Glucogenetic Genes*

Nuclear Trapping of the Forkhead Transcription Factor FoxO1 via Sirt-dependent Deacetylation Promotes Expression of Glucogenetic Genes*

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Activation of NAD-dependent deacetylases, or Sirtuins, prolongs life span and mimics the effects of caloric restriction in yeast. The FoxO subfamily of forkhead transcription factors has been shown to mediate some of the effects of Sirtuins. Here we have shown that Sirtuin activation or hydrogen peroxide treatment overrides the phosphorylation-dependent nuclear exclusion of FoxO1 caused by growth factors and causes nuclear translocation of FoxO1 in hepatocytes. Genetic measurements of nuclear fluorescence recovery after photobleaching show that FoxO1 is readily diffusible within the nucleus under normal conditions but becomes restricted within a nuclear subdomain following treatment with the prototypical Sirtuin agonist resveratrol or oxidative stress. Expression of FoxO1 target genes is accordingly increased, leading to activation of gluconeogenesis and increased glucose release from hepatocytes. Selective modulation of the FoxO1/Sirtuin interaction represents a promising therapeutic modality for metabolic disorders.

The long-standing observation that caloric restriction is associated with longevity has led to a widely held theory that metabolism and life span share common cellular pathways (1, 2). One such pathway has been proposed to involve forkhead transcription factors of the FoxO subfamily (3–5). Genetic epistasis in Caenorhabditis elegans and metabolic studies in mice indicate that FoxO genes regulate cell differentiation, transformation, and metabolism (6). In C. elegans, mutations of the FoxO ortholog Daf16 rescue the dauer state caused by mutations of the insulin/insulin-like growth factor receptor ortholog Daf2 (7, 8). Moreover, extra copies of the gene encoding the NAD-dependent deacetylase Silent Information Regulator (Sir) 2.1 prolong life span in a Daf16-dependent fashion (9), suggesting that FoxO activity is regulated via deacetylation. These twin observations provide the underpinning for investigations of the role of FoxO proteins in mammalian metabolism and life span.

FoxO activity is subject to complex regulation by growth factors and cellular stress. The former inhibit FoxO via serine/threonine phosphorylation and nuclear exclusion (10). The latter causes FoxO acetylation, thus promoting the interaction between FoxO and SirT1, the mammalian ortholog of Sir2.1 (4, 5, 11, 12). However, the effect of SirT1-dependent deacetylation on FoxO function remains somewhat controversial, with most (4, 5, 12), but not all (11), studies suggesting that deacetylation increases FoxO-dependent transcription.

In this study, we sought to uncover the mechanism by which stress-induced deacetylation affects FoxO activity. To this end, we studied FoxO translocation using live cell imaging, coupled to measurements of protein kinetics with fluorescence recovery after photobleaching (FRAP)† and fluorescence loss in photobleaching (FLIP) experiments (13). We also measured expression of FoxO1 target genes and glucose production in hepatocyte cultures. Our findings are consistent with a model in which deacetylation promotes FoxO nuclear retention and increases FoxO-dependent glucose production, thus providing evidence for a mechanism to regulate FoxO activity via subnuclear targeting.

EXPERIMENTAL PROCEDURES

DNA Constructs and Cell Transfection—We cloned a mouse FoxO1 cDNA into pEGFP-N1 (Clontech Laboratories) to generate FoxO1-EGFP. We used FuGENE 6 (2.5 μg/ml DNA) (Roche Applied Science) to obtain transiently transfected cells expressing FoxO1-EGFP. We have described the adenosine vector expressing HA-tagged wild type FoxO1 in previous publications (14). We transfected cells with adenosine vectors 24 h before the experiment.

Cell Culture—We cultured Sv40 hepatocytes or H4IIE rat hepatoma cells in α-minimal essential medium supplemented with 4% fetal calf serum, 1% Pen-Strep, and 200 ng/ml dexamethasone at 37 °C in 5% CO2 incubator. We lysed cells in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% Na orthovanadate, 10 mM NaF, 1 mM dithiothreitol, 2 mg/ml pepstatin, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 mM nicotinamide, and 2 μM trichostatin A (TsA). Immunoprecipitation was carried out using anti-FOXO1 (Santa Cruz), Sirt1 (Upstate Biotechnology), phospho-Ser253-Akt, or acetyl lysine (Cell Signaling). We employed the following concentrations of reagents: H2O2 0.5 mM, insulin 100 nM, resveratrol 10 μM, nicotinamide 10 mM, and Trichostatin A 2 μM. All were from Sigma. We used Leptomycin B (LC Laboratories) at 20 nM. We cultured HER 293 cells in minimal essential medium, 10% fetal calf serum, 1% Pen-Strep and transduced them with an adenosine vector containing HA-tagged wild type FoxO1 24 h before the experiment (14).

Image Acquisition and Analysis—We plated and observed cells

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† The abbreviations used are: FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching; GFP, green fluorescent protein; HA, hemagglutinin; PBS, phosphate-buffered saline; NAM, nicotinamide; HDAC, histone deacetylase; TsA, trichostatin A.
LabTek chambers (Nalgene). We captured confocal microscope images on a LSM 510 META microscope (Zeiss) using 488 nm laser excitation for GFP and 635 nm for rhodamine. Images were captured with a ×63 1.2 water objective and an open pinhole to collect fluorescence from the entire depth of the cell. We analyzed images with the Image J software (NIH). We measured fluorescence separately within the nucleus and the cytoplasm. To calculate relative nuclear fluorescence, we divided nuclear fluorescence by the total amount of cellular fluorescence. Time lapse sequences of images of FoxO1-GFP nuclear translocation were captured with a ×63 1.2 water objective at 10-s intervals (15). All quantitative values represent averages from at least 20 cells from five independent experiments.

Immunofluorescence Microscopy—We prepared cells for immunofluorescence microscopy by transfecting FoxO1-GFP by FuGENE 6 (Roche Applied Science) and staining for Sirt1 in LabTek chambered slides (Nalgene). We fixed cells in 4% paraformaldehyde in PBS for 15 min, washed using PBS, and treated them with 0.1 mM Tris, pH 8.1, for 10 min. Thereafter, we permeabilized cell membranes with PBS and 0.2% Triton X-100, washed in PBS, and incubated in blocking buffer containing 1% bovine serum albumin for 10 min. Subsequently, we incubated cells with Sirt1 antisera (Cell Signaling) in PBS containing 1% bovine serum albumin for 1 h at room temperature, washed in PBS, and incubated with a rhodamine-conjugated secondary antibody for 1 h at room temperature. Slides were mounted using Fluoromount-G (Southern Biotech).

FRAP Analysis—We plated, transfected, and observed cells in LabTek II chambers (Nalgene). We performed selective photobleaching of the nucleus on a LSM 510 META microscope (Zeiss) using 488 nm laser excitation for GFP and a ×63 1.2 water objective. We photobleached a defined region (outlined in the figures) at full laser power (100% power for 15 s). Thereafter, we monitored fluorescence recovery by scanning the whole cell at low laser power (4% power every 7.5 s). We observed no photobleaching during recovery. For FLIP experiments, we repeatedly imaged and bleached cells at 20-s intervals. Bleaching and imaging settings were identical to the ones used in FRAP. We generated FRAP recovery curves from background-subtracted images. We determined the total cell fluorescence for each image using Image J software (NIH) and compared the percentage of nuclear fluorescence to the initial value to account for the amount lost during the bleach pulse (15). All quantitative values represent averages from at least 10 cells from five independent experiments.

Gene Expression Studies—We isolated RNA using RNeasy mini-kit (Qiagen), followed by DNase (Qiagen) digestion to remove contaminating DNA. We carried out first strand synthesis with the SuperScript (Qiagen), followed by DNase (Qiagen) digestion to remove contaminating DNA. We carried out real-time quantitative PCR using a DNA Engine Opticon 2 System instrument (MJ Research) and DyNAamo SYBR Green quantitative PCR kit (Finnzymes). Reactions were carried out in triplicate, using β-actin or Hprt to normalize mRNA content (16). Primer sequences are available upon request. We employed standard PCR conditions.

Measurement of Glucose Release from H4IIE Hepatoma Cells—To measure glucose production, we removed the growth medium, washed cells with PBS three times, and incubated with glucose- and serum-free α-minimal essential medium for 1 h. We then washed with PBS and incubated cells in glucose- and serum-free α-minimal essential medium supplemented with 200 nM dexamethasone, 20 μM forskolin, 2 mM glutamine, 16 mM lactate, and 4 mM pyruvate (Sigma) (17). We measured glucose concentration in triplicate using the Amplex red glucose assay (Invitrogen). Results are the mean of two independent experiments performed in triplicate.

RESULTS

Hydrogen Peroxide Treatment Affects FoxO Shuttling—We first determined the cellular distribution of FoxO1 using a FoxO1-GFP fusion protein in hepatocytes. FoxO1-GFP localized primarily to the cytoplasm (70% cytoplasmic and 30% nuclear) in the presence of serum, whereas in the absence of serum the cellular distribution is reversed (70% nuclear and 30% cytoplasmic) (Fig. 1, A and B). These data show that the GFP tag does not affect FoxO distribution. Treatment of cells with H2O2 to cause oxidative stress caused up to 70% of FoxO1-GFP to translocate from the cytoplasm to the nucleus, regardless of the presence of serum (Fig. 1, A and B). Time course analysis indicated that FoxO1-GFP began to localize to the nucleus after ~45 min in H2O2-treated cells and achieved steady-state levels (nuclear percentage levels above 70%) at 120 min, with a t1/2 = 80 min (Fig. 1C). We obtained identical results in Cos7 cells (Fig. 1D). The changes in FoxO1 distribution could not be accounted for by perturbations to the fluorophor, as the localization of GFP expressed in the same cells was unaffected by oxidative stress (Fig. 1E). The delayed nuclear translocation of FoxO1-GFP during peroxide treatment may be caused by a transient increase in Akt activity (data not shown).

FIG. 1. Kinetic analysis of FoxO1-GFP translocation in response to serum, H2O2, and insulin. Quantitative (A) and qualitative (B) analysis of FoxO1-GFP localization in SV40-transformed hepatocytes treated with H2O2 (0.5 mM) for 2 h in the presence or absence of serum or with insulin (100 nM for 2 h). C, kinetics of FoxO1-GFP translocation following H2O2 treatment. D, qualitative analysis of FoxO1-GFP translocation in Cos7 cells treated with H2O2 (0.5 mM), insulin (100 nM), resveratrol (10 μM), or NAM (10 mM) and TαA (2 μM) for 2 h. E, qualitative analysis of GFP translocation in SV40-transformed hepatocytes treated with H2O2 (0.5 mM) for 2 h. F, insulin-induced Ser253 phosphorylation of FoxO1 in the presence and absence of H2O2. We transduced cells with adenovirus encoding HA-tagged FoxO1 and performed experiments under the conditions indicated.
FIG. 2. Sirtuin-mediated deacetylation regulates FoxO1 localization during H2O2 treatment. Quantitative (A) and qualitative (B) analysis of FoxO1-GFP localization in SV40-transformed hepatocytes treated with H2O2 (0.5 mM), resveratrol (Res, 10 μM), nicotinamide (NAM, 10 mM) and Trichostatin A (TsA, 2 mM). All treatments were carried out for 2 h. C, co-immunoprecipitation of FoxO1 and Sirt1 in H2O2-treated cells. We transduced SV40-transformed hepatocytes with adenovirus encoding HA-tagged FoxO1 and immunoprecipitated cell extracts with anti-HA antiserum. The immunoprecipitated proteins were then immunoblotted with either anti-Sirt1 (upper panel) or anti-FoxO1 antiserum (lower panel). D, FoxO1-GFP (green fluorescence) colocalizes with Sirt1 (red fluorescence) in cells treated with H2O2 or resveratrol. E, FoxO acetylation. We transduced SV40-transformed hepatocytes with adenovirus encoding HA-tagged FoxO1 and immunoprecipitated cell extracts with anti-HA antiserum. The immunoprecipitated proteins were then immunoblotted with either anti-acetyl-Lysine (AcK, upper panel) or anti-FoxO1 antiserum (lower panel).
This may account for the apparent discrepancy between reports of FoxO phosphorylation (18) and nuclear translocation (5) in response to H$_2$O$_2$. To further clarify this point, we measured whether insulin treatment could override the effect of H$_2$O$_2$. Addition of insulin promoted FoxO1-GFP localization to the cytoplasm (Fig. 1, A and B) with a rapid time course ($t_{1/2}$ = 13 min, data not shown) but was unable to do so in the presence of H$_2$O$_2$ (Fig. 1, A and B). Consistent with these results, we observed that insulin-induced FoxO phosphorylation was abolished in H$_2$O$_2$-treated cells (Fig. 1F).

**FoxO Acetylation and Nuclear Shutting**—To determine the mechanism by which FoxO1 translocates to the nucleus during oxidative stress, we tested the effect of agents that affect FoxO1 acetylation. The small molecule activator of NAD-dependent deacetylases (Sirtuins) (19), resveratrol (20), caused FoxO1-GFP translocation to the nucleus (Fig. 2, A and B) with a $t_{1/2}$ = 15 min (data not shown), suggesting that the interaction of FoxO1 with Sirt1 contributes to its nuclear distribution. Indeed, co-immunoprecipitation experiments indicated that Sirt1 could be detected in FoxO1 immunopellets following H$_2$O$_2$ treatment (Fig. 2C, *middle lane*). Conversely, treatment with the Sirtuin inhibitor nicotinamide (NAM) and the class I/II histone deacetylase (HDAC) inhibitor TsA prevented Sirt1 binding to FoxO1 (Fig. 2C, *right lane*). Furthermore, immunohistochemical studies showed that FoxO1-GFP colocalized with Sirt1 in the nucleus during H$_2$O$_2$ treatment (Fig. 2D). Combined treatment with resveratrol and H$_2$O$_2$ also caused nuclear translocation (Fig. 2, A and B). To determine whether the effect of oxidative stress on FoxO1 is mediated solely through Sirtuins or also through class I/II HDACs, we compared the effects of NAM and TsA on FoxO1-GFP translocation. Treatment with either TsA or NAM failed to affect FoxO1-GFP localization. TsA had no effect on H$_2$O$_2$-dependent nuclear accumulation of FoxO1, whereas NAM completely reversed the effect of H$_2$O$_2$ (Fig. 2, A and B). In addition, prior NAM treatment prevented FoxO1-GFP nuclear translocation in response to H$_2$O$_2$ (data not shown). Moreover, NAM was able to prevent resveratrol-induced nuclear localization of FoxO1-GFP, whereas TsA treatment did not affect the response to resveratrol (Fig. 2, A and B). As Sirtuins have been shown to deacetylate FoxO (4, 5, 11), we sought to determine the acetylation status of FoxO1 under different conditions. Treatment with NAM alone for 2 h increased FoxO1 acetylation, whereas incubation with insulin, H$_2$O$_2$, or resveratrol had no effect (Fig. 2E). Unlike previous data showing promiscuous FoxO deacetylation by Sirtuins and HDACs (4, 5, 11), these findings are consistent with the possibility that Sirtuins are the specific FoxO deacetylases in hepatocytes.

**Sirtuin-mediated Deacetylation Perturbs FoxO1 Nucleocytoplasmic Shuttling**—We measured the shuttling properties of FoxO1 using FRAP. In these experiments, an area of the cell is photobleached with a high intensity laser pulse and the movement of unbleached molecules from neighboring areas into the bleached area is recorded by time lapse microscopy (13). Fluorescence recovery depends on the amount of time required for total cell fluorescence to achieve steady-state distribution between different cellular compartments following the photobleach. We selectively photobleached the nuclear FoxO1-GFP pool and monitored the recovery from the cytoplasmic pool. In the presence of serum, nuclear FoxO1-GFP fluorescence recovered its prebleaching levels within 200 s (Fig. 3, A and B, *squares*). Importantly, after selective bleaching of the nuclear pool, cytoplasmic FoxO1-GFP fluorescence decreased as nuclear fluorescence increased. We then performed FRAP experiments in serum-deprived cells. Within 350 s of selectively photobleaching the nuclear FoxO1-GFP pool, the nucleus completely recovered the prebleaching fluorescence levels (Fig. 3, A and B, *triangles*), whereas the cytoplasmic pool of FoxO1-GFP fluorescence decreased. These data show that FoxO1-GFP is mobile between the two compartments and shuttles constitutively between the nucleus and the cytoplasm independently of serum. The main difference between the two conditions is that the rate of nucleocytoplasmic shuttling is slower in serum-deprived cells ($t_{1/2}$ = 350 versus 200 s), probably reflecting reduced phosphorylation-dependent nuclear export.

Next, we measured the mobility of FoxO1-GFP in resveratrol-treated cells. In the presence of resveratrol, the nucleus regained only 40% of total cellular FoxO1-GFP fluorescence observed prior to photobleaching (Fig. 3, A and B, *diamonds*), whereas 60% of FoxO1-GFP was found in the nucleus. Similarly, FRAP experiments in H$_2$O$_2$-treated cells indicated that the nucleus regained only 35% of total prebleaching fluorescence after 350 s (Fig. 3, A and B, *circles*). Although this 35% continued to shuttle between the nucleus and cytoplasm, 65% of FoxO1-GFP appeared to be sequestered in the nucleus. The decrease in the mobile fraction of FoxO1-GFP in resveratrol- and H$_2$O$_2$-treated cells suggests that FoxO1-GFP is actively retained within the nucleus.

Because NAM prevented nuclear accumulation of FoxO1-GFP in H$_2$O$_2$-treated cells, we tested whether it could restore FoxO1-GFP in the presence of H$_2$O$_2$ or resveratrol. FRAP analysis of cells treated with H$_2$O$_2$ or resveratrol showed that NAM, but not TsA (Fig. 4A), was able to restore nuclear fluorescence to prebleaching levels. This indicated that FoxO1-GFP is mobile between the nucleus and cytoplasm when Sirtuins, but not when class I/II HDACs, are inhibited. As a control to rule out interference from the fluorophor during oxidative stress on FoxO1-GFP kinetics, we performed FRAP...
experiments in cells transfected with GFP and treated with H₂O₂. Within 80 s of selectively photobleaching the nuclear pool of GFP, the nucleus recovered the prebleaching fluorescence levels (data not shown). These data indicate that GFP is freely mobile between the nucleus and the cytoplasm and that H₂O₂ does not obstruct GFP kinetics.

Measurements of FoxO Kinetics using FLIP—Because only a small percentage of FoxO1-GFP fluorescence could be detected in the nucleus after FRAP experiments in H₂O₂- and resveratrol-treated cells, we conducted FLIP experiments (13) to test whether FoxO1-GFP is bound to the nuclear compartment. By repeatedly photobleaching fluorescence in a selected area of the cell and monitoring the fluorescence in the non-photobleached regions, the mobility of a fluorescently tagged protein can be readily observed and the continuity of cellular environments determined (21). The rate of fluorescence loss is dependent on the mobility of the protein. As such, proteins that do not move between compartments show no loss (13). We repeatedly photobleached a portion of the cytoplasm in H₂O₂-treated cells and captured images between pulses. After 500 s, all cytoplasmic FoxO1-GFP fluorescence was lost (Fig. 4B). The repeated bleach pulses did not affect the majority of nuclear FoxO1-GFP fluorescence, suggesting that a large percentage of FoxO1-GFP is bound to the nuclear compartment and does not shuttle during oxidative stress. An identical FLIP experiment was conducted in cells treated with resveratrol. Similar to H₂O₂, a majority of nuclear FoxO1-GFP fluorescence was retained after the cytoplasmic signal was lost (Fig. 4B). We conclude that Sirtuin-deacetylated FoxO1 does not shuttle between the nucleus and cytoplasm but remains bound to the nuclear compartment. It appears that acetylation is a prerequisite for FoxO1 mobility between the two compartments.

Sirtuin Activation and Hepatic Glucose Metabolism—Regulation of hepatic glucose production is an important physiologic function of FoxO1 (14, 24, 25). To correlate the changes in FoxO1 distribution with its biologic effects, we measured glucose production in H4IIE rat hepatoma cells following resveratrol or insulin treatment. Incubation of cells transduced with

**Fig. 4.** Effects of HDAC and Sirtuin inhibitors on FoxO localization. A, qualitative FRAP analysis of FoxO1-GFP in cells under various conditions. Concentrations of H₂O₂, Res, NAM, and TsA are identical to those in Fig. 1. We obtained images before photobleaching and at the indicated time points thereafter. The photobleached area is outlined. We plotted fluorescence intensities of nuclear FoxO1-GFP as a function of time under various conditions and normalized them to prebleach values. B, a portion of the cytoplasm was photobleached repeatedly in cells expressing FoxO1-GFP in the presence of H₂O₂ or resveratrol. Between each pulse, the entire field of view was imaged at low laser power to determine the extent of fluorescence outside the bleached region as a result of photobleaching within the region.

**Fig. 5.** FoxO1 is immobile in the nucleus after H₂O₂ and resveratrol treatment. Qualitative nuclear FRAP analysis of FoxO1-GFP in cells treated with H₂O₂ (0.5 mM), Res (10 μM), or Leptomycin-B (20 nM). Images were obtained before photobleaching and at the indicated time points thereafter. Photobleached areas are outlined by rectangles.
wild type FoxO1 adenovirus in glucose-free medium supplemented with the glucogenetic precursors lactate and pyruvate. Basal conditions are indicated by a solid line with diamonds, insulin treatment with squares, and resveratrol treatment with triangles. Similar experiments were conducted in cells transduced with a phosphorylation defective, constitutively nuclear FoxO1 (ADA-FoxO1) (B) and with a dominant-negative FoxO1 lacking the transactivation domain (Δ256-FoxO1) (C). Effects of wild type, ADA, and Δ256 FoxO1 on the expression of glucose-6-phosphatase (G6pc) (D), phosphoenolpyruvate carboxykinase (Pck1) (E), and insulin-like growth factor-binding protein 1 (Igfbp1) (F) in the absence or presence of resveratrol. All experiments were performed at least twice, and determinations of glucose production and gene expression were carried out in triplicate.

**DISCUSSION**

The present studies provide evidence for a deacetylation-based mechanism by which FoxO1 becomes tightly associated with a nuclear subdomain. This association with the nuclear compartment increases FoxO1-dependent transcription and is reflected in increased glucose release from cultured hepatoma cells. The main finding of this work is that Sirtuin activation by resveratrol or oxidative stress renders FoxO1 immobile within the nuclear compartment and promotes FoxO1-dependent transcription of genes important for hepatic glucose production. As Sirtuins have been shown to deacetylate FoxO proteins (4, 5, 11), we conclude that deacetylation targets FoxO for nuclear retention. The ability of oxidative stress, as well as resveratrol, to increase transcription mediated by a constitutively nuclear, phosphorylation-defective FoxO1 mutant (ADA) should be emphasized. These findings provide evidence that nuclear localization per se is necessary, but not sufficient, for full transcriptional activation by FoxO1 and are consistent with the nuclear FRAP experiments, indicating that FoxO1 is freely mobile within the nucleus under basal conditions but becomes restricted in resveratrol- or H2O2-treated cells. Although we (26) and others (27–30) have considered the phosphorylation-defective mutant FoxO as constitutively active, these data suggest otherwise. We therefore propose that the
definition of “constitutively nuclear” be adopted for this mutant.

The effect of resveratrol could be mimicked in our experimental system by H$_2$O$_2$ treatment, a reagent that is commonly employed to induce oxidative stress by increasing intracellular concentrations of reactive oxygen species. It should be emphasized that the effects of H$_2$O$_2$ in cultured cells are complex. At early time points, H$_2$O$_2$ is known to have insulin-mimetic effects (31), which include increased Akt activity and FoxO1 nuclear exclusion (18). With prolonged incubation, there is down-regulation of insulin signaling and FoxO1 localization becomes predominantly nuclear (5).

Previous studies have dealt with the effects of deacetylation on FoxO1-dependent transcription. Most (4, 32), but not all (11), authors have found that deacetylation promotes FoxO-dependent transcription. However, these studies focused primarily on the effects of FoxO proteins on the cell cycle. The present data lend further support to the idea that deacetylation is a positive modulator of FoxO1 function by providing evidence that FoxO1-dependent regulation of hepatic glucose production is also affected by resveratrol, a small molecule activator of Sirtuins. Our findings are at odds with those of Motta et al. (11), who found increased G6pc and Pck1 expression in mice lacking Sirt1. However, it is unclear whether their data were obtained in fasted or fed mice. Moreover, as the Sirt1$^{-/-}$ mice were hyperglycemic, it is possible that the increase in Pck1 and G6pc is secondary to hyperglycemia rather than Sirt1 ablation. Further work will be required to establish a consensus on this point. Moreover, it should be noted that Sirt1 has also been shown to promote Pgc1a-dependent gluconeogenesis, a conclusion that is fully consistent with our findings (33). This is a key question if therapies based on Sirtuin modulation are to be pursued for the treatment of metabolic disorders. For example, if Sirtuin activation increases hepatic glucose production in vivo as it does in cultured cells, then hepatic Sirtuins should be inhibited to treat diabetes. On the other hand, in other tissues such as pancreatic $\beta$ cells Sirtuin activation may indeed prove beneficial.

Deacetylation of transcription factors is generally associated with reduced activity. For example, Sirt1-mediated deacetylation dampens p53-dependent transcription (34–36). This process involves changes in p53 degradation (37), but currently there is no information on its effect on p53 subnuclear kinetics. Conversely, it is unclear whether deacetylation affects FoxO-dependent transcription by altering its stability. The finding that deacetylation increases the nuclear residence time of FoxO seemingly supports the idea that deacetylation increases FoxO-dependent transcription, but it should be emphasized that the net outcome of this modification is likely to be rather complex as FoxO can be both an activator (27) and a suppressor of gene expression (16, 38). Further studies with site-directed mutants that prevent or mimic acetylation will be required to address the specific effects of this important post-translational modification on the pleiotropic actions of FoxO proteins.

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