Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase

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The Sir2 deacetylase modulates organismal life-span in various species. However, the molecular mechanisms by which Sir2 increases longevity are largely unknown. We show that in mammalian cells, the Sir2 homolog SIRT1 appears to control the cellular response to stress by regulating the FOXO family of Forkhead transcription factors, a family of proteins that function as sensors of the insulin signaling pathway and as regulators of organismal longevity. SIRT1 and the FOXO transcription factor FOXO3 formed a complex in cells in response to oxidative stress, and SIRT1 deacetylated FOXO3 in vitro and within cells. SIRT1 had a dual effect on FOXO3 function: SIRT1 increased FOXO3's ability to induce cell cycle arrest and resistance to oxidative stress but inhibited FOXO3's ability to induce cell death. Thus, one way in which members of the Sir2 family of proteins may increase organismal longevity is by tipping FOXO-dependent responses away from apoptosis and toward stress resistance.

Although all species have a defined life-span, simple modifications of the environment, including caloric restriction or sublethal levels of stress, can substantially affect organismal longevity. A pivotal regulator of organismal life-span is the Sir2 (sensing information regulator 2) gene (1). Deletion of Sir2 in yeast abolishes the increase in life-span induced by caloric restriction or sublethal levels of stress, indicating that Sir2 is a mediator of signals that promote longevity (2, 3). Expression of extra copies of Sir2 is sufficient to increase life-span in yeast (4) and in Caenorhabditis elegans (5), suggesting that the role of Sir2 in extending organismal life-span is conserved through evolution.

Sir2 is a nicotinamide adenine dinucleotide (NAD)–dependent histone deacetylase (6, 7). In mammals, there are seven members of the Sir2 family, termed sirtuins (SIRTs), among which SIRT1 is the closest homolog of yeast Sir2 (8). We will here refer to mammalian Sir2 as SIRT1. In addition to deacetylating histones, SIRT1 also deacetylates other proteins, including MyoD and the tumor suppressor p53 (9–13). However, whether these proteins mediate the effects of the Sir2 family on longevity or whether Sir2 has other important substrates that account for its effect on longevity is still unclear.

In C. elegans, the ability of Sir2 to extend life-span depends on the presence of Daf-16, a member of the FOXO family of Forkhead transcription factors (5, 14, 15). We hypothesized that, in higher organisms, FOXO transcription factors might be direct substrates of SIRT1. In mammals, there are four evolutionarily conserved FOXO family members (FOXO1, FOXO3, FOXO4, and FOXO6) that are negatively regulated by the insulin-phosphoinositide-3 kinase (PI3K)–Akt signaling pathway (16–19). Mammalian FOXO factors control various biological functions, including cell cycle arrest at the G1-S and G2-M checkpoints (20, 21), detoxification of reactive oxygen species (ROS) (22, 23), repair of damaged DNA (21), and apoptosis (16, 24). Because the ability to detoxify ROS and to repair damage is correlated with increased organismal longevity in many species (25), these particular functions of FOXO transcription factors may be relevant to FOXO’s ability to control longevity.

We sought to identify the conditions under which FOXO transcription factors are highly acetylated in mammalian cells, because these might be conditions in which SIRT1 regulates FOXO transcription factors. We treated cells with various extracellular stimuli and performed Western blot experiments with antibodies to acetylated lysine (Fig. 1A). These experiments revealed that the FOXO family member FOXO3 is acetylated within cells and that the acetylation of FOXO3 is increased in response to hydrogen peroxide (H2O2) and slightly enhanced in response to heat shock (Fig. 1A) (26). In contrast, FOXO3 acetylation was not affected by ultraviolet (UV) irradiation or growth factor stimulation (Fig. 1A) (26). Thus, FOXO3 acetylation appears to be specifically induced in response to oxidative stress stimuli. Consistent with this observation, we found that protein acetylases, including p300/CREB-associated factor (PCAF), interact with FOXO3 in a stress-inducible manner (fig. S1).

We tested whether SIRT1 might be recruited to FOXO3 to possibly regulate FOXO3 acetylation. In the absence of stress stimuli, when growth factors are present, SIRT1 is a nuclear protein (10, 11), whereas FOXO transcription factors are localized in the cytoplasm (16, 18). However, immunostaining experiments revealed that, when growth factors are present, oxidative stress stimuli, including H2O2, menadione, and heat shock, triggered the translocation of FOXO3 from the cytoplasm to the nucleus (Fig. 1B). In the absence of growth factors, FOXO3 was already in the nucleus, and under these conditions oxidative stress stimuli did not affect FOXO3 subcellular localization (Fig. 1B) (27). In contrast to FOXO3, SIRT1 was always nuclear, in the absence or presence of stress stimuli (26). Thus, under conditions of oxidative stress, FOXO3 and SIRT1 are both present within the nucleus.

To determine whether FOXO3 and SIRT1 interact in response to oxidative stress, we performed coimmunoprecipitation experiments in cells that expressed a hemagglutinin epitope (HA)–tagged form of FOXO3 and a Flag-tagged version of SIRT1 (28). SIRT1 and FOXO3 interacted, and this interaction was increased in response to several types of oxidative stress stimuli (H2O2, menadione, and heat shock), but not in response to UV or gamma irradiation or to growth factors (Fig. 1C).

We tested whether the interaction between FOXO3 and SIRT1 was detectable when both proteins were expressed at their endogenous levels. Immunoprecipitation experiments with an antibody to FOXO3 or a control preimmune immunoglobulin G (IgG) revealed that endogenous FOXO3 interacted with endogenous SIRT1 in 293T cells and in cerebellar granule neurons and that this interaction increased in response to oxidative stress (Fig. 1, D and E) (26). These results indicate that the interaction between endogenous FOXO3 and SIRT1 is detectable in primary cells and immortalized cell lines and that the FOXO3–SIRT1 interaction is enhanced in response to oxidative stress.

We next asked if the presence of FOXO3 in the nucleus was sufficient to promote the
interaction of FOXO3 with SIRT1. A mutant form of FOXO3 that is constitutively nuclear because its three Akt phosphorylation sites have been mutated to alanines (16) did not interact with SIRT1 in the absence of stress stimuli (Fig. 1F). Thus, the presence of FOXO3 in the nucleus appears not to be sufficient to promote the interaction between SIRT1 and FOXO3. We have found by mass spectrometry that FOXO3 is acetylated at five different lysine residues and phosphorylated at eight serine or threonine residues in the presence of stress stimuli (fig. S2). Therefore, a combination of stress-induced FOXO3 acetylation and phosphorylation events may promote the interaction between SIRT1 and FOXO3.

The observations that SIRT1 and FOXO3 interact and that FOXO3 is acetylated raised the possibility that, within this protein complex, FOXO3 is a substrate of SIRT1. To determine if SIRT1 directly deacetylated FOXO3, we first acetylated FOXO3 in vitro by incubating FOXO3 with PCAF or the p300 acetylase, in the presence of acetyl–coenzyme A (acetyl-CoA), and then used the acetylated form of FOXO3 as a substrate for recombinant SIRT1 (rSIRT1) (28). rSIRT1 deacetylated FOXO3 that had been acetylated by PCAF or p300 in vitro only in the presence of the cofactor NAD (Fig. 2A).

We next tested whether SIRT1 deacetylated FOXO3 that had been acetylated within cells in response to oxidative stress. We purified FOXO3 from 293T cells that were treated in the presence of H2O2 and incubated the purified acetylated form of FOXO3 with rSIRT1 (Fig. 2B). rSIRT1 deacetylated the stress-induced acetylated form of FOXO3 in vitro only in the presence of NAD. The deacetylation of FOXO3 by rSIRT1 was partially inhibited by BML-210, an inhibitor of SIRT1, but not by Trichostatin (TSA), an inhibitor of class I and class II histone deacetylases (HDACs) (Fig. 2B). Thus, SIRT1 appears to deacetylate FOXO3 in vitro.

To determine if SIRT1 might contribute to FOXO3 deacetylation within cells, we incubated 293T cells with SIRT1 chemical inhibitors (nicotinamide, BML-210, and splitomycin) and with TSA. We then immunoprecipitated Flag-FOXO3 from these cells and analyzed the immune complex by Western blot with antibodies to acetylated lysine (28). Treatment with SIRT1 inhibitors alone or with TSA alone had no effect on FOXO3 acetylation (Fig. 2C). By contrast, incubation of cells with TSA and nicotinamide together

**Fig. 1.** Interaction of SIRT1 and FOXO3 in response to oxidative stress. (A) Acetylation of FOXO3 in cells in response to stress stimuli. 293T cells expressing the Flag-tagged form of FOXO3 were treated with H2O2 (500 μM, 1 hour), UV radiation (500 J/m², 1 hour), or heat shock (HS, 42°C, 1 hour). The acetylation of immunoprecipitated FOXO3 was assessed by Western blot with the antibodies to acetylated lysine (Acetyl-K) and total levels of FOXO3 by Western blot with an antibody to Flag. (B) Localization of FOXO3 in response to stress. CCl39 fibroblasts were transfected with the HA-tagged form of FOXO3 and were treated with heat shock (42°C) in the absence or presence of growth factors. Immunofluorescence experiments were done with an antibody to HA. Data represent the mean and error of two independent experiments. C, cytoplasmic FOXO3; N, nuclear FOXO3. (C) Interaction of transfected FOXO3 and SIRT1. 293T cells were cotransfected with the HA-tagged version of FOXO3 and a Flag-tagged version of human SIRT1. Cells were treated with a variety of stimuli, including UV radiation (500 J/m², 1 hour), H2O2 (500 μM, 1 hour), menadione (Me, 20 μM, 1 hour), heat shock (42°C, 1 hour), gamma irradiation (γIR, 20 gray, 1 hour), or IGF-1 (100 ng/ml, 1 hour). Flag-tagged SIRT1 was immunoprecipitated (IP), and the immune complexes were assessed for the presence of FOXO3 by Western blot (WB) with the antibody to HA. The amounts of HA-FOXO3 present in the cell extracts were analyzed with the antibody to HA and amounts of immunoprecipitated SIRT1 with the antibody to Flag. (D) Interaction of endogenous FOXO3 and SIRT1. 293T cells were stimulated by H2O2 (500 μM, 1 hour). Endogenous FOXO3 was immunoprecipitated with a mouse polyclonal antibody to FOXO3 (FOXO3) or a control preimmune mouse serum (PI). The immune complexes were assessed for the presence of SIRT1 by Western blot with an antibody to SIRT1. Amounts of immunoprecipitated FOXO3 were assessed by Western blot with an antibody to FOXO3. Amounts of endogenous SIRT1 in the cell extracts were determined with an antibody to SIRT1. (E) Effects of stress on the interaction between FOXO3 and SIRT1. 293T cells were either nonstimulated or stimulated by H2O2 (500 μM, 1 hour), and FOXO3 and SIRT1 interaction was analyzed as in (D). The fact that antibodies to endogenous FOXO3 appear to coimmunoprecipitate less endogenous Sir2 in response to oxidative stress than the antibodies to Flag-FOXO3 (C) may be explained by a difference in the affinity of these two different antibodies for their antigenic determinants. (F) Interaction of mutant FOXO3 with SIRT1. 293T cells were cotransfected with the Flag-tagged form of wild-type SIRT1 and with the HA-tagged form of either wild-type FOXO3 (WT) or a mutant of FOXO3 in which all three phosphorylation sites have been replaced by alanines (TM). Cells were incubated in the presence (+) or absence (−) of H2O2 (500 μM, 1 hour). The immune complexes were analyzed as in (C).
led to an increase in the acetylation of ectopically expressed or endogenous FOXO3 and of endogenous p53 (Fig. 2, C and D). These results suggest that both SIRT1 and Class I and II HDACs contribute to FOXO3 deacetylation within cells.

To address more definitively whether endogenous SIRT1 has a role in deacetylating FOXO3 in vivo, we assessed FOXO3 acetylation in mouse embryonic fibroblasts (MEFs) derived from SIRT1 knockout mice or from wild-type littermates by immunoprecipitating endogenous FOXO3 and immunoblotting with antibodies to acetylated lysine (13) (Fig. 2E). Acetylation of endogenous FOXO3 was enhanced in SIRT1–/- MEFs compared to wild-type MEFs (Fig. 2E), suggesting that endogenous SIRT1 does influence FOXO3 acetylation in vivo. We also assessed the acetylation of FOXO3 in wild-type or SIRT1 knockout cells, by immunoprecipitating acetylated proteins with two different antibodies that recognize many acetylated sites in proteins (Fig. 2F and fig. S3) and by detecting the presence of FOXO3 in the immune complex with antibodies to FOXO3. Amounts of acetylated FOXO3 were greater in two independent lines of SIRT1–/- MEFs (KO1 and KO2) than that in wild-type cells (Fig. 2F and fig. S3). These data indicate that SIRT1 is one of multiple FOXO3 deacetylases in mammalian cells.

FOXO transcription factors transactivate a series of target genes that have critical roles in the cellular response to stress stimuli. FOXO targets include genes that control repair of damaged DNA (GADD45) (21), ROS detoxification (Mn superoxide dismutase and catalase) (22, 23), cell cycle arrest (p27KIP1) (20), and cell death (Fas Ligand and BIM) (16, 24).

To gain insight into the effect of SIRT1 on FOXO3-dependent gene transcription, we used a fibroblast cell line that expresses an inducible form of FOXO3 consisting of a fusion between the active form of FOXO3 (a T32A/S253A/S315A triple mutant) and the ligand-binding domain of the estrogen receptor (FOXO3-ER) (21). In the absence of ligand, FOXO3-ER is maintained in an inactive state in these cells. Addition of 4-hydroxysteromifen (4-OHT), an artificial ligand for the estrogen receptor, triggers the rapid activation of FOXO3-ER (21). In control cell lines that do not express FOXO3-ER, 4-OHT has no effect on the expression of FOXO3 target genes (21).

Addition of 4-OHT to FOXO3-ER-expressing cells moderately increased the expression of FOXO3 was assessed by immunoblotting with antibodies to acetylated lysine, amounts of FOXO3 with an antibody to FOXO3, and endogenous SIRT1 with an antibody to SIRT1. The acetylation of endogenous p53 was detected with an antibody to acetylated p53. Molecular size markers (kD) are indicated. (F) MEFs derived from wild-type or two independent littermate SIRT1–/- embryos (KO1 and KO2) were incubated in the presence of TSA for 2 hours. Endogenous acetylated proteins were immunoprecipitated with antibodies to acetylated lysines (Cell Signaling and Technology). The presence of FOXO3 in the immune complex and FOXO3 levels in the extract were assessed by Western blot with antibodies to FOXO3. The presence of endogenous SIRT1 was detected by Western blot with an antibody to SIRT1. The control panel represents the IgG that corresponds to the anti-acetylated antibodies and controls for the addition of the same quantity of anti-acetylated antibodies in each sample.
Expression of the FOXO3 targets GADD45 and p27 (Fig. 3, A and B). The increased expression of GADD45 and p27 was inhibited in the presence of nicotinamide and TSA, a combination of inhibitors for SIRT1 and Class I and II HDACs that led to the greatest acetylation of endogenous FOXO3 (Fig. 3, A and B). However, FOXO3-induced expression of the cell-death gene BIM was not inhibited by treatment of cells with nicotinamide and TSA, indicating that not all FOXO3 target genes are affected in the same way by deacetylase inhibitors. Consistent with the possibility that deacetylases may differentially affect various FOXO3 target genes, we found that SIRT1 repressed FOXO3-dependent expression of a luciferase reporter gene that is driven by the promoter of the gene encoding the death cytokine Fas ligand (Fig. S4). Accordingly, in preliminary experiments using Affymetrix gene arrays, we found that SIRT1 expression in FOXO3-ER-expressing fibroblasts promoted increased expression of one set of genes and repressed expression of another (29).

To assess further the role of endogenous SIRT1 in FOXO3-dependent gene expression, we analyzed the expression of FOXO3 target genes in wild-type or SIRT1−/− MEFs that were treated with LY 294002 (LY), a chemical compound that inhibits PI3K, thereby leading to the activation of endogenous FOXO3 (16). Quantitative real-time polymerase chain reaction (PCR) (28) showed that LY treatment of MEFs led to the increased expression of the stress resistance gene GADD45 (Fig. 3C). This response to LY was reduced in two independent lines of SIRT1−/− fibroblasts (Fig. 3C). Thus, endogenous SIRT1 appears to contribute to increased expression of the stress response gene GADD45 in response to inhibition of the PI3K pathway. In contrast, amounts of BIM mRNA were variable, and on average, LY treatment increased BIM expression to a similar extent in wild-type and SIRT1−/− cells (Fig. 3D). These results provide further evidence that SIRT1 does not regulate all FOXO3 target genes in the same manner.

The finding that the presence of SIRT1 enhanced expression of a FOXO3 target involved in stress resistance (GADD45) but appeared to somewhat diminish expression of proapoptotic FOXO targets (Fas ligand and BIM) led us to consider the possibility that SIRT1 could modulate the balance between stress resistance and cell death within cells. We generated FOXO3-ER stable cell lines that expressed either an empty vector or wild-type mouse SIRT1 (Fig. S5). The activation of FOXO3-ER induced cell cycle arrest at the G1-S phase transition (28) (Fig. 4A). In cells expressing extra copies of SIRT1, the ability of FOXO3 to induce cell cycle arrest at the G1-S transition was enhanced compared to that in cells expressing the empty vector. To determine if endogenous SIRT1 was necessary for FOXO3-mediated cell cycle arrest, we compared effects of FOXO3 on cell cycle arrest in MEFs derived from either SIRT1−/− mice or their wild-type littermates (Fig. 4B). We infected SIRT1−/− or wild-type MEFs with a retrovirus that encodes an active form of FOXO3 and assessed cell cycle progression. The constitutively active form of FOXO3 still induced cell cycle arrest in wild-type and in SIRT1−/− MEFs. However, the effect of the constitutively active form of FOXO3 on cell cycle arrest was diminished in SIRT1−/− MEFs (Fig. 4B). In addition, LY, whose presence leads to endogenous FOXO activation, was also less effective in inducing cell cycle arrest in SIRT1−/− MEFs compared to wild-type MEFs (Fig. S6). These results indicate that endogenous SIRT1 may potentiate FOXO3’s ability to induce cell cycle arrest, possibly allowing more time for cells to detoxify ROS and to repair damaged DNA.

FOXO transcription factors can also promote apoptosis (16, 24). We therefore tested the effects of SIRT1 on FOXO-induced cell death in primary cultures of cerebellar granule neurons (Fig. 4C). Expression of FOXO3 in neurons led to a modest increase in cell death, and this effect decreased when exogenous SIRT1 was also expressed (Fig. 4C). We also tested whether SIRT1 affects FOXO3-mediated cell death in nonneuronal cell types. In fibroblasts, activation of
SIRT1 in inhibiting stress-induced cell death in induced by FOXO3 in the presence of stress (caspase 3) (Fig. 4D) (26). Expression of extra copies of wild-type SIRT1 inhibited cell death induced by FOXO3 in the presence of stress stimuli (Fig. 4D). Consistent with a role for SIRT1 in inhibiting stress-induced cell death in fibroblasts, we found that SIRT1−/− MEFs were more sensitive to H2O2-induced cell death than were wild-type MEFs (Fig. 5E).

We have shown that in mammalian cells, in response to oxidative stress, the deacetylase SIRT1 forms a protein complex with the Forkhead transcription factor FOXO3 that contributes to deacetylation of FOXO3. SIRT1 differentially affected FOXO3 function, potentiating FOXO3’s effect on cell cycle arrest and DNA repair target genes but attenuating FOXO3-dependent apoptosis in the presence of stress stimuli. By deacetylating FOXO3 transcription factors, SIRT1 might tip FOXO-dependent responses away from cell death and toward stress resistance. Given that increased cellular stress resistance is correlated with extended organismal longevity, such an action might explain the effects of the Sir2 family of proteins on organismal longevity. Nevertheless, predicting the effect of FOXO deacetylation on mammalian longevity is difficult, because FOXO transcription factors have distinct functions in different tissues.

SIRT1’s effects on FOXO3 are reminiscent of SIRT1’s effects on the tumor suppressor p53 (9, 10). Under conditions of cellular stress, SIRT1 deacetylation of p53 leads to an inhibition of apoptosis (9, 10, 13). Given that SIRT1 also reduces FOXO3-induced apoptosis in the presence of stress stimuli, it is possible that FOXO3 and p53 somehow function together to mediate the effects of SIRT1. Consistent with this possibility, we find that FOXO3 and p53 interact with one another specifically under conditions of oxidative stress (fig. S7). A better understanding of the network of regulation between SIRT1, FOXO transcription factors, and p53 may help revealing important mechanisms that control stress resistance and organismal longevity.

References and Notes
26. A. Brunet, M. E. Greenberg, unpublished data.
27. In these experiments, the PKB inhibitor LY was included to suppress autocrine insulin-like growth factor 1 (IGF-1) signaling, thereby uncoupling Akt from upstream signaling pathways. The use of LY, or differences between cell lines, may explain the difference between our findings and those of Nemoto and Finkel (23) regarding stress-dependent FOXO3 localization in the absence of growth factors.
28. Materials and methods are available as supporting material on Science Online.
29. Y. Lin, M. E. Greenberg, unpublished data.
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Fig. 4. Differential effects of SIRT1 on FOXO-mediated biological responses. (A) Rat1 fibroblasts expressing FOXO3-ER, together with either empty vector (−) or mouse SIRT1 (+) were incubated in the presence or absence of 500 nM 4-OHT for 24 hours. Cell cycle progression was assessed by bromodeoxyuridine (BrdU) incorporation. More than 1000 cells were scored per data point. The data represent the mean and error bars of four independent experiments. Results are expressed as the number of cells that have undergone cell cycle arrest divided by the number of cells undergoing cell cycle arrest in the control (the FOXO3-ER cell line, in the absence of 4-OHT). Statistical significance was determined by ANOVA. (B) Wild-type MEFs (WT) or SIRT1−/− MEFs (KO) were infected with a control retrovirus (−) or with a retrovirus that encodes the constitutively active form of FOXO3 (+). Cell cycle progression was assessed by BrdU incorporation. The results are expressed as fold cell-cycle arrest induced by FOXO3 encoded retrovirus compared to that induced by the control retrovirus in wild-type MEFs versus SIRT1−/− MEFs. Data represent the mean and error bars of three independent experiments conducted in duplicate. Statistical significance was determined by ANOVA. (C) Cerebellar granule neurons were cotransfected with empty vector control (−) or the constitutively active mutant of FOXO3 (+) together with an empty vector (−) or SIRT1 wild-type (+) and a vector encoding β-galactosidase. Twenty-four hours after transfection, cells were incubated in the presence of IGF-1 for an additional 24 hours. Transfected cells were stained with the antibody to β-galactosidase, and DNA was stained with the Hoechst compound. Experiments were performed in duplicate, and 200 cells per coverslip were counted in a blinded fashion. Data correspond to the mean and error of three independent experiments. Statistical significance was determined by ANOVA. (D) Rat1 fibroblasts expressing FOXO3-ER, together with an empty vector (−) or mouse SIRT1 (+) were incubated in the presence or absence of 1 µM etoposide (ETO), in the presence or absence of 500 nM 4-OHT for 16 hours. Apoptosis was detected by Western blot analysis with an antibody to the cleaved form of caspase 3. This experiment is representative of three independent experiments.