FoxO1 protects against pancreatic β cell failure through NeuroD and MafA induction

Yukari Ido Kitamura,[1,5] Tadahiro Kitamura,[1,5] Jan-Philipp Kruse,[2,3] Jeffrey C. Raum,[4] Roland Stein,[4] Wei Gu,[3] and Domenico Accili[1,*]

[1]Naomi Berrie Diabetes Center, Department of Medicine
[2]Integrated program in Molecular, Cellular and Biophysical Studies
[3]Institute of Cancer Genetics, College of Physicians & Surgeons of Columbia University
Columbia University, New York, New York 10032
[4]Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee 37232
[5]These authors contributed equally to this work.
*Correspondence: da230@columbia.edu

Summary

Diabetes causes pancreatic β cell failure through hyperglycemia-induced oxidative stress, or "glucose toxicity." We show that the forkhead protein FoxO1 protects β cells against oxidative stress by forming a complex with the promyelocytic leukemia protein Pml and the NAD-dependent deacetylase Sirt1 to activate expression of NeuroD and MafA, two *Insulin2* (*Ins2*) gene transcription factors. Using acetylation-defective and acetylation-mimicking mutants, we demonstrate that acetylation targets FoxO1 to Pml and prevents ubiquitin-dependent degradation. We show that hyperglycemia suppresses MafA expression in vivo and that MafA inhibition can be prevented by transgenic expression of constitutively nuclear FoxO1 in β cells. The findings provide a mechanism linking glucose- and growth factor receptor-activated pathways to protect β cells against oxidative damage via FoxO proteins.

Introduction

Forkhead transcription factors of the FoxO subfamily play important roles in cellular differentiation, proliferation, and metabolism. Because of their pivotal role in diverse cellular processes, there exist complex mechanisms to regulate FoxO-dependent transcription (Accili and Arden, 2004). Hormones and growth factors inhibit FoxO via phosphorylation by a host of serine-threonine kinases (Kops and Burgering, 1999). In contrast, oxidative stress leads to changes of the acetylation status of FoxO proteins. Evidence from overexpression studies indicates that FoxO is acetylated by the nuclear receptor coactivators Cbp and p300 and deacetylated by histone deacetylases (HDACs) and NAD-dependent deacetylases (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004).

An important target tissue of FoxO signaling is the pancreatic β cell. This cell is the archetypal metabolic sensor, as it adjusts insulin secretion to prevailing glucose levels. Failure of β cell function is commonly seen in type 2 diabetes, but the nature of the underlying metabolic abnormality is unclear (Bell and Polonsky, 2001). A widely held theory is that chronic exposure to elevated glucose concentrations causes a deterioration of β cell function ("glucose toxicity"). Glucose toxicity is thought to arise as a consequence of chronic oxidative stress, when intracellular glucose concentrations exceed the glycolytic capacity of the β cell. Under these conditions, glucose is shunted to enolization pathways, leading to superoxide formation (Robertson et al., 2003). β cells are ill equipped to dispose of hydroxyl radicals, as their complement of antioxidant enzymes, such as catalase, Mn-superoxide dismutase (Mn-SOD), and glutathione peroxidase, is low (Lenzen et al., 1996; Welsh et al., 1995). It has been proposed that an increase in hydroxyl radicals impairs expression of the *Ins2* gene transcription factor Pdx1 (Poitout et al., 1996; Sharma et al., 1995). This leads, in turn, to decreased *Ins2* gene transcription and increased apoptosis.

We have previously shown that FoxO1 haploinsufficiency restores β cell mass and prevents diabetes in *Insulin receptor substrate 2* (*Irs2*) knockout mice(Kitamura et al., 2002). We have also shown that overexpression of constitutively nuclear FoxO1 in β cells prevents compensatory β cell hyperplasia in insulin-resistant mice (Nakae et al., 2002). These data provide evidence that FoxO1 regulates β cell mass, but it remains unclear whether it also regulates β cell function.

In this study, we have uncovered a new role of FoxO1 in the protection against β cell failure. Exposure of βTC-3 cells to hydrogen peroxide, an inducer of superoxide formation, targets FoxO1 to Pml-containing nuclear subdomains and increases expression of the *Ins2* gene transcription factors NeuroD and MafA in a FoxO1-dependent manner. Acetylation is required to target FoxO1 to nuclear Pml bodies, while deacetylation by the NAD-dependent deacetylase Sirt1 is required to induce gene expression. Using acetylation-defective and "constitutively acetylated" FoxO mutants, we show that acetylation affects the balance between protein stability and DNA transcription. Thus, acetylation-defective mutants are more transcriptionally active than wild-type (wt) FoxO1 but are more rapidly ubiquitinated and degraded, while mutations that mimic the acetylated state increase stability but impair FoxO1 transcription. Consistent with the idea that FoxO1 plays a role in the regulation of MafA expression in vivo, we show that FoxO1 and MafA levels are concordantly decreased in diabetic animals. Expression of a constitutively nuclear FoxO1 transgene in β cells prevents MafA downregulation in response to hyperglycemia.

These data indicate that FoxO1 orchestrates a compensatory response, aimed at preserving β cell function under conditions of metabolic stress.

Results

Nuclear translocation of FoxO1 in response to oxidative stress

In most cell types, FoxO1 shuttles between nucleus and cytoplasm. Cultured pancreatic βTC-3 cells behave unlike other cell types, as in these cells FoxO1 is constitutively cytoplasmic, presumably reflecting tonic activation of insulin receptor signaling by endogenously produced insulin (Harbeck et al., 1996). Thus, endogenous FoxO1 is constitutively phosphorylated at the main phosphorylation sites, S253 and S316, (Figure 1A) and localizes to the cytoplasm in cultured βTC-3 cells (Figure 1B). We asked whether metabolic challenges affect FoxO1 localization. However, removal of serum and/or glucose from the medium for up to 72 hr did not affect FoxO1 localization or phosphorylation (Figures 1A and 1B). In contrast, incubation of β cells with H_2O_2 for up to 24 hr to induce oxidative stress resulted in nuclear redistribution of FoxO1 in ~30% of cells (Figure 1C and Table 1). To demonstrate the physiological relevance of this observation, we determined FoxO1 subcellular localization in primary islet cell cultures, which are composed by >90% of β cells (data not shown). As in βTC-3 cells, endogenous FoxO1 is cytoplasmic in primary β cells. H_2O_2 promoted Foxo translocation in ~45% of primary islet cells and culture in 25 mM glucose in ~20% of cells (Figure 1D and Table 1). Thus, H_2O_2 treatment of βTC-3 cells faithfully recapitulates the effects of hyperglycemia in β cells.

Exposure to oxidative stress during the S phase of the cell cycle results in apoptosis (Chen et al., 2000). Conversely, exposure to oxidative stress in the G1 or G2/M phase leads to "stress-induced premature senescence" (Frippiat et al., 2001), presumably to prevent somatic mutations induced by DNA damage. Following treatment with H_2O_2, we observed a sharp increase in the number β cells undergoing premature senescence in primary islet cell cultures. The increase in cellular senescence was mimicked by constitutively nuclear FoxO1 and was partially blunted by dominant-negative FoxO1 (Figures 1E and 1F), consistent with the idea that FoxO1 nuclear translocation promotes cellular survival under stress.

FoxO1 induces NeuroD expression in response to H_2O_2

To evaluate the functional consequences of FoxO1 redistribution, we measured expression of the *Ins2* gene transcription factor NeuroD in βTC-3 cells exposed to H_2O_2. This protein is expressed in uncommitted pancreatic endocrine precursor cells and then becomes restricted to differentiated β cells, where it promotes *Ins2* expression (Jensen et al., 2000; Schwitzgebel et al., 2000). *NeuroD* knockout mice develop diabetes shortly after birth, due to a >70% decrease in endocrine cell number (Miyata et al., 1999; Naya et al., 1997). In cultured βTC-3 cells, we found only occasional NeuroD-positive cells, consistent with the observation that NeuroD is not absolutely required for β cell function and *Ins2* gene transcription (Huang et al., 2002). Following H_2O_2 treatment, most βTC-3 cells that scored positive for nuclear FoxO1 also demonstrated NeuroD expression (Figure 2A). A similar induction of NeuroD expression could be brought about by overexpression of constitutively nuclear ADA-FoxO1 (Figures 2B and 2C). We observed a similar increase of *NeuroD* mRNA in primary islet cultures exposed to H_2O_2 or 25 mM glucose (Figure S1 available with this article online). During embryonic endocrine cell differentiation, activation of NeuroD is associated with growth arrest (Jensen et al., 2000). However, in cultured βTC-3 cells, we found that 97% of the scarce NeuroD-positive cells are Ki67 positive, indicating that they are actively replicating (Figure 2D). In contrast, following H_2O_2 treatment, only 5.7% of FoxO1/NeuroD-positive cells were Ki67 positive, while the remainder (>94%) were Ki67 negative, indicating that they are growth arrested. As growth arrest is an important FoxO function (Medema et al., 2000), this experiment provides further evidence for activation of the FoxO transcriptional program.

To distinguish between direct and indirect effects of FoxO1 on *NeuroD* transcription, we performed gel shift, promoter activity, and chromatin immunoprecipitation (ChIP) assays. Incubation of nuclear extracts from βTC-3 cells with a labeled oligonucleotide spanning five forkhead sites in the *NeuroD* promoter gave rise to a gel-retarded band that could be specifically supershifted by FoxO1 antiserum (Figure 2E). Moreover, expression of ADA-FoxO1 resulted in a 6-fold induction of *NeuroD* promoter activity, which was abolished by point mutations of the FoxO1 consensus sites (Figure 2F). ChIP assays demonstrated that FoxO1 binds to a forkhead site of the *NeuroD* promoter in intact chromatin in an H_2O_2-dependent manner (Figure 2G).

FoxO1 promotes MafA expression

MafA is an *Ins2* gene transcription factor that is expressed at the beginning of the "secondary transition" in β cells. The onset of MafA expression coincides with β cell acquisition of glucose competence, i.e., the ability to respond to changes in glucose concentrations with insulin secretion (Matsuoka et al., 2004; Olbrot et al., 2002). Similar to the findings for NeuroD expression, we detected increased MafA levels in response to H_2O_2 treatment or ADA-FoxO1 transduction (Figure 3A). Similarly, H_2O_2 or 25 mM glucose treatment increased MafA levels in primary islets (Figure S1). Coexpression of FoxO1 with a reporter gene driven by the *MafA* promoter increased luciferase activity ~20-fold compared to control vector in transient transfection studies (Figure 3B), and ChIP assays showed that FoxO1 binds to a forkhead element in the *MafA* promoter in an H_2O_2-dependent manner (Figure 3C). These data indicate that *MafA* is a direct FoxO target.

Prolonged culture of β cells with high glucose decreases MafA binding to the *Ins2* promoter (Poitout et al., 1996), but there is no information on MafA protein levels in diabetes. We analyzed MafA immunoreactivity in pancreas sections from mice with a range of different glucose levels. In wt mice, virtually all β cells expressed MafA, as did β cells of mice with hypoglycemia (*Insr* Δ80)(Kitamura et al., 2004) (Figure 3D). In contrast, we observed a decrease in MafA expression in two models of diabetes, one with mild hyperglycemia (~150 mg/dl) caused by overexpression of constitutively nuclear FoxO1 in liver (FoxO307, Figure 3D) (Nakae et al., 2002) and one with marked hyperglycemia (>400 mg/dl) caused by insulin receptor mutations (L2, Figure 3C) (Okamoto et al., 2004). These findings indicate that MafA expression is exquisitely sensitive to increases in plasma glucose levels.

We then asked whether constitutive nuclear expression of

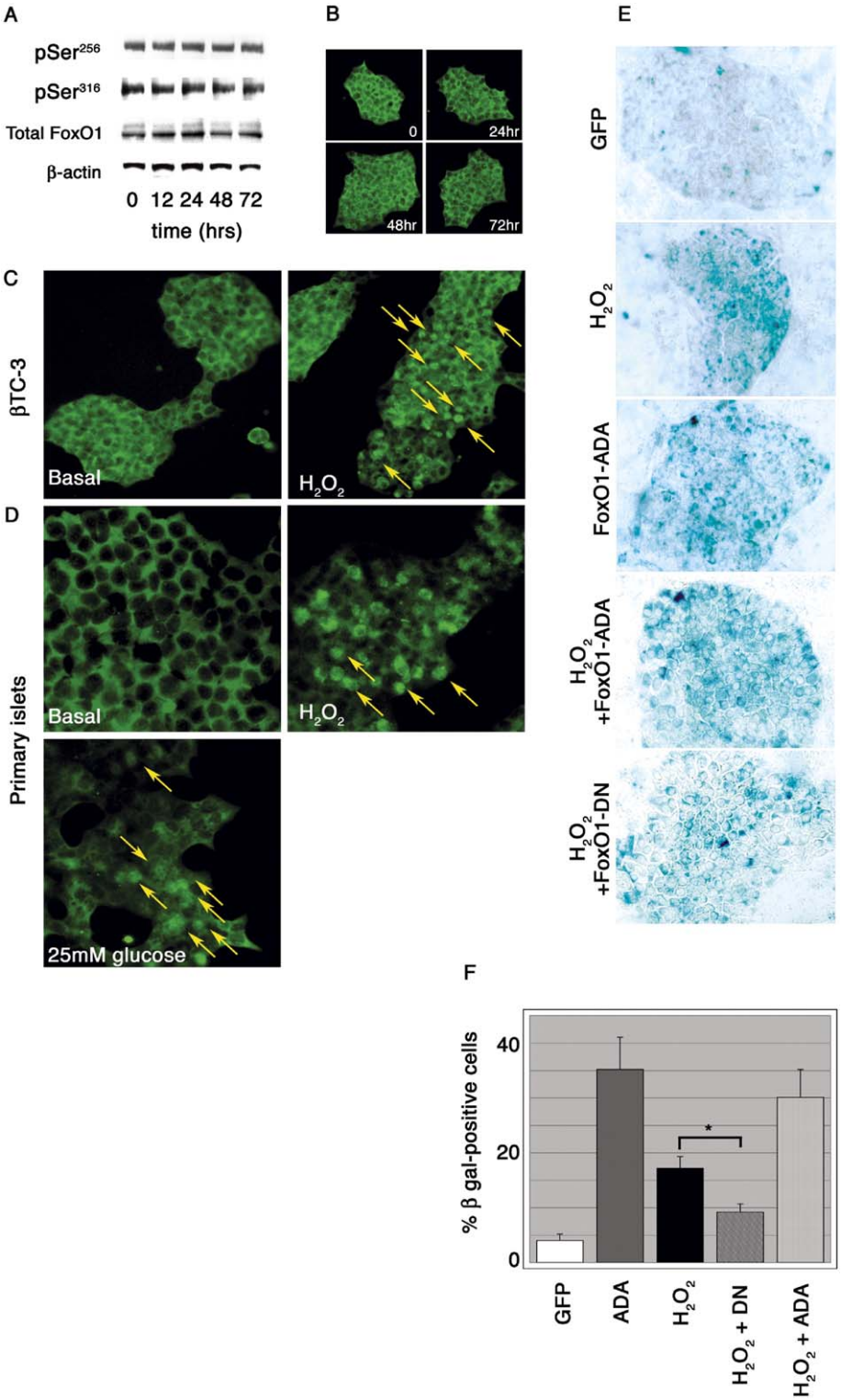


Figure 1. FoxO1 translocates to the nucleus in response to H_2O_2

A) We analyzed FoxO1 phosphorylation by Western blotting with antisera against phospho-Ser256, phospho-Ser316, and anti-FoxO1.

B) Immunocytochemical analysis of FoxO1 distribution in βTC-3 cells following serum or glucose depletion.

C) We treated βTC-3 cells with or without 50 μM H_2O_2 for 12 hr, then performed immunocytochemistry to determine FoxO1 distribution. Arrows indicate cells with nuclear FoxO1.

D) We isolated islets (consisting of >90% β cells) and cultured them in the presence or absence of 25 mM glucose or 50 μM H_2O_2 for 24 hr prior to performing immunocytochemistry as indicated above to detect endogenous FoxO1.

E) We cultured primary islets in the presence or absence of 50 μM H_2O_2 for 7 days or transduced them with FoxO1-ADA, FoxO1-DN (Δ256), or GFP adenovirus at a MOI of 1 and then continued culture for the same length of time. At the end of the experiment, we measured senescence by SA β-galactosidase activity assays to visualize lysosomes.

F) We counted the number of SA β-galactosidase-positive cells in more than 20 islet cell clusters. We present the data as the mean percentage of SA β-galactosidase-positive cells (±SEM). An asterisk indicates $p < 0.05$ by one factor-ANOVA.

FoxO1 in β cells could prevent MafA downregulation by hyperglycemia. We analyzed mice expressing a constitutively nuclear FoxO1 transgene in both liver and β cells (FoxO305) (Nakae et al., 2002). Despite similar plasma glucose levels to FoxO307 mice (~150 mg/dl), FoxO305 mice retained normal MafA expression by immunohistochemistry (Figure 3D) and mRNA levels (Figure S2). To examine this point further, the same FoxO305 mice were rendered more severely diabetic by intercrossing them with insulin-resistant mice (L1, Figure 3C) (Okamoto et al., 2004). The resulting mice had glucose levels

Table 1. FoxO1 nuclear translocation in response to oxidative stress

Condition	Nuclear	Nuclear/ Cytoplasmic	Cytoplasmic
βTC-3 basal	0	0	100
βTC-3 H_2O_2	24 ± 5	5 ± 2	70 ± 5
Primary islets basal	0	0	100
Primary islets H_2O_2	30 ± 5	15 ± 3	55 ± 4
Primary islets 25 mM glucose	10 ± 2	8 ± 3	80 ± 5

Quantification of the effects of H_2O_2 and hyperglycemia on FoxO1 subcellular localization. We quantitated the number of cells with nuclear, cytoplasmic, and combined nuclear/cytoplasmic patterns of FoxO1 expression. Values are expressed as percentage of all scored cells. Each experiment was conducted at least three times.

~400 mg/dl (H. Okamoto and D.A., unpublished data). MafA expression was preserved in 70% of β cells, as opposed to 10% of β cells in diabetic mice with similar glucose levels (L2, Figure 3D). If FoxO1 is required to induce MafA in vivo and MafA is reduced in diabetes, the expectation would be that FoxO1 levels should also be decreased in diabetic animals. As shown in Figure 3E, we observed a profound decrease in FoxO1 expression in two models of adult-onset type 2 diabetes, one caused by β cell dysfunction (*Irs2*$^{-/-}$)(Withers et al., 1998) and one caused by insulin resistance (L2) (Okamoto et al., 2004). These data are consistent with the hypothesis that FoxO1 participates in the regulation of MafA expression in vivo.

FoxO1 is targeted to Pml bodies

The observations that oxidative damage increases expression of FoxO1 target genes, while FoxO1 levels are decreased in diabetes, are apparently at odds. To reconcile them, we hypothesized that, as FoxO1 is retained in the nucleus, it is also more rapidly degraded. If so, nuclear retention in response to oxidative stress would only afford a temporary reprieve against the metabolic onslaught of chronic hyperglycemia. The next set of experiments was designed to uncover the posttranslational modifications that determine the balance between FoxO1-dependent transcription and protein stability.

Oxidative stress causes extensive posttranslation protein modifications, including acetylation and ubiquitination (Brooks and Gu, 2003). As shown in several studies (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004), FoxO1 was transiently acetylated in response to H_2O_2 treatment. In vivo coimmunoprecipitation studies and in vitro assays with purified FoxO1-GST indicate that FoxO1 is acetylated by the nuclear coactivators Cbp/p300 and deacetylated by the NAD-dependent deacetylase (Sirtuin) Sirt1 (Figure S3). It has been suggested that acetylation promotes FoxO-dependent transcription (Daitoku et al., 2004; Motta et al., 2004). However, this point remains controversial, with some data suggesting that acetylation increases (Motta et al., 2004), and others that it decreases FoxO activity (Brunet et al., 2004; Daitoku et al., 2004; van der Horst et al., 2004). We hypothesized that acetylation is required to target FoxO to transcriptionally active nuclear domains, rather than directly promote FoxO-dependent transcription.

The promyelocytic leukemia-associated protein Pml forms characteristic nuclear subdomains (Pml nuclear bodies), where several transcriptional regulators reside (Guo et al., 2000; Pearson and Pelicci, 2001). Pml is also a mediator of stress-induced premature senescence (Pearson and Pelicci, 2001). Therefore, we investigated whether FoxO1 associates with Pml during oxidative stress. We detected Pml in FoxO1 immunoprecipitates following treatment with H_2O_2 (Figure 4A). Immunohistochemical studies in βTC-3 cells indicated that these two proteins are found in different cellular compartments in basal conditions but colocalize in a punctate nuclear pattern following H_2O_2 treatment (Figure 4B). These results indicate that FoxO1 engages in protein-protein interactions with Pml in response to oxidative stress. We next examined whether inhibition of FoxO1 or Pml by siRNA would affect NeuroD expression. The FoxO1 and Pml siRNAs effectively abolished expression of the two proteins (Figure 4C). Consistent with the hypothesis that Pml and FoxO1 are required for NeuroD induction, we saw that NeuroD levels decreased in cells expressing either siRNA (Figure 4C).

To determine whether acetylation is required for FoxO1 binding to Pml, we introduced mutations that affect FoxO1 acetylation. We replaced six lysine residues corresponding to proposed FoxO acetylation sites (K242, K245, K259, K262, K271, and K291) (Brunet et al., 2004; Daitoku et al., 2004) with arginine to prevent acetylation (henceforth KR series), and with glutamine to mimic it (Luo et al., 2004) (henceforth KQ series) (Figure S4). To rule out the competing effects of nuclear exclusion, we introduced the mutations both in wt and in a phosphorylation-defective mutant (ADA-FoxO1). However, the results are identical with both sets of constructs. Moreover, neither the 6KR nor the 6KQ mutant showed alterations of nuclear exclusion in response to insulin treatment (data not shown). Consistent with the observation that acetylation is required to target FoxO1 to Pml, the 6KR mutant failed to bind Pml in response to H_2O_2, while the 6KQ mutant was constitutively bound to it even in the absence of H_2O_2 (Figure 4D). We obtained similar data in Sirt1 coimmunoprecipitations (Figure 4E). In contrast, neither the 6KR nor the 6KQ mutant bound to Cbp (Figure 4F). These data indicate that acetylation of these residues is required for the interaction of FoxO1 with Pml and Sirt1.

Pml targets FoxO1 for ubiquitin-mediated degradation

Acetylation affects protein degradation via ubiquitination (Ito et al., 2002; Jeong et al., 2002; Li et al., 2002). Accordingly, we observed that less FoxO1 was ubiquitinated in response to H_2O_2 (Figures 5A and 5B) and that protein half-life was extended from ~4 hr to >12 hr in cells treated with H_2O_2 or deacetylase inhibitors (Figure S5).

To determine whether acetylation affects ubiquitin-dependent degradation, we determined the ubiquitination of the 6KR and 6KQ mutants. Consistent with the idea that acetylation inhibits degradation via the ubiquitin pathway, we observed decreased ubiquitination of the 6KQ mutant and increased ubiquitination of the 6KR mutant (Figure 5C). Moreover, we observed that FoxO1 ubiquitination increased in cells coexpressing FoxO1 and Pml or Sirt1 and decreased in cells coexpressing FoxO1 and Cbp (Figure 5D). Finally, we observed that FoxO1 levels decreased in cells overexpressing Pml in a Pml-dose-dependent manner (Figure 5E, left panel). This effect was blocked by MG132 (Figure 5E, right panel), indicating that it requires FoxO targeting to the proteosomal compartment.

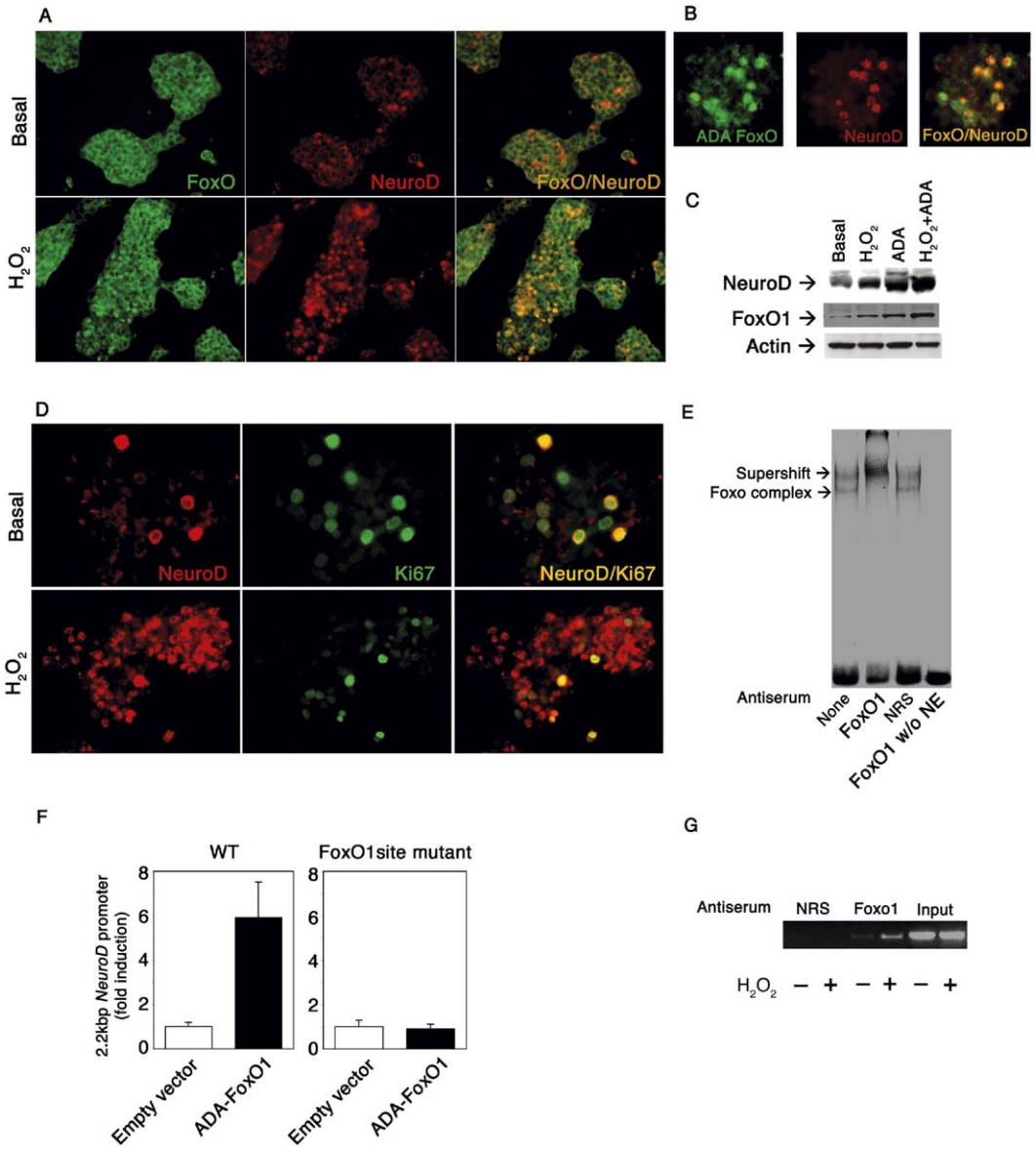


Figure 2. FoxO1 activates NeuroD expression

A) We treated βTC-3 cells with or without 50 μM H_2O_2 for 24 hr, then performed immunostaining for FoxO1 (green) and NeuroD (red).

B) We transduced βTC-3 cells with HA-tagged FoxO1-ADA adenovirus and performed immunostaining with anti-HA antiserum (green) and NeuroD (red) following treatment.

C) We determined expression of FoxO1 and NeuroD by Western blotting under the conditions indicated in **(A)**.

D) Expression of NeuroD (red) and Ki67 (green) in βTC-3 cells following H_2O_2 treatment for 24 hr is shown.

E) FoxO1 binds to the *NeuroD* promoter in vitro. We incubated βTC-3 nuclear extracts with an oligonucleotide spanning five forkhead sites in the *NeuroD* promoter. We performed electromobility gel shift assays in the presence of anti-FoxO1 antiserum or normal rabbit serum.

F) FoxO1 increased *NeuroD* promoter activity. We cotransfected βTC-3 cells with a wt 2.2 kb *NeuroD* promoter/pGL3 basic vector and ADA-FoxO1 (left panel) or a mutant 2.2 kb promoter with point mutations of the forkhead binding sites (right panel). Bars indicate the mean fold-increase over basal values (+SEM).

G) ChIP assays of the *NeuroD* promoter are shown. We transduced βTC-3 cells with FoxO1 adenovirus and performed ChIP assays with the indicated antisera. The crosslinked DNA was amplified by PCR using a set of primers spanning the forkhead binding sites in the *NeuroD* promoter. Input represents DNA extracted from chromatin prior to immunoprecipitation.

These data indicate that Pml or Sirt1 bound FoxO1 is more rapidly ubiquitinated, suggesting that these interactions target FoxO1 for degradation.

We finally asked whether changes in acetylation affect FoxO1 transcription. We measured in vivo DNA binding using ChIP assays with the *NeuroD* promoter. In these experiments, we used the constitutively nuclear FoxO-ADA mutant to rule out the effects of nuclear translocation. We observed increased binding of the 6KR mutant to the *NeuroD* promoter and decreased binding of the 6KQ mutant (Figure 5F). Moreover, the

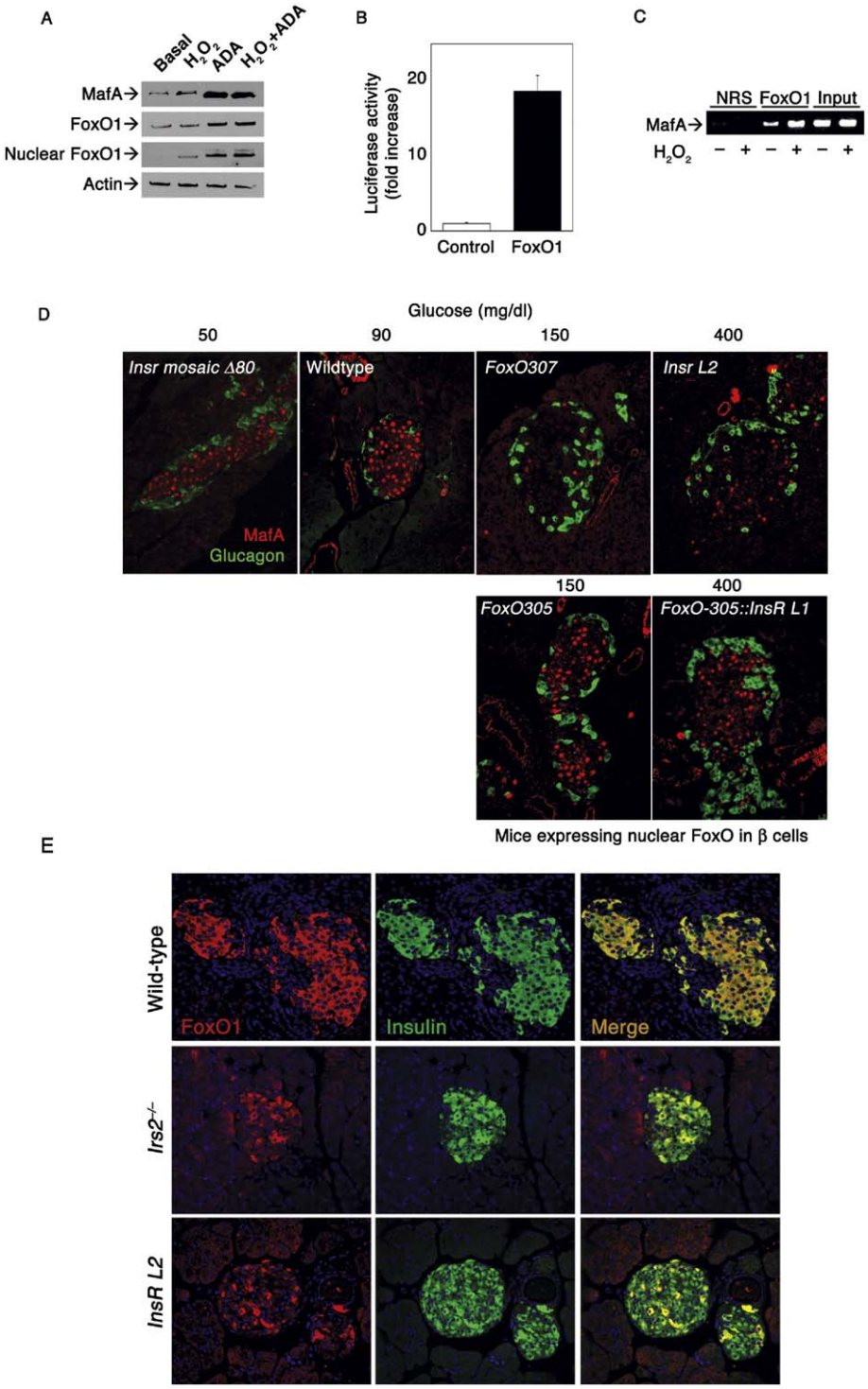


Figure 3. FoxO1 promotes MafA expression in βTC-3 cells and in transgenic mice

A) Western blot of FoxO1 and MafA in βTC-3 cells. We treated βTC-3 cells with H_2O_2 for 24 hr or transduced them with HA-tagged FoxO1-ADA as indicated in Figure 2, and determined expression of FoxO1 and MafA by Western blotting. To measure nuclear FoxO1 levels, we isolated nuclei from βTC-3 cells under the conditions indicated.

B) FoxO1 increased *MafA* promoter activity. We cotransfected βTC-3 cells with a wt *MafA* promoter/pGL3 basic vector and control vector or FoxO1, then measured luciferase activity. Means ± SEM of three experiments are shown.

C) FoxO1 is recruited to the *MafA* promoter. We incubated βTC-3 cells in the absence or presence of H_2O_2 (50 μM) and performed ChIP assay with a FoxO1 antiserum. For PCR, we used a set of primers corresponding to forkhead binding sites in the *MafA* promoter.

D) We determined MafA expression in different mouse models of diabetes. We prepared paraffin sections from insulin receptor mosaic mice (*Insr* Δ80), wt, FoxO1 transgenics (*FoxO305* and *FoxO307*), insulin receptor transgenic knockout (*L2*), or *FoxO305* transgenics intercrossed with insulin receptor transgenic knockouts (*FoxO305::L1*). We performed double immunohistochemistry with MafA (red) and glucagon (green).

E) FoxO expression in diabetic animals is shown. We prepared paraffin sections from wt, *Irs2*$^{-/-}$, and insulin receptor transgenic knockout (L2) mice. We performed double immunohistochemistry with FoxO1 (red) and insulin (green).

6KR mutant elicited stronger activity of a FoxO reporter gene in luciferase assays than the 6KQ mutant (Figure 5G). Thus, acetylation decreased and deacetylation increased FoxO1-dependent gene expression.

Bimodal regulation of FoxO1 in response to H_2O_2

We summarize these data as a model in Figure 6. Under normal conditions, FoxO1 is rapidly excluded from the nucleus in β cells. In response to hyperglycemia (or H_2O_2), FoxO1 is acetylated. Acetylation prevents ubiquitination—thus resulting in acetyl-FoxO1 nuclear accumulation—and targets FoxO to Pml bodies but hinders FoxO-dependent transcription. Upon associating with the Pml body, FoxO is deacetylated by Sirt1, leading to increased transcription. However, the deacetylated protein is also more efficiently ubiquitinated. Thus, the tradeoff of increased transcription is rapid degradation. The ostensible

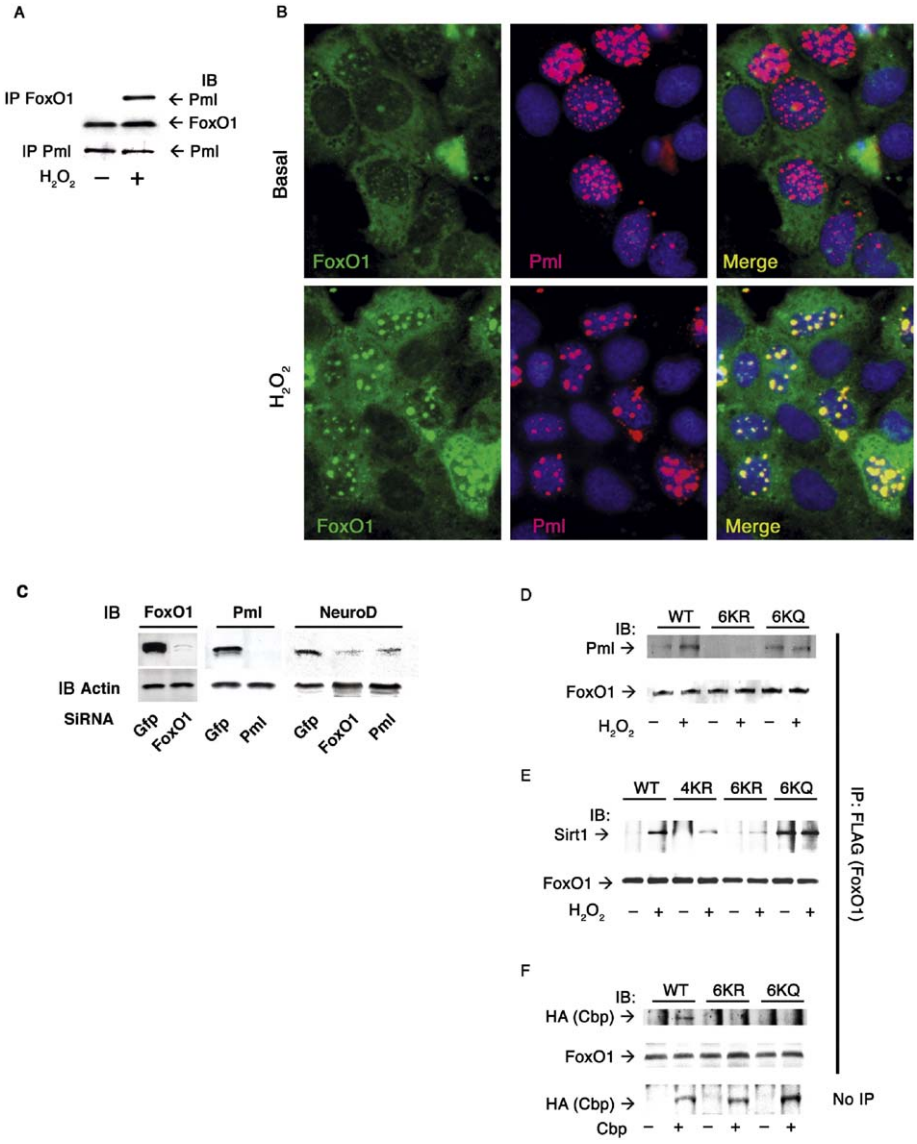


Figure 4. FoxO1 binds to Pml in H_2O_2-treated βTC-3 cells

A) FoxO1 binds to Pml following H_2O_2 treatment. We cotransfected FLAG-Pml with HA-FoxO1-ADA in βTC-3 cells. After treatment with H_2O_2 for 24 hr, we immunoprecipitated cell extracts with FoxO1 (HA-tag antibody) and performed Western blotting with anti-FLAG (Pml) (upper panel) or anti-FoxO1 antibody (middle panel), or we carried out immunoprecipitation and Western blotting with anti-FLAG antibody to normalize for Pml content (lower panel).

B) Endogenous FoxO1 colocalizes with Pml following H_2O_2 treatment. Immunohistochemical analysis of endogenous FoxO1 localization in βTC-3 cells exposed to H_2O_2 for 24 hr is shown. To visualize Pml, we transfected FLAG-tagged Pml.

C) FoxO1 and Pml inhibition by siRNA. Expression of FoxO1 (left panel), Pml (middle panel), and NeuroD (right panel) in βTC-3 cells transfected with the indicated siRNAs.

D) Acetylation-deficient FoxO1 (6KR) fails to bind to Pml in response to H_2O_2, while acetylation-mimicking mutant (6KQ) is constitutively bound to it in βTC-3 cells.

E) Coimmunoprecipitation of mutant FoxO1 and Sirt1. Similar to **(D)**, acetylation-deficient FoxO1 fails to bind to Sirt1, while acetylation-mimicking mutant is constitutively bound to it.

F) Interaction of FoxO1 with Cbp is prevented by mutations affecting FoxO1 acetylation. We cotransfected βTC-3 cells with FLAG-tagged FoxO1 and HA-tagged Cbp and performed coimmunoprecipitations as indicated.

goal of this mechanism is to prevent unchecked FoxO-dependent transcription, which has the potential to result in apoptosis (Brunet et al., 1999) or cellular atrophy (Sandri et al., 2004; Stitt et al., 2004).

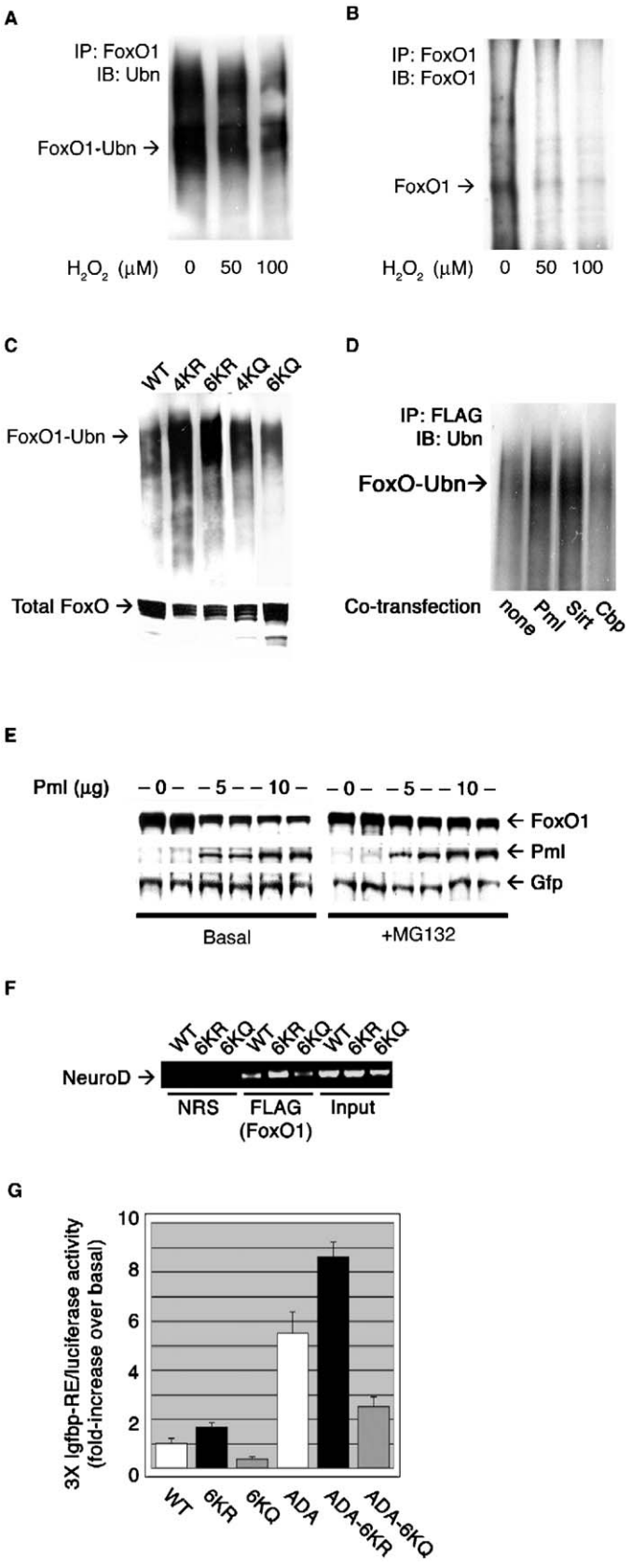
Discussion

The integration of metabolic signals, such as nutrients and glucose—acting via oxidative stress—with growth factor/hormonal signals in the life cycle of the β cell has long bedeviled the diabetes field. The main conclusion of these studies is that these competing signals are integrated by FoxO1 to protect against β cell failure.

A model of FoxO1 function in β cells

FoxO1 activates gene expression in response to oxidative stress to preserve insulin secretion and promote cell survival. Metabolic stress overrides phosphorylation-dependent nuclear exclusion and causes nuclear retention via acetylation. The net outcome is to increase expression of *Ins2* gene transcription factors (NeuroD and MafA), prevent β cell replication under conditions that could eventuate in apoptosis, and promote β cell senescence. Acetylation serves the twin purposes of increasing FoxO1 stability and targeting FoxO1 to Pml nuclear bodies, where it is deacetylated by Sirt1 to become transcriptionally active. As unchecked FoxO1 transcription can be detrimental to the cell (Accili and Arden, 2004), deacetylated FoxO1 is rapidly degraded. Thus, FoxO1 translocation in response to oxidative stress can be envisaged to allow β cells to withstand acute metabolic changes, for example transient oscillations of glucose levels. However, since deacetylated FoxO1 is degraded, this mechanism is unable to preserve β cell function in the face of continuing stress, as indicated by the loss of FoxO1 expression in diabetic animals.

The present findings allow us to reconcile some unexplained features of FoxO1 function in mature β cells and integrate them



into current models of diabetes pathophysiology (Accili, 2004). We have previously shown that a constitutively nuclear FoxO1 impairs compensatory β cell hyperplasia in insulin-resistant states but fails to impair β cell function, despite decreased Pdx1 levels. While FoxO1 β cell transgenics are glucose intolerant at an early age and display mild hyperglycemia (120–150 mg/dl), they fail to become more severely diabetic with age (Nakae et al., 2002). These seemingly irreconcilable effects can now be explained by FoxO1' s ability to promote MafA expression. Moreover, as in these transgenic mice FoxO1 expression fails to be downregulated with the onset of hyperglycemia (because it is driven by a heterologous promoter), the mutant FoxO1 becomes protective against further deterioration of β cell function.

FoxO targets in the β cell

The identification of *NeuroD* as a FoxO1 target is noteworthy, as thus far the only known *NeuroD* activator was Neurogenin3 (Huang et al., 2000), which, however, is not expressed in the adult pancreas. Activation of *NeuroD* appears to recapitulate an earlier differentiation stage of the β cell progenitor, when cells turn on *NeuroD* expression and become mitotically arrested (Jensen et al., 2000). The increase in MafA expression in response to FoxO1 nuclear translocation should be viewed as central to the β cell compensatory response, as hyperglycemia has been shown to impair MafA-dependent *Ins2* gene expression (Matsuoka et al., 2003). In this respect, we show that MafA expression correlates with FoxO1 expression and decreases in genetic models of diabetes, providing a critical test of the hypothesis that MafA expression is regulated by glucose through FoxO1 (Poitout et al., 1996).

Figure 5. Mutations of acetylation sites affect FoxO1 ubiquitination and DNA binding

A) FoxO1 is ubiquitinated in response to 50 μm H_2O_2 treatment. βTC-3 cells were incubated with the indicated concentrations of H_2O_2 for 24 hr, and FoxO1 was detected by blotting with anti-ubiquitin (Ubn) antiserum following FoxO1 immunoprecipitation.

B) To determine that the Ubn bound high-molecular-weight species was indeed FoxO1 and not other proteins, we performed FoxO1 immunoblotting following FoxO1 immunoprecipitation.

C) Ubiquitination of wt and acetylation site mutants of FoxO1 is shown. We cotransfected βTC-3 cells with the FLAG-tagged FoxO1 constructs indicated at the top of the panel and ubiquitin. Following immunoprecipitation with anti-FLAG antiserum, we performed immunoblots with anti-ubiquitin antiserum. The lower panel shows a control immunoblot with anti-FLAG antiserum to normalize protein amount in each lane.

D) Ubiquitination of with-type FoxO1 in βTC-3 cells cotransfected with FLAG-tagged FoxO1 and Pml, Sirt, or Cbp is shown.

E) Coexpression of FoxO1 and Pml results in increased proteasome-dependent FoxO1 degradation. We compared FoxO1 protein levels in βTC-3 cells that had been cotransfected with increasing amounts (5 or 10 μg) of plasmid DNA encoding Pml in the absence and presence of the proteasome inhibitor MG132 (25 μm). We carried out MG132 treatment for 12 hr. Total DNA amount was kept constant by adding vector DNA or GFP.

F) Acetylation-deficient FoxO1 mutant binds more avidly to the forkhead site of the NeuroD promoter. We performed experiments as indicated in Figure 2, except that we used 6KR and 6KQ FoxO1 mutants.

G) Reported promoter activity of acetylation site-mutant FoxO1s is shown. We used a synthetic FoxO1 target sequence derived from the Igf binding protein-1 promoter to direct expression of a luciferase reporter gene. We carried out both sets of experiments in βTC-3 cells. Bars indicate the mean fold-increase over basal values (+SEM).

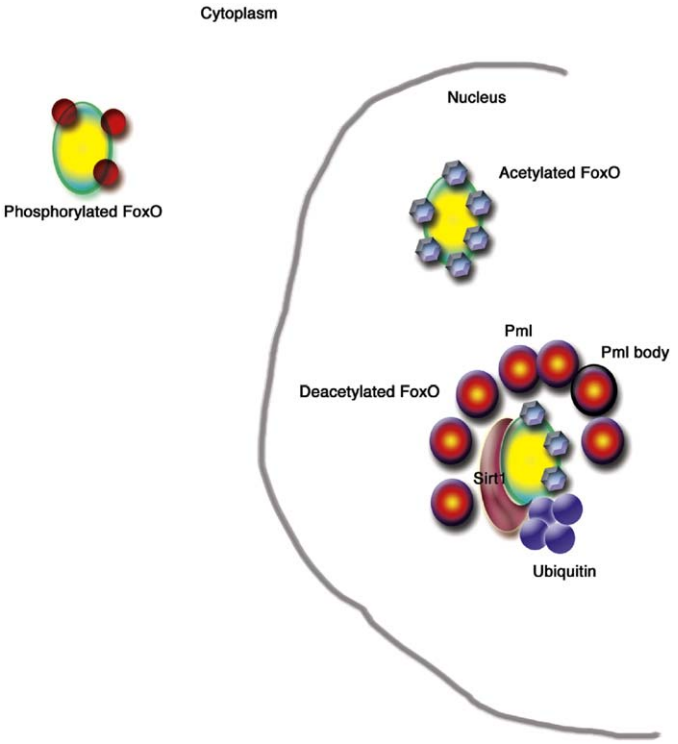


Figure 6. A model of FoxO1 function in β cells

FoxO1 nuclear retention protects β cells from oxidative stress-induced damage. Acetylated FoxO is retained in the nucleus, where it engages Pml and Sirt1. Deacetylation of FoxO1 by Sirt1 promotes FoxO1-dependent transcription and accelerates FoxO1 degradation. Because FoxO becomes rapidly degraded when deacetylated, this mechanism has the potential to protect β cells against acute metabolic distress but not against the chronic changes associated with prolonged hyperglycemia, a common cause of oxidative stress that has been shown to bring about β cell failure.

The identification of Pml as a FoxO-interacting protein is consistent with the role of Pml to regulate premature senescence (Pearson and Pelicci, 2001), apoptosis (Quignon et al., 1998), and targeting of transcriptional coactivators (Doucas et al., 1999). We suggest that FoxO1 acetylation by p300/Cbp and deacetylation by Sirt1 takes place within Pml bodies. Interestingly, this model is opposite to the mechanism by which deacetylation controls p53 function (Luo et al., 2001; Vaziri et al., 2001). Our data raise the possibility that Pml regulates metabolic functions via interactions with FoxO proteins.

Conclusions

It will be important to investigate whether genetic or pharmacological manipulations of FoxO1 can be used to improve β cell failure in diabetic patients, to preserve islet function prior to transplant, or to drive differentiation of uncommitted endocrine progenitors into β cells.

Experimental procedures

Antibodies

We purchased anti-insulin antibodies from Linco Research; anti-acetyllysine, anti-Sirt1, and anti-acetylated histoneH3 antibodies from Upstate Biotechnology; anti-FoxO1, anti-FoxO1 pSer256, and anti-FoxO1 pSer316 antibodies from Cell Signaling; anti-p300 (N-15), anti-CBP (C-20), anti-NeuroD (G-20 or N-19), anti-Pml (PG-M3), anti-FoxO1 (H-128), anti-Bad (C-7), anti-c-Myc (9E10) and anti-ubiquitin (P4D1) antibodies from Santa-Cruz; anti-HA (12CA5) antibody from Boehringer Mannheim; anti-glucagon and anti-Flag (M2) antibodies from Sigma; anti-Ki67 antibody from Castra; anti-Actin (Ab-1) antibody from Oncogene. For FoxO1 immunostaining, we used a cocktail of three antisera from Cell signaling, Santa-Cruz, and the previously described antipeptide antiserum 3587 (Nakae et al., 2002).

Expression vectors and adenoviral vectors

We have previously described expression vectors encoding Myc-tagged FoxO1, HA-tagged FoxO1-ADA (constitutively nuclear) and FoxO1-Δ256 (dominant negative) (Nakae et al., 2002), FLAG-tagged p300 and Pml (Zhong et al., 1999), HA-tagged Cbp (Gu et al., 1997), His-HA-ubiquitin (Li et al., 2003), and adenoviral vectors encoding HA-tagged wt and FoxO1-ADA (Nakae et al., 2001). We purchased the mouse Sirt1 expression vector (Sir2α/pUSEamp) from Upstate Biotechnology. To generate the 4KR and 4KQ mutants, we replaced K242, K245, K259, and K262 with arginine or glutamine. To generate the 6KR and 6KQ mutants, we replaced two additional lysine residues (K271 and K291) with arginine or glutamine. We carried out site-directed mutagenesis using the Quick Change kit (Stratagene).

Cell culture, transfection, and viral transduction

We cultured βTC-3 cells in high glucose DMEM supplemented with 15% horse serum and 2.5% FCS. In some experiments, we infected βTC-3 cells with FoxO1 adenovirus 6 hr before treatments at MOIs between 1 and 30. All experiments were repeated at least three times.

Immunoprecipitation and Western blotting

We lysed cultured cells in RIPA buffer containing 10 μM TSA, 10 mM NAM, and protease inhibitors (Roche). After centrifugation, cell extracts were diluted with Co-IP buffer (50 mM Tris, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 5 mM $MgCl_2$), immunoprecipitated, and analyzed by immunoblotting.

Gel shift and luciferase assays

We performed gel shift assays as described previously (Kitamura et al., 2002). We used the following oligonucleotide probe: GGTATTTAAACGCAA CACCCAGAGTAAATGAAAAAAAACAAAAACAAAACAAAAAAACAA (–1831 to –1772 of the *NeuroD* promoter). In some experiments, we incubated anti-FoxO1 or nonimmune sera with nuclear extracts for 1 hr prior to adding radiolabeled probe into the reaction mixture. We performed luciferase assays as previously (Kitamura et al., 2002), using a wt 2.2 kb NeuroD promoter fragment cloned into a pGL3 basic vector (Stratagene) or a mutant promoter with single base pair changes in the forkhead binding sites (AAACA →AGACA) at positions –1795, –1790, and –1784. For the *MafA* promoter studies, we cloned a DNA fragment encompassing nt –8118 to –7748 of the mouse *MafA* promoter (Zhao et al., 2005) into pGL3 basic and performed cotransfection assays as indicated for *NeuroD*.

Chromatin immunoprecipitation assays

We performed ChIP assay in hepatocytes or βTC-3 cells following previously described methods (Nakae et al., 2003). For the NeuroD promoter, we used the following primers: 5′-GCAGCAAAGCATGAACATTCA-3′ and 5′-ACTGGCTGAGAGCAGAGCAAGCAAG-3′ (–2119 ~ –2099 and –1743 ~ –1717 of the mouse NeuroD promoter). For the MafA promoter, we used the following primers: 5′-CACCCCAGCGAGGGCTGATTTAATT-3′ and 5′-AGCAAGCACTTCAGTGTGCTCAGTG-3′ (–8118 to –8094 and –7772 to –7748 of the mouse MafA promoter). All experiments were repeated at least three times.

Immunofluorescence

We performed immunostaining with anti-FoxO1 (1:30), anti-NeuroD (1:100), or anti-Ki67 (1:1000) antibodies as described (Kitamura et al., 2002). For colocalization experiments with FoxO1 and Pml, we transfected FLAG-tagged Pml and co-stained with anti-Flag (1:1,000) and anti-Cbp (1:30) antibodies. For immunohistochemistry, we used 5 μm-thick paraffin sections and performed immunofluorescence as described previously (Kitamura et al., 2002). In some experiments, we performed antigen retrieval before incubation of primary antibodies. We used anti-insulin, anti-glucagon, anti-FoxO1, anti-MafA, and anti-Sirt1 antibodies at dilutions of 1:2,000, 1:2,000,

1:30, 1:500, and 1:100, respectively. We visualized the immune complex with FITC- or CY3-conjugated secondary antibodies.

Premature senescence

We isolated islets from 2-month-old C57/B6 mice and cultured them as described previously (Kitamura et al., 2002). Five days after isolation, adherent cells organized as monolayers. At this point, we treated the cells with 50 μM H_2O_2 or low amount of adenovirus expressing FoxO1-ADA, FoxO1-DN, or GFP (MOI = 1), then cultured the cells for 7 days in normal culture medium. To measure premature senescence, we fixed and stained cells for SA β-galactosidase activity as described (Dimri et al., 1995).

Mouse models

The following strains of mice have been described in previous publications: *Irs2*$^{-/-}$ develop diabetes as a result of decreased β cell mass (Withers et al., 1998). FoxO305 and FoxO307 develop diabetes secondary to increased expression of hepatic glucogenetic genes, *Pck1* and *G6pc* (Nakae et al., 2002). Insulin receptor transgenic knockouts express insulin receptors exclusively in liver, β cells and brain (L1) or liver and β cell (L2); as a result, L1 mice are insulin resistant but not diabetic, while L2 are insulin resistant and diabetic (Okamoto et al., 2004). Insulin receptor mosaics (Δ80) carry a mosaic deletion of *Insulin receptor* in ~80% of somatic cells and are growth retarded and hypoglycemic (Kitamura et al., 2004). We obtained L1 × 305 mice by intercrosses of FoxO305 and *Insulin receptor* transgenic knockouts L1. We performed PCR genotyping as described.

Supplemental data

Supplemental data include five figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/3/153/DC1/.

Acknowledgments

This work was supported by NIH grants DK57539 and DK64819 to D.A. and DK42502 to R.S., by DK63608 (Columbia Diabetes and Endocrinology Research Center) and DK07563 (Vanderbilt Molecular Endocrinology Training Program), and by the Berrie Program in Cell-Based Therapies at Columbia University. T.K. is the recipient of a Uehara Memorial Foundation Research Fellowship. We thank Qun Xu and Qiong Li for skilled technical assistance with immunohistochemistry and members of the Accili, Gu, and Stein laboratories for stimulating discussions.

Received: December 13, 2004
Revised: March 29, 2005
Accepted: August 12, 2005
Published: September 13, 2005

References

Accili, D. (2004). Lilly lecture 2003: the struggle for mastery in insulin action: from triumvirate to republic. Diabetes *53*, 1633–1642.

Accili, D., and Arden, K.C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell *117*, 421–426.

Bell, G.I., and Polonsky, K.S. (2001). Diabetes mellitus and genetically programmed defects in beta-cell function. Nature *414*, 788–791.

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., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y., et al. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011–2015.

Chen, Q.M., Liu, J., and Merrett, J.B. (2000). Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts. Biochem. J. *347*, 543–551.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., Miyagishi, M., Nakajima, T., and Fukamizu, A. (2004). Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc. Natl. Acad. Sci. USA *101*, 10042–10047.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.

Doucas, V., Tini, M., Egan, D.A., and Evans, R.M. (1999). Modulation of CREB binding protein function by the promyelocytic (PML) oncoprotein suggests a role for nuclear bodies in hormone signaling. Proc. Natl. Acad. Sci. USA *96*, 2627–2632.

Frippiat, C., Chen, Q.M., Zdanov, S., Magalhaes, J.P., Remacle, J., and Toussaint, O. (2001). Subcytotoxic H2O2 stress triggers a release of transforming growth factor-beta *1*, which induces biomarkers of cellular senescence of human diploid fibroblasts. J. Biol. Chem. *276*, 2531–2537.

Gu, W., Shi, X.L., and Roeder, R.G. (1997). Synergistic activation of transcription by CBP and p53. Nature *387*, 819–823.

Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W., and Paolo Pandolfi, P. (2000). The function of PML in p53-dependent apoptosis. Nat. Cell Biol. *2*, 730–736.

Harbeck, M.C., Louie, D.C., Howland, J., Wolf, B.A., and Rothenberg, P.L. (1996). Expression of insulin receptor mRNA and insulin receptor substrate 1 in pancreatic islet beta-cells. Diabetes *45*, 711–717.

Huang, H.P., Liu, M., El-Hodiri, H.M., Chu, K., Jamrich, M., and Tsai, M.J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol. Cell. Biol. *20*, 3292–3307.

Huang, H.P., Chu, K., Nemoz-Gaillard, E., Elberg, D., and Tsai, M.J. (2002). Neogenesis of beta-cells in adult BETA2/NeuroD-deficient mice. Mol. Endocrinol. *16*, 541–551.

Ito, A., Kawaguchi, Y., Lai, C.H., Kovacs, J.J., Higashimoto, Y., Appella, E., and Yao, T.P. (2002). MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. EMBO J. *21*, 6236–6245.

Jensen, J., Heller, R.S., Funder-Nielsen, T., Pedersen, E.E., Lindsell, C., Weinmaster, G., Madsen, O.D., and Serup, P. (2000). Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes *49*, 163–176.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J., and Kim, K.W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell *111*, 709–720.

Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W.H., 3rd, Wright, C.V., White, M.F., Arden, K.C., and Accili, D. (2002). The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J. Clin. Invest. *110*, 1839–1847.

Kitamura, T., Kitamura, Y., Nakae, J., Giordano, A., Cinti, S., Kahn, C.R., Efstratiadis, A., and Accili, D. (2004). Mosaic analysis of insulin receptor function. J. Clin. Invest. *113*, 209–219.

Kops, G.J., and Burgering, B.M. (1999). Forkhead transcription factors: new insights into protein kinase B (c- akt) signaling. J. Mol. Med. *77*, 656–665.

Lenzen, S., Drinkgern, J., and Tiedge, M. (1996). Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. Free Radic. Biol. Med. *20*, 463–466.

Li, M., Luo, J., Brooks, C.L., and Gu, W. (2002). Acetylation of p53 inhibits its ubiquitination by Mdm2. J. Biol. Chem. *277*, 50607–50611.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science *302*, 1972–1975.

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.

Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R.G., and Gu, W. (2004). Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. Proc. Natl. Acad. Sci. USA *101*, 2259–2264.

Matsuoka, T.A., Zhao, L., Artner, I., Jarrett, H.W., Friedman, D., Means, A., and Stein, R. (2003). Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. Mol. Cell. Biol. *23*, 6049–6062.

Matsuoka, T.A., Artner, I., Henderson, E., Means, A., Sander, M., and Stein, R. (2004). The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. Proc. Natl. Acad. Sci. USA *101*, 2930–2933.

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.

Miyata, T., Maeda, T., and Lee, J.E. (1999). NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev. *13*, 1647–1652.

Motta, M.C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551–563.

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., 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., Arden, K.C., and Accili, D. (2003). The forkhead transcription factor foxo1 regulates adipocyte differentiation. Dev. Cell *4*, 119–129.

Naya, F.J., Huang, H.P., Qiu, Y., Mutoh, H., DeMayo, F.J., Leiter, A.B., and Tsai, M.J. (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev. *11*, 2323–2334.

Okamoto, H., Nakae, J., Kitamura, T., Park, B.C., Dragatsis, I., and Accili, D. (2004). Transgenic rescue of insulin receptor-deficient mice. J. Clin. Invest. *114*, 214–223.

Olbrot, M., Rud, J., Moss, L.G., and Sharma, A. (2002). Identification of beta-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. Proc. Natl. Acad. Sci. USA *99*, 6737–6742.

Pearson, M., and Pelicci, P.G. (2001). PML interaction with p53 and its role in apoptosis and replicative senescence. Oncogene *20*, 7250–7256.

Poitout, V., Olson, L.K., and Robertson, R.P. (1996). Chronic exposure of betaTC-6 cells to supraphysiologic concentrations of glucose decreases binding of the RIPE3b1 insulin gene transcription activator. J. Clin. Invest. *97*, 1041–1046.

Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J.C., and de The, H. (1998). PML induces a novel caspase-independent death process. Nat. Genet. *20*, 259–265.

Robertson, R.P., Harmon, J., Tran, P.O., Tanaka, Y., and Takahashi, H. (2003). Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes *52*, 581–587.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S.H., and Goldberg, A.L. (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell *117*, 399–412.

Schwitzgebel, V.M., Scheel, D.W., Conners, J.R., Kalamaras, J., Lee, J.E., Anderson, D.J., Sussel, L., Johnson, J.D., and German, M.S. (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development *127*, 3533–3542.

Sharma, A., Olson, L.K., Robertson, R.P., and Stein, R. (1995). The reduction of insulin gene transcription in HIT-T15 beta cells chronically exposed to high glucose concentration is associated with the loss of RIPE3b1 and STF-1 transcription factor expression. Mol. Endocrinol. *9*, 1127–1134.

Stitt, T.N., Drujan, D., Clarke, B.A., Panaro, F., Timofeyva, Y., Kline, W.O., Gonzalez, M., Yancopoulos, G.D., and Glass, D.J. (2004). The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol. Cell *14*, 395–403.

van der Horst, A., Tertoolen, L.G., de Vries-Smits, L.M., Frye, R.A., Medema, R.H., and Burgering, B.M. (2004). FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J. Biol. Chem. *279*, 28873–28879.

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.

Welsh, N., Margulis, B., Borg, L.A., Wiklund, H.J., Saldeen, J., Flodstrom, M., Mello, M.A., Andersson, A., Pipeleers, D.G., Hellerstrom, C., et al. (1995). Differences in the expression of heat-shock proteins and antioxidant enzymes between human and rodent pancreatic islets: implications for the pathogenesis of insulin-dependent diabetes mellitus. Mol. Med. *1*, 806–820.

Withers, D.J., Sanchez-Gutierrez, J., Towery, H., Burks, D.J., Ren, J.-M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G.I., et al. (1998). Disruption of IRS-2 causes type 2 diabetes in mice. Nature *391*, 900–904.

Zhao, L., Guo, M., Matsuoka, T.A., Hagman, D.K., Parazzoli, S.D., Poitout, V., and Stein, R. (2005). The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. J. Biol. Chem. *280*, 11887–11894.

Zhong, S., Hu, P., Ye, T.Z., Stan, R., Ellis, N.A., and Pandolfi, P.P. (1999). A role for PML and the nuclear body in genomic stability. Oncogene *18*, 7941–7947.