CHAPTER

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FOXO4 Is Acetylated upon Peroxide Stress and Deacetylated by the Longevity Protein hSir2^{SIRT1}*

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FOXO transcription factors have important roles in metabolism, cellular proliferation, stress tolerance, and aging. FOXOs are negatively regulated by protein kinase B/c-Akt-mediated phosphorylation. Here we show that FOXO factors are also subject to regulation by reversible acetylation. We provide evidence that the acetyltransferase CREB-binding protein (CBP) binds FOXO resulting in acetylation of FOXO. This acetylation inhibits FOXO transcriptional activity. Binding of CBP and acetylation are induced after treatment of cells with peroxide stress. Deacetylation of FOXOs involves binding of the NAD-dependent deacetylase hSir2^{SIRT1}. Accordingly, hSir2^{SIRT1}-mediated deacetylation precludes FOXO inhibition through acetylation and thereby prolongs FOXO-dependent transcription of stress-regulating genes. These data demonstrate that acetylation functions in a second pathway of negative control for FOXO factors and provides a novel mechanism whereby hSir2^{SIRT1} can promote cellular survival and increase lifespan.

The Forkhead box, class O subfamily of forkhead transcription factors (FOXO)[1] consists of the functionally related proteins FOXO1, FOXO3a, and FOXO4 (also known as FKHR, FKHRL1, and AFX, respectively; Ref. 1). The growth factor-stimulated phosphatidylinositol 3-kinase-protein kinase B (PKB)/c-Akt pathway negatively regulates FOXO factors by phosphorylation-mediated nuclear exclusion (2–4). This pathway is evolutionarily conserved between *Caenorhabditis elegans* and humans. DAF-16, the *C. elegans* homologue of mammalian FOXO, is also controlled by phosphatidylinositol 3-kinase/PKB signaling. DAF-16 regulates daver formation in larvae, and responses to various environmental stresses and longevity in adult worms (5–8). In parallel, mammalian FOXO transcription factors have been implicated in regulating metabolism, cell cycle progression, and stress tolerance (9, 10; reviewed in Ref. 11).

In *C. elegans*, overexpression of the NAD-dependent deacetylase Sir2 (silent information regulator 2) increases lifespan, which requires DAF-16 (12). The *Sir2* family of genes is a highly conserved group of genes with seven human homologues, of which the *SIRT1* gene encodes the closest homologue of yeast and *C. elegans* Sir2, hence named hSir2^{SIRT1} (13). Recently, deacetylation of p53 by hSir2^{SIRT1} has been demonstrated, and it has been suggested that this functions in increasing cellular resistance against stress. However, subsequent studies (14) showed that in HEK293T cells, which lack functional p53, activation of hSir2^{SIRT1} by resveratrol treatment still increases cellular resistance against gamma-radiation, thus suggesting alternative pathways. As DAF-16 is necessary for lifespan extension by Sir2, FOXOs may well function in such an alternative pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Treatment—HEK293T, DL23 (DLD-1 human colon carcinoma cells expressing a conditionally active version of FOXO3a; Ref. 10), and A14 cells (human insulin receptor overexpressing mouse NIH3T3 cells (15) and C2C12 mouse myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum. In all experiments, cells were cultured in the presence of 10% fetal bovine serum, unless stated otherwise. HEK293T cells were transiently transfected using FuGENE6 reagent according to the manufacturer's suggestions (Roche Applied Science). A14 cells were transfected using the calcium phosphate method. Total amounts of transfected DNA were equalized using pBluescript II KS(+). 1 μM trichostatin A (ICN), 5 mM nicotinamide (Nam, Supelco), 1 μg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 500 nM 4-hydroxy-tamoxifen (4-OHT), and 20–500 μM hydrogen peroxide (H_2O_2, Merck) were added as indicated. All experiments were performed at least three times and representative results are shown.

Plasmids, Oligonucleotides, and Recombinant Proteins—pMT2-HA-FOXO4, pRP261-GST-FOXO4-DB (3), pcDNA3-HA-FOXO3a.A3 (10), pcDNA3.1-hSir2^{SIRT1} (16), pBabe-puro (17), 6× DBE (18), and p27GL-1609 (19) have been described before. pcDNA3.1-myc-FOXO4 was created by ligating a Klenow-blunted SalI/NotI fragment from pMT2-HA-FOXO4 into Klenow-blunted BamHI/NotI digested pcDNA3.1-myc. GLOFLAG3-FLAG-FOXO4 was created by ligating a Klenow-blunted SalI/NotI fragment from pMT2-HA-FOXO4 into mungbean-blunted BamHI/NotI-digested GLOFLAG3. pcDNA3.1-myc-hSir2^{SIRT1} and pRP265-GST-hSir2^{SIRT1} were created by ligating a BamHI/XbaI fragment from pcDNA3.1-hSir2^{SIRT1} into BamHI/XbaI-digested pcDNA3.1-myc and pRP265, respectively.

pRc/RSV-CBP-HA and pRP265-GST-p300-HAT were kindly provided by R. Giles (20) and R. Vries (21), respectively. Gal4-DBD-CBP-HA constructs were a kind gift of E. Kalkhoven (22). pRL-TK (Tk-Renilla luciferase) was purchased from Promega.

siRNA duplexes to down-regulate hSir2^{SIRT1} expression (duplex 1,

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[1] The abbreviations used are: FOXO, Forkhead box, class O subfamily of forkhead transcription factors; Nam, nicotinamide; EGF, epidermal growth factor; 4-OHT, 4-hydroxy-tamoxifen; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; MnSOD, manganese superoxide dismutase; HA, hemagglutinin; PKB, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, short interfering RNA; DBE, Daf-16 family protein binding element; RIPA, radioimmune precipitation assay buffer.

sense sequence, CAACUUGUACGACGAAGACdTdT; duplex 2, sense sequence, GCUGAUGAACCGCUUGCUAdTdT; duplex 3, sense sequence, CGUCUUAUCCUCUAGUUCUdTdT) were purchased from Dharmacon RNA Technologies and cotransfected using OligofectAMINE according to the manufacturer (Invitrogen).

GST-FOXO4-DB, GST-p300-HAT, and GST-tagged hSir2^{SIRT1} were purified from bacteria using a standard GST-fusion protein-purification protocol (23).

Antibodies—Monoclonal 12CA5 and 9E10 antibodies were produced using hybridoma cell lines. Monoclonal antibodies recognizing the FLAG-M2 epitope, p27^{kip1}, and GAPDH were obtained from Sigma, Transduction Laboratories, and Chemicon, respectively. Polyclonal antibodies recognizing acetylated lysine residues (α-AcLys), actin, CBP, Gal4-DBD, MnSOD, and pS473-PKB were obtained from Cell Signaling Technologies, Santa Cruz Biotechnology (3×), Stressgen BioReagents, and New England Biolabs, respectively. Polyclonal antibodies recognizing FOXO3a were purchased from Sigma and Santa Cruz Biotechnology. Polyclonal antibodies recognizing FOXO4 (19) and PKB (15) have been described before. Polyclonal antibody recognizing hSir2^{SIRT1} was raised in a rabbit using an N-terminally ^{6}His-tagged fragment consisting of amino acids 506–747 of hSir2^{SIRT1}.

Western Blot Analysis—Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies.

FOXO Activity—To determine the expression of endogenous p27^{kip1} and MnSOD, HEK293T cells were transfected with empty vector or HA-FOXO4 together with pBabe-puro. Either constructs encoding Gal4-DBD-CBP-HA or myc-hSir^{SIRT1} were cotransfected or cells were treated using hydrogen peroxide or Nam for twenty-four hours. Twenty-four hours before harvest, 1 μg/ml puromycin was added to the cultures to select for transfected cells. Cells were lysed in 1× Laemmli sample buffer, and samples were analyzed by Western blot analysis. If indicated, DL23 cells treated for twenty-four hours with 4-OHT and/or Nam were used.

For luciferase assays, either A14 cells transiently transfected with a reporter construct bearing six canonical FOXO binding sites (6× DBE-luciferase) or a reporter cell line (A14-p27luc) stably expressing the human p27^{kip1}-promoter linked to luciferase (p27GL-1609) were used. Cells were treated as above. Luciferase counts were normalized using Tk-Renilla-luciferase. Samples were analyzed according to the manufacturer's instructions (Promega). To control for effects on basal transcription, the fold induction of luciferase expression was determined by dividing luciferase ratios from samples transfected with FOXO4 by the ratios from parallel samples transfected with empty vector. Statistical analysis was performed using a Student's t test. $p < 0.05$ was considered significant.

Flow cytometric analysis to determine the percentage of cells in the G_1-phase or S-phase of the cell cycle was essentially done as described (3). Cells were either transfected with HA-FOXO3a.A3 and hSir2^{SIRT1} or treated with 4-OHT (500 nM) and/or Nam (5 mM). Statistical analysis was performed using a Student's t test. $p < 0.05$ was considered significant.

For the measurement of reactive oxygen species, DL23 and A14 cells were loaded with 10 μg/ml CM-H_2DCFDA probe (Molecular Probes) in HEPES buffered saline for 5 min at 37 °C. Cells were incubated in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10 mM bicarbonate, pH 7.2, without phenol red at 37 °C and treated with 150 μM H_2O_2. Fluorescence was collected with a Leica DMR microscope. Excitation was done with a monochromator (SPEX Industries) at 490 nm (slit = 8 nm), >515 nm dichroic mirror, and a >515 nm emission filter. Ultra-low light conditions were applied only during image collection, under which no photo-activation of the cellular H_2DCFDA could be observed. Images were collected by integration of 20 frames at 10-s interval (fixed gain, intensifier/camera/grabber, during all experiments). Off-line analysis was done by quantification of the average pixel intensities of at least five individual cells/image during time in at least two separate experiments. Statistical analysis was performed using a Student's t test. $p < 0.05$ was considered significant.

Co-immunoprecipitation—For exogenous proteins, HEK293T cells were transfected with the indicated constructs. For CBP/FOXO4 co-immunoprecipitations, cells were left untreated or were treated with insulin plus EGF or hydrogen peroxide only for 30 min (see Fig. 4*A*) or 60 min (see Fig. 4*C*) or with insulin plus EGF for 30 min before adding hydrogen peroxide for 30 min (see Fig. 4*A*, as indicated). For hSir2^{SIRT1}/FOXO4 co-immunoprecipitations, cells were treated with Nam (5 mM) or hydrogen peroxide (200 μM) as indicated. Forty hours after transfection, cells were lysed in RIPA buffer. Immunoprecipitation samples were analyzed by Western blot analysis. For endogenous proteins, C2C12 or HEK293T cells were treated using 500 μM H_2O_2 for 1 h and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 5% glycerol, 5 mM EDTA, 100 mM NaCl, protease inhibitors). Immunoprecipitations were carried out essentially as described for exogenous proteins.

Acetylation—HEK293T cells were transfected with the indicated constructs. Cells were treated with Nam for 1 h or with hydrogen peroxide for the indicated times. Forty hours after transfection, cells were lysed in acetylation buffer (50 mM Tris-HCl, pH 7.5, 0.4% Triton X-100, 5 mM EDTA, 100 mM NaCl, protease inhibitors) or RIPA buffer. FOXO4 immunoprecipitation samples were analyzed by Western blot analysis.

In Vitro and in Vivo Deacetylation—For *in vitro* deacetylation, 2.5 μg GST-FOXO4-DB were incubated with 0.05 μCi [^{14}C]-acetyl-CoA (ICN) and 2.0 μg GST-p300-HAT in reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 150 mM NaCl (21) for 45 min at 30 °C. Subsequently, deacetylation buffer (50 mM Tris-HCl, pH 8.8, 4 mM $MgCl_2$, 0.2 mM dithiothreitol, 2 μM trichostatin A plus 1 mM NAD^+ if indicated (24) and 2.0 μg of GST-tagged wt-hSir2^{SIRT1} were added to a final volume of 100 μl. Samples were incubated for another 90 min at 37 °C, and the reaction was terminated using 5× Laemmli sample buffer. Samples were separated by SDS-PAGE and analyzed by autoradiography.

For *in vivo* deacetylation, HEK293T cells were transfected with the indicated constructs. Forty hours posttransfection cells were lysed in RIPA buffer, and myc-FOXO4 was immunoprecipitated from cleared lysates using 9E10 antibody. Immunoprecipitates and total lysates were subjected to Western blotting.

RESULTS

Nicotinamide Inhibits FOXO Transcriptional Activity—To investigate the possibility that hSir2^{SIRT1} controls FOXO function, we analyzed the effect of Nam, an inhibitor of the Sir2 family of deacetylases (25), on FOXO4 transcriptional activity. Nam inhibited FOXO4-mediated transcription of a luciferase reporter construct bearing six canonical FOXO DNA-binding sites (Fig. 1*A*; Ref. 18). Previously, we have shown that FOXO transcriptionally regulates the expression of the cell-cycle inhibitor p27^{kip1} (19). In support of this, expression of FOXO4 in HEK293T cells increased p27^{kip1} expression (Fig. 1*B*). Importantly, Nam inhibited the expression of the endogenous *p27^{kip1}* gene induced by FOXO4 (Fig. 1*B*). Inhibition of p27^{kip1} expression by Nam in these cells was not due to PKB activation, as Nam treatment under these conditions did not change phosphorylation of PKB on Ser-473, which is indicative of PKB activity. Also, in A14 cells stably expressing the luciferase gene driven by the p27^{kip1} promoter (A14-p27luc), FOXO4-induced luciferase expression was reduced by Nam (data not shown). These data demonstrate that the Sir2-inhibitor Nam inhibits FOXO4 transcriptional activity, possibly via hSir2^{SIRT1}.

Next we investigated whether Nam also affects the biological activity of FOXO transcription factors. Previously, we have employed DL23 cells, DLD-1 human colon carcinoma cells expressing an inducible conditionally active version of FOXO3a and lacking functional p53, and demonstrated in these cells transcriptional regulation of p27^{kip1} expression and induction of a G_1 cell-cycle arrest by FOXO (10). Treatment of DL23 cells with 4-OHT induced p27^{kip1} expression (Fig. 1*C*, *left panel*). This induction was reduced by treatment of these cells with Nam (Fig. 1*C*, *left panel*). Furthermore, FOXO3a activation imposed a cell-cycle arrest, which was partially relieved by treatment with Nam, in keeping with its effect on p27^{kip1} expression (Fig. 1*C*, *right panel*). In addition to imposing a cell-cycle arrest, FOXO activation increases cellular resistance against oxidative stress by transcriptional regulation of the gene encoding manganese superoxide dismutase (MnSOD) (10). Activation of FOXO in DL23 cells by 4-OHT treatment increased MnSOD expression, and, similar to FOXO-induced p27^{kip1} expression, MnSOD levels were reduced in cells treated with Nam (Fig. 1*C*, *left panel*). In keeping with MnSOD mediating FOXO-induced cellular resistance against oxidative

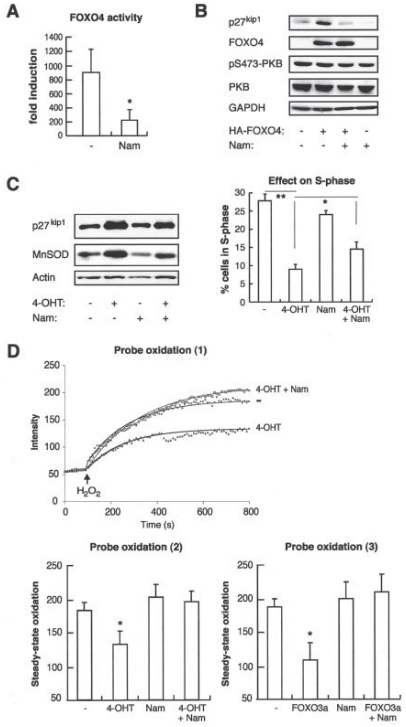

FIG. 1. **Nam inhibits the transcriptional and biological activity of FOXO4.** *A*, A14 cells were transfected with empty vector or a construct encoding HA-FOXO4 together with 6× DBE-luciferase and treated with Nam. All samples were assayed in triplicate. Luciferase counts were normalized using Tk-Renilla-luciferase (luciferase ratio), and the fold induction of luciferase expression was determined by dividing luciferase ratios from samples transfected with FOXO4 by the ratios from parallel samples transfected with empty vector to control for effects on basal transcription. *, $p < 0.05$. *B*, HEK293T cells were transfected with empty vector or a construct encoding HA-FOXO4 and treated with Nam. Lysates of transfected cells were assayed for $p27^{kip1}$, HA-FOXO4, pS473-PKB, and PKB expression. GAPDH expression was used to monitor equal protein loading. *C*, DL23 cells were treated for twenty-four hours using Nam and/or 4-OHT. Total lysates were analyzed by Western blotting for the expression of $p27^{kip1}$, MnSOD, and actin (*left panel*). The percentage of DL23 cells in S-phase was determined by flow cytometry of bromodeoxyuridine-stained cells. Histograms were analyzed using ModFit LT software (*right panel*). *, $p < 0.05$; **, $p < 0.001$. *D*, the kinetics of CM-H_2CDFA probe oxidation in DL23 cells (*upper* and *lower left panels*) and A14 cells (*lower right panel*) was measured after H_2O_2 (150 μM) addition (average of 4–5 cells/experiment). Curves were fitted with a single exponent according to $f(x) = e^{-kt}$, using χ^2 minimalization. All measurements had similar half-lives ($\tau = 172 \pm 14$ s) (*left panel*). The line representing the control + Nam was omitted from the graph for reasons of clarity (it was in between the control and 4-OHT + Nam lines). Steady-state oxidation levels were measured at 800 s (*lower panels*). *, $p < 0.05$. The experiments presented are representative of at least three independent experiments.

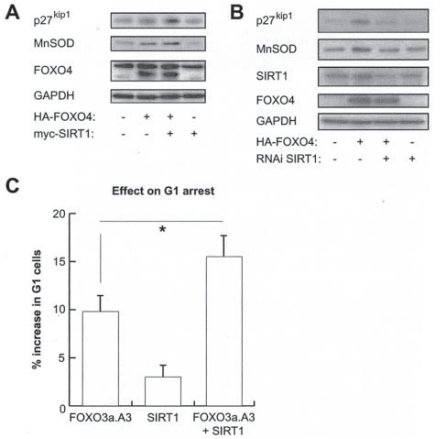

FIG. 2. **hSir2^{SIRT1} enhances FOXO4 transcriptional and biological activity.** *A*, HEK293T cells were transfected as described for Fig. 1*B* together with a myc-hSir2^{SIRT1} construct. $p27^{kip1}$, MnSOD, and HA-FOXO4 expressions were examined by Western blotting. GAPDH expression was used to monitor equal loading. *B*, HEK293T cells were transfected with three different hSir2^{SIRT1}-targeting siRNA oligonucleotides and/or HA-FOXO4. Forty hours after transfection, cells were harvested and total lysates were assayed for $p27^{kip1}$, MnSOD, hSir2^{SIRT1}, and FOXO4 expression. GAPDH was used to check for equal loading. *C*, A14 cells were transfected with empty vector or HA-FOXO3a.A3 together with hSir2^{SIRT1}. Cells were harvested forty hours posttransfection, and the increase in percentage of cells in the G_1-phase of the cell cycle as compared with untransfected cells was determined by flow cytometry of propidium iodide-stained cells. Histograms were analyzed using ModFit LT software. *, $p < 0.05$. The experiments presented are representative of at least three independent experiments.

stress, H_2O_2-induced oxidative stress was reduced in cells treated with 4-OHT, whereas Nam treatment inhibited the effect of FOXO3a (Fig. 1*D*, *upper* and *lower left panels*). In addition, A14 cells expressing active FOXO3a after retroviral transduction displayed increased resistance against H_2O_2-induced oxidative stress, and this was also reversed by Nam treatment (Fig. 1*D*, *lower right panel*). These results show that inhibition of Sir2 function by Nam treatment inhibits FOXO-induced gene expression of $p27^{kip1}$ and MnSOD and, consequently, FOXO-induced cell-cycle arrest and resistance against oxidative stress.

hSir2^{SIRT1} Stimulates FOXO Transcriptional Activity—Next we determined whether the deacetylase inhibitor Nam indeed exerts its inhibitory effect on FOXO-mediated transcription through hSir2^{SIRT1}. We first tested the effect of hSir2^{SIRT1} expression on FOXO4 activity. To this end, hSir2^{SIRT1} and FOXO4 were transfected into HEK293T cells, and the expression of endogenous $p27^{kip1}$ and MnSOD was determined. Both genes were up-regulated by hSir2^{SIRT1} in a FOXO-dependent manner (Fig. 2*A*). Also, in A14-p27luc cells, FOXO4-induced luciferase expression was stimulated by co-expression of hSir2^{SIRT1} (data not shown). To further demonstrate involvement of hSir2^{SIRT1} in regulating FOXO4 transcriptional activity, we used siRNA to hSir2^{SIRT1}, which resulted in reduced levels of endogenous hSir2^{SIRT1} (Fig. 2*B*). In keeping with the inhibitory effect of Nam on FOXO activity, knock-down of hSir2^{SIRT1} impaired FOXO4-induced $p27^{kip1}$ expression. Expression of MnSOD, another transcriptional target of FOXOs (10), was also reduced by siRNA to hSir2^{SIRT1}. As regulation of $p27^{kip1}$ is important in mediating a FOXO-induced cell-cycle arrest, we also analyzed the effect of hSir2^{SIRT1} on FOXO-induced cell-cycle arrest. In keeping with the effect of Nam (see Fig. 1*C*), hSir2^{SIRT1} increased the percentage of cells arrested in G_1 (Fig. 2*C*). Thus, these data indicate that hSir2^{SIRT1} enhances the transcriptional activity of FOXO and thereby

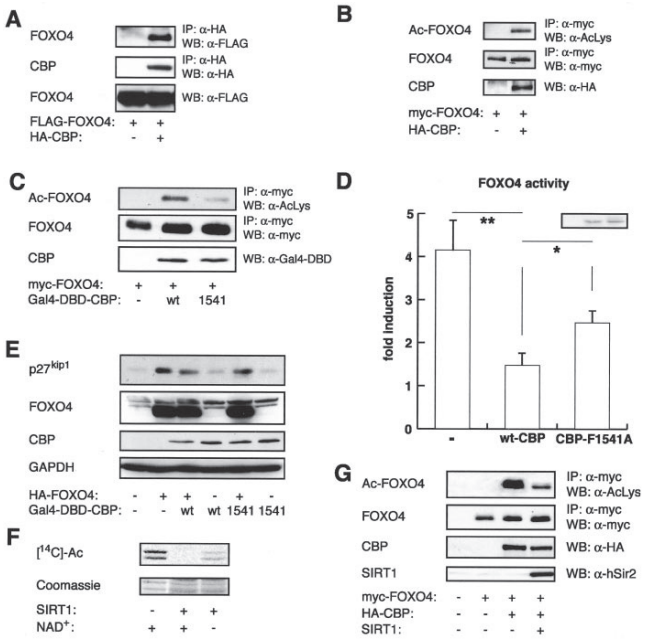

FIG. 3. **CBP inhibits FOXO4 transcriptional activity through acetylation and hSir2^{SIRT1} *deacetylates FOXO4.*** *A*, HEK293T cells were transfected with constructs encoding HA-CBP and FLAG-FOXO4. CBP immunoprecipitates and total lysates were analyzed by Western blotting using anti-HA (12CA5) and anti-FLAG-M2 antibodies. *B*, HEK293T cells were transfected with constructs encoding HA-CBP and myc-FOXO4. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-acetylated lysine (α-*AcLys*), anti-myc (9E10), and anti-HA (12CA5) antibodies. *C*, HEK293T cells were cotransfected with a construct encoding HA-FOXO4 together with empty vector, GAL4-DBD-wt-CBP, or an acetyltransferase-impaired mutant (CBP-F1541A). Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-acetylated lysine (α-*AcLys*), anti-myc (9E10), and anti-Gal4-DBD antibodies. *D*, A14-p27luc cells were transfected with the constructs used in Fig. 2*C*. Samples were analyzed as described for Fig. 1*A*. Similar results were obtained with two other independent A14-p27luc cell lines. *Inset*, CBP expression. *, $p < 0.05$; **, $p < 0.01$. *E*, HEK293T cells were transfected as described for Fig. 1*B* together with the CBP constructs used in Fig. 2, *C* and *D*. p27^{kip1}, HA-FOXO4, and GAL4-DBD-CBP expression were examined by Western blotting. GAPDH expression was used to monitor equal loading. *F*, bacterially expressed GST-FOXO4-DB was acetylated using GST-p300-HAT and [^{14}C]acetyl CoA. Subsequently, bacterially expressed GST-hSir2^{SIRT1} was added in the presence or absence of NAD$^+$ for 90 min. Samples were assayed by Coomassie blue staining and autoradiography. *G*, HEK293T cells were transfected with constructs encoding myc-FOXO4, HA-CBP, and hSir2^{SIRT1}. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-acetylated lysine (α-*AcLys*), anti-myc (9E10), anti-HA (12CA5), and anti-hSir2^{SIRT1} antibodies. The experiments presented are representative of at least three independent experiments.

modulates its biological activity.

CBP Acetylates FOXO and Inhibits FOXO Transcriptional Activity—As Sir2 deacetylases are generally thought to act as repressors of transcription via deacetylating histones (26) we hypothesized that hSir2^{SIRT1} directly deacetylates FOXO transcription factors. Acetylation of proteins is mediated by acetyltransferases such as the related proteins p300 and CBP; binding between p300/CBP and FOXO as well as FOXO acetylation have been reported (27–29). To confirm these findings, HA-CBP and FLAG-FOXO4 were co-expressed in HEK293T cells. Indeed FLAG-FOXO4 was found to bind to HA-CBP (Fig. 3*A*). More importantly, binding of CBP to FOXO4 induced acetylation of FOXO4 (Fig. 3*B*). Also, co-expression of CBP and FOXO3a resulted in acetylation of FOXO3a (data not shown), suggesting that CBP is able to mediate acetylation of other FOXO transcription factors. Similarly, co-expression of p300 and FOXO4 induced acetylation of FOXO4 (data not shown), indicating that CBP and p300 are functionally equivalent in FOXO4 acetylation.

Because inhibiting hSir2^{SIRT1} activity by either Nam or siRNA-mediated knockdown of hSir2^{SIRT1} inhibited transcriptional activity of FOXO, we next determined the effect of CBP-mediated acetylation of FOXO4 on its transcriptional activity. In A14-p27luc cells expressing wt-CBP, FOXO4 activity was considerably impaired as compared with control cells expressing FOXO4 only (Fig. 3*D*). This repression was due to the acetyltransferase activity of CBP as co-expression of the CBP-mutant F1541A, which possesses reduced but not absent acetyltransferase activity (Fig. 3*C*; Ref. 30), impaired FOXO4 transcriptional activity to a smaller extent. We also analyzed the effect of CBP expression on the endogenous FOXO target gene p27^{kip1}. Consistent with the reporter assays, wt-CBP inhibited the induction of p27^{kip1} expression by FOXO4, whereas the F1541A mutant displayed a smaller effect because of residual activity (Fig. 3*E*). Taken together, these data indicate that CBP inhibits FOXO4 transcriptional activity by acetylation, in agreement with the observed inhibition of transcriptional activity of FOXO4 by the deacetylase inhibitor Nam.

hSir2^{SIRT1} Deacetylates FOXO4—As CBP and p300 can acetylate FOXO transcription factors, we tested whether hSir2^{SIRT1} could deacetylate FOXO4 *in vitro*. To this end, bacterially expressed FOXO4-DNA-binding domain (GST-FOXO4-

DB) was *in vitro* acetylated by a GST-tagged p300 acetyltransferase domain in the presence of [^{14}C]acetyl-CoA (21). Subsequently, the reactions were incubated with GST-tagged hSir2^{SIRT1} in the absence or presence of the essential cofactor NAD^+ (24). GST-hSir2^{SIRT1} completely deacetylated GST-FOXO4-DB in an NAD-dependent manner (Fig. 3*F*). The incomplete inhibition of deacetylase activity by omitting NAD^+ was possibly due to traces of NAD^+ in the GST-hSir2^{SIRT1} purification. Next, we tested deacetylation by hSir2^{SIRT1} *in vivo*. In HEK293T cells, a clear deacetylation of CBP-acetylated FOXO4 was observed upon cotransfection with hSir2^{SIRT1} (Fig. 3*G*). Taken together, these results show that hSir2^{SIRT1} can directly deacetylate FOXO4 both *in vitro* and *in vivo* and thereby stimulate FOXO transcriptional and biological activity.

Hydrogen Peroxide Induces Acetylation of FOXO and Inhibits FOXO Activity—To investigate whether extracellular signaling could regulate acetylation/deacetylation of FOXO4, we tested the effects of insulin/insulin-like growth factor-1, a known regulator of FOXO function, and hydrogen peroxide. Hydrogen peroxide is known to promote acetylation of proteins like p53 and histones (31, 32), and FOXO transcription factors have been shown to be important in regulating stress responses (10). First, we investigated whether hydrogen peroxide could affect binding of CBP to FOXO4. Therefore, HA-CBP was immunoprecipitated from HEK293T cells co-expressing myc-FOXO4. Increasing amounts of hydrogen peroxide enhanced the interaction between HA-CBP and myc-FOXO4 (Fig. 4*A*), suggesting that peroxide stress may induce acetylation of FOXO4 by CBP. Furthermore, activation of phosphatidylinositol 3-kinase/PKB signaling by treating cells with insulin and epidermal growth factor decreased basal binding between HA-CBP and myc-FOXO4. As CBP is localized exclusively within the nucleus, the decreased binding observed is likely due to the fact that phosphatidylinositol 3-kinase/PKB signaling induces relocalization of myc-FOXO4 to the cytosol. Importantly, activation of phosphatidylinositol 3-kinase/PKB signaling could not prevent the increase of HA-CBP/myc-FOXO4 complex formation induced by hydrogen peroxide treatment, indicating that the effect of peroxide is dominant over growth factor stimulation in this respect. Next, we determined whether endogenous FOXO and CBP could interact. We detected endogenous interaction between CBP and both FOXO4 and FOXO3a. Similar to the transient expression studies, H_2O_2 treatment increased the interaction between FOXO and CBP, although the magnitude differed (Fig. 4*B*). Finally, we tested whether enhanced binding of CBP to FOXO4 after treatment of cells with hydrogen peroxide resulted in increased acetylation of FOXO4. Thus, HEK293T cells transfected with a myc-FOXO4 construct were treated with different concentrations of peroxide and followed up for several periods of time. FOXO4 was immunoprecipitated and acetylation was determined by Western blotting. Hydrogen peroxide treatment indeed induced acetylation of FOXO4 (Fig. 4*C*). Also, treatment of cells cotransfected with FLAG-FOXO4 and HA-CBP with hydrogen peroxide resulted in increased acetylation in the fraction of FLAG-FOXO4 bound to HA-CBP (Fig. 4*D*). As noted, we detected acetylation by endogenous acetyltransferases only at later timepoints than would be suggested by the binding of CBP to FOXO4 (Fig. 4, *A* and *B*). As we used a pan-acetyllysine-antibody, we attribute this result to the low sensitivity of the antibody toward FOXO4, precluding detection of a low stoichiometry. This is further suggested by the observation that upon overexpression of CBP, we detected acetylation at shorter timepoints after peroxide treatment. Therefore, increased binding of FOXO4 to CBP after hydrogen peroxide treatment likely leads to increased acetylation.

As hydrogen peroxide induces acetylation of FOXO4, we expected that peroxide would have the same effect as CBP or Nam on FOXO4 transcriptional activity. To study this, FOXO4 was transfected in HEK293T cells, and the expression of endogenous p27^{kip1} was determined upon treatment of these cells with various concentrations of hydrogen peroxide for twenty-four hours. Indeed, hydrogen peroxide inhibited p27^{kip1} expression (Fig. 4*E*). Inhibition of p27^{kip1} expression was not due to activation of PKB by hydrogen peroxide, as under these conditions we could not detect any changes in the Ser-473 phosphorylation of PKB. Taken together, these data indicate that hydrogen peroxide inhibits FOXO4 transcriptional activity through induction of FOXO4 acetylation.

Hydrogen Peroxide Stimulates Binding of hSir2^{SIRT1} to FOXO—Finally, because acetylation of FOXO4 occurs in conjunction with binding of the acetyltransferase CBP, we also investigated whether deacetylation by hSir2^{SIRT1} involves direct binding. Therefore, we expressed myc-hSir2^{SIRT1}, HA-FOXO4, and treated cells with Nam or hydrogen peroxide for one hour to induce acetylation of HA-FOXO4. Complex formation between hSir2^{SIRT1} and FOXO4 could only be detected after peroxide treatment (Fig. 4*F*), suggesting that hSir2^{SIRT1} binds to acetylated FOXO4. On the contrary, Nam was not able to stabilize the interaction of hSir2^{SIRT1} with FOXO4, indicating that the effect of Nam on FOXO transcriptional activity can not be accounted for by mere binding of hSir^{SIRT1} to FOXO. We also tested whether FOXO and hSir2^{SIRT1} interacted endogenously. Antibody quality precluded the detection of a clear interaction between FOXO4 and hSir2^{SIRT1}, but in HEK293T cells, an interaction between endogenous FOXO3a and hSir2^{SIRT1} was detected, and this interaction was weakly induced by peroxide treatment (Fig. 4*G*). Taken together, these data show that hSir2^{SIRT1} directly binds to FOXO, which is induced by peroxide stress.

DISCUSSION

Our results suggest a model in which FOXO transcription factors are subject to regulation by reversible acetylation. Acetylation of FOXOs results in inhibition of their transcriptional and biological activities, which is reversed by the longevity protein hSir2^{SIRT1}. Inhibition through acetylation of FOXO transcription factors is functionally equivalent to the previously described inhibition by PKB-mediated phosphorylation.

We show that the acetyltransferase CBP binds to and acetylates FOXO4, thereby inhibiting its transcriptional activity. Previous reports on CBP/p300 binding to FOXO (27, 28) suggested that CBP/p300 binding is required for activation of FOXO-dependent transcription. However, there are several important differences between these and our studies that may explain this apparent discrepancy. First, Nasrin *et al.* (28) inferred a role for CBP/p300 in the activation of FOXO because they observed that the viral protein E1A, which can inhibit CBP co-activator function, also inhibits FOXO transcriptional activity on an insulin-like growth factor BP1 promoter. However, E1A is known to have a plethora of targets within the transcriptional machinery and thus, by itself, a block by E1A is not sufficient proof of the involvement of CBP/p300 (for a review, see Ref. 33). Second, whereas we have also analyzed the effect of CBP on the regulation of endogenous gene transcription, other studies have relied on transient reporter assays in which a proper chromatin context is likely to be lacking. Third, the role of CBP/p300 can be promoter-context-dependent, and accordingly, different FOXO-responsive genes may respond differently to the presence of CBP/p300. Differential responsiveness could relate to the fact that we have to discriminate between the effect of CBP/p300 as histone acetyltransferase and as acetyltransferase acting on FOXO itself. It could be

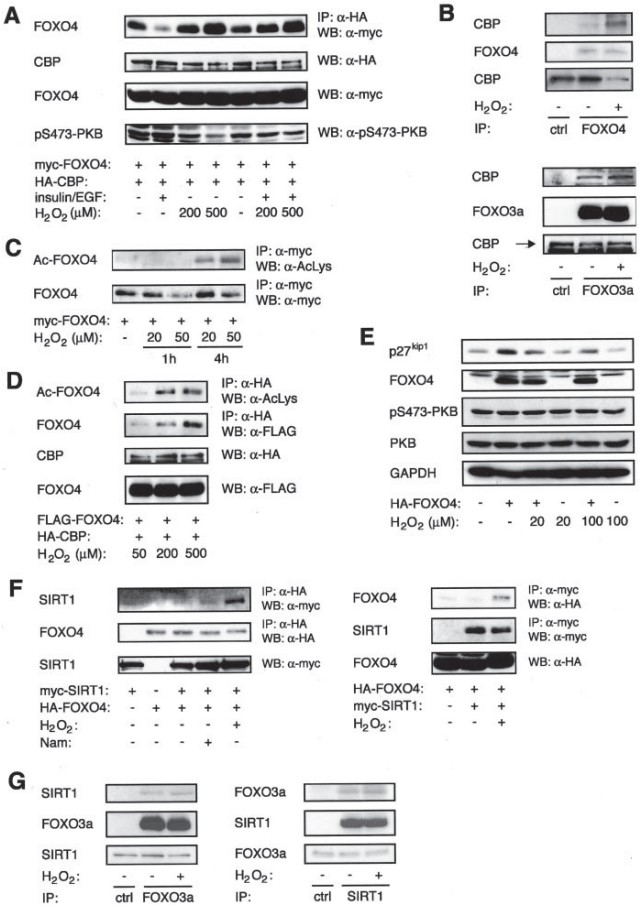

FIG. 4. **Peroxide stress induces FOXO4 acetylation, thereby inhibiting FOXO4 transcriptional activity, and induces hSir2^{SIRT1} binding to FOXO.** *A*, HEK293T cells were transfected with constructs encoding HA-CBP and myc-FOXO4. Cells were treated with the indicated concentrations of peroxide or insulin plus EGF for 30 min, and CBP was immunoprecipitated from cellular lysates. *Two lanes* at *far right*, insulin and EGF were given 30 min before adding peroxide. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-HA (12CA5), anti-myc (9E10), and anti-pS473-PKB antibodies. *B*, C2C12 and HEK293T cells were treated for 1 h with 500 μM H_2O_2. Endogenous FOXOs (*FOXO4* from C2C12 in *upper panel*, *FOXO3a* from HEK293T in *lower panel*) were immunoprecipitated, and immune complexes were assayed for the presence of CBP. Total lysates were also checked for CBP expression. *C*, HEK293T cells were transfected with a construct encoding myc-FOXO4 and treated with the indicated concentrations of hydrogen peroxide for 1 or 4 h. FOXO4 immunoprecipitation samples were analyzed by Western blotting using anti-acetyllysine (α-*AcLys*) and anti-myc (9E10) antibodies. *D*, the experiment was performed essentially as in *A*. Immunoprecipitates were first subjected to anti-acetyllysine (α-*AcLys*) antibody before probing for FOXO4. *E*, HEK293T cells were transfected as in Fig. 1*B* and treated with peroxide. p27^{Kip1}, HA-FOXO4, pS473-PKB, and PKB expression were examined by Western blotting. GAPDH expression was used to monitor equal loading. *F*, HEK293T cells were transfected with constructs encoding myc-hSir2^{SIRT1} and HA-FOXO4. Cells were treated with 200 μM hydrogen peroxide or 5 mM Nam. Either HA-FOXO4 (*left panel*) or myc-hSir2^{SIRT1} (*right panel*) was immunoprecipitated from cellular lysates. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-HA (12CA5) and anti-myc (9E10) antibodies. *G*, HEK293T cells were treated for 1 h with 500 μM H_2O_2. Endogenous FOXO3a (*left panel*) or hSir2^{SIRT1} (*right panel*) were immunoprecipitated, and immune complexes were assayed for the presence of hSir2^{SIRT1} and FOXO3a, respectively. Total lysates were also checked for hSir2^{SIRT1} and FOXO3a expression, respectively. The experiments presented are representative of at least three independent experiments.

possible that, with respect to different promoters, these activities of CBP/p300 may synergize or antagonize. Because CBP/p300-mediated histone acetylation is generally considered to contribute to transcriptional activation, and as we observe inhibition of FOXO transcriptional activity, we conclude that in the context of the promoters analyzed here, the direct acetylation of FOXO acts as an inhibitory signal.

The binding of CBP to FOXO4 as well as the acetylation of FOXO4 is induced upon treatment of cells with hydrogen peroxide. Furthermore, hSir2^{SIRT1} can bind to acetylated FOXO4

and consequently deacetylate FOXO4. Thus, hSir2^{SIRT1} can counteract the inhibition of FOXO induced by acetylation. Recently, regulation of p53 acetylation by hSir2^{SIRT1} has been described (16, 24), and this has been implicated in mediating cellular protection against genotoxic stresses. However, apoptosis induced by loss of hSir2^{SIRT1} is only partially rescued by dominant-negative p53 (16). Furthermore, in cells lacking functional p53, resveratrol, a presumed activator of hSir2^{SIRT1}, can still enhance protection against genotoxic stress (14). These observations suggest alternative p53-independent pathways involved in cellular protection by hSir2^{SIRT1}. Recently, we have shown that FOXO activation results in increased resistance against oxidative stress (10), and here we show that this is regulated by hSir2^{SIRT1}. We like to propose that FOXOs play an important role in the hSir2^{SIRT1}-mediated, p53-independent protection against stress. Therefore, by preventing negative regulation of FOXO due to acetylation after increased oxidative stress, hSir2^{SIRT1} can enhance cellular defenses against oxidative stress provided for by FOXO. This may be especially relevant under conditions of endogenous oxidative stress occurring during normal metabolic activities. Oxidative stress is considered to be a prime parameter of aging in all living organisms. Therefore, the results presented in this report provide a rationale for the observed increase in longevity induced by *C. elegans* Sir2, which is FOXO-dependent.

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