford, E., Haigis, M., Liszt, G., and Guarente, L. (2004). Calorie restriction extends yeast life span by lowering the level of NADH. Genes Dev. *18*, 12–16.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137–148.

Mahmud, D.L., Amlak, M., Deb, D.K., Platanias, L.C., Uddin, S., and Wickrema, A. (2002). Phosphorylation of forkhead transcription factors by erythropoietin and stem cell factor prevents acetylation and their interaction with coactivator p300 in erythroid progenitor cells. Oncogene *21*, 1556–1562.

McBurney, M.W., Yang, X., Jardine, K., Hixon, M., Boekelheide, K., Webb, J.R., Lansdorp, P.M., and Lemieux, M. (2003). The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol. Cell. Biol. *23*, 38–54.

Medema, R.H., Kops, G.J., Bos, J.L., and Burgering, B.M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature *404*, 782–787.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature *424*, 277–283.

Nadal, A., Marrero, P.F., and Haro, D. (2002). Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1. Biochem. J. *366*, 289–297.

Nakae, J., Kitamura, T., Silver, D.L., and Accili, D. (2001). The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J. Clin. Invest. *108*, 1359–1367.

Nakae, J., Biggs, W.H., III, Kitamura, T., Cavenee, W.K., Wright, C.V., Arden, K.C., and Accili, D. (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat. Genet. *32*, 245–253.

Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W.H., III, Arden, K.C., and Accili, D. (2003). The forkhead transcription factor Foxo1 regulates adipocyte differentiation. Dev. Cell *4*, 119–129.

Nakamura, N., Ramaswamy, S., Vazquez, F., Signoretti, S., Loda, M., and Sellers, W.R. (2000). Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. Mol. Cell. Biol. *20*, 8969–8982.

Nasrin, N., Ogg, S., Cahill, C.M., Biggs, W., Nui, S., Dore, J., Calvo, D., Shi, Y., Ruvkun, G., and Alexander-Bridges, M.C. (2000). DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. Proc. Natl. Acad. Sci. USA *97*, 10412–10417.

Nemoto, S., and Finkel, T. (2002). Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. Science *295*, 2450–2452.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994–999.

Ramaswamy, S., Nakamura, N., Sansal, I., Bergeron, L., and Sellers, W.R. (2002). A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer Cell *2*, 81–91.

Rena, G., Prescott, A.R., Guo, S., Cohen, P., and Unterman, T.G. (2001). Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14–3-3 binding, transactivation and nuclear targetting. Biochem. J. *354*, 605–612.

Shore, D. (2000). The Sir2 protein family: A novel deacetylase for gene silencing and more. Proc. Natl. Acad. Sci. USA *97*, 14030–14032.

Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA circles–a cause of aging in yeast. Cell *91*, 1033–1042.

Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C., and Boeke, J.D. (2000). A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA *97*, 6658–6663.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., McCormick, F., Tempst, P., et al. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science *279*, 710—714.

Tang, T.T., Dowbenko, D., Jackson, A., Toney, L., Lewin, D.A., Dent, A.L., and Lasky, L.A. (2002). The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J. Biol. Chem. *277*, 14255–14265.

Tissenbaum, H.A., and Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature *410*, 227–230.

Tomizawa, M., Kumar, A., Perrot, V., Nakae, J., Accili, D., Rechler, M.M., and Kumaro, A. (2000). Insulin inhibits the activation of transcription by a C-terminal fragment of the forkhead transcription factor FKHR. A mechanism for insulin inhibition of insulin-like growth factor-binding protein-1 transcription. J. Biol. Chem. *275*, 7289–7295.

Tran, H., Brunet, A., Grenier, J.M., Datta, S.R., Fornace, A.J., Jr., DiStefano, P.S., Chiang, L.W., and Greenberg, M.E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science *296*, 530–534.

Tran, H., Brunet, A., Griffith, E.C., and Greenberg, M.E. (2003). The many forks in FOXO's road. Sci. STKE. *172*, RE5.

Tzivion, G., Shen, Y.H., and Zhu, J. (2001). 14–3-3 proteins; bringing new definitions to scaffolding. Oncogene *20*, 6331–6338.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita,

T.K., Guarente, L., and Weinberg, R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149–159.

Weindruch, R., and Walford, R.L. (1988). The retardation of aging and disease by dietary restriction. (Springfield, IL: Thomas press).

Weinmann, A.S., Bartley, S.M., Zhang, T., Zhang, M.Q., and Farnham, P.J. (2001). Use of chromatin immunoprecipitation to clone novel E2F target promoters. Mol. Cell. Biol. *20*, 6820–6832.

Wells, J., Boyd, K.E., Fry, C.J., Bartley, S.M., and Farnham, P.J. (2000). Target gene specificity of E2F and pocket protein family members in living cells. Mol. Cell. Biol. *20*, 5797–5807.