SIRT1 Deacetylation and Repression of p300 Involves Lysine Residues 1020/1024 within the Cell Cycle Regulatory Domain 1*

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The SIR2 family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases modulates diverse biological functions in different species, including longevity, apoptosis, cell cycle exit, and cellular differentiation. SIRT1, the closest mammalian ortholog of the yeast SIR2 (silent information regulator 2) gene, represses several transcription factors, including p53, NFkB and forkhead proteins. The p300 protein serves as a rate-limiting transcriptional cointegrator of diverse transcription factors either to activate or to repress transcription through modular subdomains. Herein, SIRT1 physically interacted with and repressed p300 transactivation, requiring the NAD-dependent deacetylase activity of SIRT1. SIRT1 repression involved the CRD1 transcriptional repression domain of p300. Two residues within the CRD1 domain (Lys-1020 and Lys-1024) were required for SIRT1 repression and served as substrates for SIRT1 deacetylation. These residues also serve as acceptor lysines for modification by the ubiquitin-like SUMO protein. The SUMO-specific protease SSP3 relieved SIRT1 repression of p300. SSP3 antagonism of SIRT1 required the SUMO-deconjuncting function of SSP3. Thus, p300 serves as a deacetylase substrate for SIRT1 through a conserved SUMO consensus motif. Because p300 is a limiting transcriptional cofactor, deacetylation and repression of p300 by SIRT1 may serve an important integration point during metabolism and cellular differentiation.

p300 and its related ortholog cAMP-response element-binding protein-binding protein (CBP) are transcriptional integrators regulating numerous signaling pathways by facilitating transcriptional activity of a broad array of transcription factors (1, 2). p300/CBP have been implicated in numerous disease processes, including several forms of cancer, cardiac hypertrophy, and Huntington’s disease (3–9). The relative abundance of p300 is rate-limiting in coactivation and corepression of many transcription factors, thus p300 serves to integrate diverse signaling pathways involved in metabolism and cellular differentiation (1, 2, 10). Orchestration of these activities by p300 involves a scaffolding function to tether transcription factors to target promoters and enzymatic activity through a histone acetyltransferase domain (11, 12). In addition to histones, several substrates including transcription factors are acetylated by p300 (1, 2). p300 function itself is also subject to regulation via a number of post-translational modifications including phosphorylation, methylation, sumoylation, and acetylation (13–18).

The modular organization of p300 likely contributes to the assembly of multicomponent transcription coactivator complexes. p300 contains several distinct conserved motifs, including a bromo domain, a glutamine-rich region, three cysteine-histidine (CH)1-rich regions (CH1, CH2, and CH3), and a transcriptional repression domain (CRD1) (cell cycle regulatory domain 1) (1, 2, 19). The bromo domain plays a role in protein-protein interactions. Association with chromatin through the bromo domain facilitates histone acetylation and activation of transcription on chromatin templates in vitro (20–22). The CH domains serve as docking modules for transcription factors (1, 2). The glutamine-rich C terminus interacts with several coactivators, including the steroid receptor coactivators (1, 2). Both the N- and C-terminal regions of p300 activate transcription, and the histone acetyltransferase domain resides in the central portion of the protein (1, 2). In addition to coactivation, p300 encodes CRD1, located between amino acids 1004 and 1044, which is targeted by the cell cycle regulatory protein p21(CDH1/phosphatase A1) (19). The CRD1 domain contains within it a site for sumoylation (13) and functions in a

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1 The abbreviations used are: CH, cysteine-histidine; AADPR, acetyladenosine diphosphate ribose; ADPR, adenosine diphosphate ribose; CMV, cytomegalovirus; CRD, cell cycle regulatory domain; DBD, DNA binding domain; FACS, fluorescence-activated cell sorting; GDI, guanine nucleotide dissociation inhibitor; GFP, green fluorescence protein; HDAC, histone deacetylase; HEK, human embryonic kidney; HPLC, high pressure liquid chromatography; LC, liquid chromatography; LUC, luciferase; MEF, mouse embryonic fibroblasts; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RSV, Rous sarcoma virus; SIR, silent information regulator; SSP, SUMO-specific protease; TSA, trichostatin A; MVE, murine stem cell virus; IRES, internal ribosome entry segment.
promoter-selective manner (19, 23). Transcriptional repression is mediated through three classes of histone deacetylases (HDACs) distinguished by their structural and catalytic homology to yeast transcriptional repressors. Class I HDACs are homologous to the yeast Rpd3p and include mammalian HDAC1–3 and HDAC8, whereas class II HDACs are related to the yeast Hda1p proteins and include mammalian HDAC4–7 and HDAC9–10 (24–26). Class III HDACs are represented by the silent information regulator 2 (SIR2) family of protein deacetylases also referred to as sirtuins. The HDACs in class I and class II are characterized by their sensitivity to the inhibitor trichostatin A (TSA). In contrast, the HDAC activity of the class III sirtuins is not inhibited by TSA, but is NAD-dependent and inhibited by nicotinamide (27–32).

The yeast SIR2 gene regulates transcriptional silencing at telomeres, ribosomal DNA, and the silent mating loci. SIR2 extends the replication life span in Saccharomyces cerevisiae (33), and in Caenorhabditis elegans increased dosage of the worm SIR2.1 gene extends the life span of mother cells after caloric restriction (34, 35). The SIR2 gene family is highly conserved with seven mammalian homologs, SIRT1–7 (36). SIRT1, the closest mammalian ortholog of the yeast SIR2 gene, functions as an NAD-dependent deacetylase of a number of nonhistone substrates including p53 (37–39). The enzymatic activity of SIR2 is regulated by the availability of the oxidized form of NAD+ allowing SIR2 to function in part as a redox or metabolic sensor (30). SIRT1 represses p53-mediated transcription and deacetylates the p53 protein at lysine 382, impairing its ability to activate the apoptotic program (37–39).

Recently, several other nonhistone substrates of SIRT1 have been identified, including RelA/p65, PCAF, MyoD, and the FOXO (forkhead box class O) subfamily of transcription factors (FOXO4, FOXO3) (37–44). p300 functions as a limiting coactivator of most of these SIRT1 substrates. Furthermore, SIRT1 antagonizes p300-mediated acetylation and acetylation of p53, RelA/p65, and FOXO3/4 (38–43). We therefore investigated the mechanism by which SIRT1 may directly regulate p300 function. Herein, SIRT1 physically associated with and repressed p300 transcriptional activity. Mutational analysis demonstrated that SIRT1 repression of p300 required residues Lys-1020 and Lys-1024 within the CRD1 transcriptional repression domain. We show that the lysine 1020 and lysine 1024 residues are in vitro substrates for mSIR2α, a SIRT1 homolog expressed in mouse. These lysine residues required for SIRT1 repression form a consensus site required for p300 sumoylation. SIRT1 repression of p300 was attenuated by the SUMO deconjugase SSP3, suggesting that p300 residues lysine 1020 and lysine 1024 are the sites of both sumoylation and SIRT1 repression. The results reveal a novel interplay between the NAD-dependent deacetylation function of SIRT1 and sumoylation in the regulation of p300 function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—The HEK 293 and HEK 293T cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. The cells were maintained in a humidified atmosphere with 5% CO2 at 37 °C. Nicotinamide (Sigma) was dissolved freshly in water prior to use at a concentration of 400 mM and for cell culture was used at a final concentration of either 5 or 10 mM.

**Antibodies**—The antibodies used for Western blotting and immunoprecipitation were as follows: mouse monoclonal anti-Gal4 (DBD) RRK5C1 (Santa Cruz Biotechnology, Santa Cruz, CA; 2.5 μg of antibody/500 μg of protein for immunoprecipitation), rabbit polyclonal anti-Gal4 (DBD) (Santa Cruz Biotechnology; 1:1,500 for Western blotting), rabbit polyclonal anti-acetylated lysine antibody (Cell Signaling Technology; 1:1,000 for Western blotting), rabbit polyclonal anti-Myc (A-14) (Santa Cruz Biotechnology; 1:800 for Western blotting), mouse monoclonal anti-Myc (9E10) (Santa Cruz Biotechnology; 1:2,000 for Western blotting), mouse monoclonal anti-SIRT1, clone 2G1/7 (Upstate Biotechnology, Lake Placid, NY; 1:5,000 for Western blotting). An antibody against guanine nucleotide dissociation inhibitor (GDI) (a generous gift from Dr. Cyrus Bicket, Washington University, St. Louis, MO) (45) was used as an internal control for protein abundance (1:4,000 for Western blotting).

**Reporter Genes and Expression Vectors**—The report constructs used were: the PG5 luciferase reporter vector that contains five Gal4 DNA binding sites (PG5LUC, Promega, Madison, WI); pRL-CMVLuc (CMVLuc, Promega) and BSV5-galactosidase (RSV5-gal), which both serve as control constitutive reporters. The human wild-type and mutant (H363Y) SIRT1 expression vectors in pcDNA3 (38), the Myc-tagged wild-type and mutant (H363Y) SIRT1 expression constructs in pcDNA3.1 (a kind gift from Dr. T. Kozarides) (37), and pcMV6-x4 expression vectors for SUMO-specific protease 3 (SSP3) and an inactive mutant SSP3 (CS48A) (13) have been described previously. All of the Gal4-p600 constructs are derivatives of the pVR-1012Gal4-p600 expression vector that expresses full-length p600 fused to the Gal4-DBD (13, 19).

**Luciferase Assays**—HEK 293 and HEK 293T cells were seeded at a density of 1.5 × 10⁴ cells/well in Dulbecco’s modified Eagle’s medium in a 24-well plate on the day prior to transfection. The following day the cells were transiently transfected with the appropriate combination of the expression vectors, and control vectors using the FuGENE system (Roche Applied Science) (47), and phosphatase inhibitors (20 mM NaF and 1 mM orthovanadate) (24). Lysates were cleared by centrifugation at 4 °C for 15 min. The protein concentration was measured by the Bio-Rad assay and the protein lysates diluted to 1 μg/μl in lysis buffer. Immunoprecipitation assays were then performed on 500–1,200 μg of total protein. Lysates were preclariated by the addition of 30 μl of a 50% slurry of protein G-agarose beads/500 μg of protein, by centrifugation at 4 °C for 1 h. The lysate was then incubated overnight with the anti-Gal4-DBD RRK5C1 mouse monoclonal antibody (Santa Cruz; 2.5 μg of antibody/500 μg of protein) by rocking at 4 °C for 1 h. To the immunoprecipitate was added, per 500 μg of protein, 30 μl of a 50% slurry of protein G-agarose beads and incubated overnight at 4 °C with rocking. The beads were washed three times with buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.1% Tween 20. The immunoprecipitates were subjected to the equivalent empty expression vector control. Statistical analyses were performed using Student’s t test, and significant differences were established as p < 0.05.

**Western Blots**—Western blotting was performed as described previously (47). Whole cell lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After transfer, the membranes were blocked in 3% skim milk overnight. For immunodetection of proteins the membrane was incubated with the appropriate primary antibody at room temperature for 1 h. The blocked membrane was then washed three times with 0.1% Tween 20 phosphate-buffered saline (PBS-T) and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) (1:3,000). The same membranes were washed three times with PBS-T, and immunoreactive proteins were visualized by the enhanced chemiluminescence system (Amersham Biosciences).

**Immunoprecipitation Assays**—Immunoprecipitation and Western blotting were performed as described previously (48). HEK 293T cells were plated at a density of 2 × 10⁴ cells in 10-cm plates in Dulbecco’s modified Eagle’s medium the day before transfection. Cells were transfected with expression vectors and control vector using Superfect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Transfections were conducted with the expression vector for the Gal4 DNA binding domain alone (Gal4-DDD), and luciferase activity is shown relative to the Gal4-DVD-DVV construct at 1. Western analysis was normalized for transfection efficiency using a β-galactosidase reporter (RSV5-gal) as an internal control. Experimental data are mean of at least two independent experiments with luciferase activity normalized for β-galactosidase activity, conducted in triplicate. In parallel, transfections were conducted with the expression vector for the Gal4 DNA binding domain alone (Gal4-DDD), and luciferase activity is shown relative to the Gal4-DVD-DVV construct at 1. Western analysis was normalized for transfection efficiency using a β-galactosidase reporter (RSV5-gal) as an internal control. Experimental data are mean of at least two independent experiments with luciferase activity normalized for β-galactosidase activity, conducted in triplicate.
eluted by boiling for 5 min in SDS sample buffer (100 mM Tris-HCl, 10 mM diithiothreitol, 4% SDS) and subjected to SDS-PAGE analysis and Western blotting as described above.

Deacetylation Assays of the p300 mCRD1 Peptide—Deacetylation assays were performed as described previously (39) in 50 mM potassium phosphate, pH 6.6. Mouse SIR2α enzyme (mSIR2α) (Upstate Biotechnology) (2 µg) was added to a 100-µl reaction mixture containing 500 µM NAD and 50 µM p300 peptide with the sequence ERSTEL(AcK)TEI-(AcK)KED (Bio-Synthesis, Texas). Reactions were incubated at 28 °C for 5 h. At 0, 1, 2, 3, and 5 h time points reactions were assayed for production of either ADPR (adenosine diphosphate ribose), AADPR (acetyladenosine diphosphate ribose) and nicotinamide by high pressure liquid chromatography (HPLC) or for peptide deacetylation by HPLC. For the production of ADPR, AADPR, and nicotinamide, the reaction mixture was separated on a Waters C-18 semipreparative column (4.2 × 50-mm C-8 column) at 25 °C (200 µl/min) with 50 mM ammonium acetate, pH 5.0. The order of elution is shown in Fig. 6A at 260 nm. For peptide HPLC, the chromatograms were obtained on a Vydac C-18 analytical column for peptide and nucleic acids, with a gradient elution (1.5 ml/min) of 15% acetonitrile, 0.1% trifluoroacetic acid, to 40% acetonitrile at 30 min. Detection of peptide products was obtained at 215 nm. The HPLC system used was a Waters 600 pump with a 717 autosampler (temperature-controlled) and a 2487 UV-visible detector. Peptide peaks were identified further by MS analysis.

LC/MS/MS Analysis—The reaction solution was loaded onto a Vydac 1.0 × 50-mm C-8 column (The Separations Group, Hesperia, CA). A Hewlett-Packard 1100 HPLC equipped with a degasser and a binary pump was used to degas the solvents and pump the solvents to generate acetonitrile gradients at flow rate of 50 µl/min. Solvent A was water containing 0.1% trifluoroacetic acid, and solvent B was 80% (v/v) acetonitrile containing 0.1% trifluoroacetic acid. The sample was desalted at 1% B for 20 min, and the peptides were separated by a 5-min 1–15% B gradient followed by a 25-min 15–40% B gradient. The column effluent was delivered directly to a LCQ quadruple ion trap mass spectrometer (ThermoFinnigan, Riviera Beach, FL) equipped with an electrospray ionization source. The MS was performed in the data-dependent mode, which detected the intensity of the ions in the m/z range of 700–1,500, and switched to the CID mode to acquire a MS/MS spectrum when certain criteria were met. The mass isolation window for CID mode was set as 3 mass units, and the relative collision energy spectrum when certain criteria were met. The mass isolation window of 700–1,500, and switched to the CID mode to acquire a MS/MS spectrum when certain criteria were met.

RESULTS

**SIRT1 Repression of p300**

**Transactivation Requires SIRT1 Deacetylase Activity**—p300 does not bind to DNA on its own and is recruited to promoters by sequence-specific DNA-binding proteins to facilitate transcriptional activation through its intrinsic histone acetyltransferase activity. To determine the effect of SIRT1 on p300-dependent transactivation, a heterologous system was used. The intrinsic transactivation function of p300 was assessed by fusing the p300 coding sequence in-frame with the DBD of the yeast transcriptional activator Gal4 (Gal4-p300) (19). Gal4-p300 activation was assessed using a luciferase reporter gene driven by multimeric Gal4 DNA binding sites (PG5LUC). HEK 293 cells were cotransfected with the PG5LUC reporter vector and an expression construct for full-length Gal4-p300(1–2414), with either an expression vector for wild-type SIRT1 or, as a control, the corresponding empty vector (pcDNA3) (Fig. 1A). The Gal4-p300 expression vector increased PG5LUC activity up to 150-fold compared with the control vector Gal4-DBD (Fig. 1A). Activation by full-length Gal4-p300(1–2414) was repressed in a dose-dependent manner by increasing doses of the SIRT1 expression vector (Fig. 1A). To assess further the specificity of SIRT1 repression, the activity of a constitutively active CMV-driven promoter linked to the luciferase gene (CMV/LUC) was assessed. CMV/LUC activity was not affected by increasing amounts of the SIRT1 expression vector (Fig. 1B). To determine whether SIRT1 repression of p300 required the NAD-dependent deacetylase function of SIRT1, a deacetylase-defective mutant of SIRT1(H363Y) was used as well as a chemical inhibitor, nicotinamide (Fig. 1C). The SIRT1(H363Y) mutant protein contains a base substitution that converts the invariant catalytically active histidine at amino acid residue 363 to a tyrosine (H363Y) (38). The inactive SIRT1(H363Y) protein did not repress p300 activity, indicating that the deacetylase activity of p300 was required for SIRT1 repression of Gal4-p300 (Fig. 1C). Nicotinamide, an inhibitor of the NAD-dependent deacetylase activity of SIRT1 (27–32), abrogated SIRT1 repression of Gal4-p300 transactivation at a dosage of 5 mM but had no effect on Gal4-p300 activity in the presence of the empty vector or the inactive SIRT1(H363Y) mutant (Fig. 1C). Taken together these data suggest that SIRT1 can specifically repress the transactivation ability of p300 and that the deacetylase activity of SIRT1 is required for repression.

**SIRT1 Interacts with p300**—To determine whether SIRT1 physically associates with p300, HEK 293T cells were transfected with either Gal4-tagged p300 or Myc-tagged SIRT1 or both (Fig. 1D). Immunoprecipitation with an anti-Gal4 antibody and immunoblotalning with anti-Gal4 showed Gal4-p300 expression in lanes 4–6 (Fig. 1D, top panel), as expected. Likewise, immunoblotting of whole cell lysates with an anti-Myc antibody to the Myc epitope tag of SIRT1 showed expression of SIRT1 in lanes 2 and 3 and lanes 5 and 6, as expected (Fig. 1D, bottom panel). When Gal4-p300 was expressed (Fig. 1D, lanes 4–6), the cellular extracts immunoprecipitated with an anti-Gal4 antibody and the immunoprecipitated material analyzed by immunoblotting with anti-Myc, a SIRT1 signal was observed in the precipitate for both the wild-type and mutant Myc-SIRT1 (Fig. 1D, lanes 5 and 6, middle panel), but not the empty vector control (Fig. 1D, lane 4, middle panel). In contrast, transfection of Myc-SIRT1 in the absence of cotransfected Gal4-p300 did not result in a signal for Myc-SIRT1 in the precipitate (Fig. 1D, lanes 2 and 3, middle panel). Thus, p300 interacts with both wild-type SIRT1 and the inactive SIRT1 mutant (H363Y).

The C Terminus of p300(1239–2414) Is Sufficient for SIRT1 Binding—p300 contains several conserved regions, which constitute most of the known functional domains in the proteins. The conserved motifs in p300 include a bromo domain, a glutamine-rich, three CH-rich regions (CH1, CH2, and CH3), and...
a transcriptional repression domain (CRD1) (Fig. 2C, 1, 2). To identify the regions of p300 required for SIRT1 binding, HEK 293T cells were transfected with either pcDNA3Myc-SIRT1, empty vector (pcDNA3), or a panel of Gal4-p300 expression vectors (Fig. 2C). Immunoprecipitation was performed with equal amounts of protein extracts using a Gal4-specific antibody. With sequential immunoblotting for Gal4 and the Myc epitope of the SIRT1 vector, SIRT1 binding was observed in the Gal4 immunoprecipitates only from cells cotransfected with the Myc-SIRT1 vector (Fig. 2A, A and B), but not with the control empty vector (data not shown) or control IgG (Fig. 2A, lane 1). SIRT1 was coimmunoprecipitated by the two CRD1 internal deletion constructs (ΔH9004851–1045 and ΔH90041004–1045) to an extent similar to that of the full-length p300 (Fig. 2B, lane 6 versus 7 and 8). A reduced level of SIRT1 binding was observed with the N terminus of p300(192–1004, 192–1044) (Fig. 2B, lane 6 versus 9 and 10). SIRT1 bound all C-terminal p300 fragments but not to the N-terminal fragment 1–1301 (Fig. 2A, lane 2 versus 3–5). The most C-terminal fragment of Gal4-p300 coimmunoprecipitated SIRT1, indicating that the region 1239–2414 is sufficient to accommodate interaction of SIRT1 with p300 (Fig. 2A, lane 5).

**SIRT1 Repression of p300 Maps to Amino Acids 852–1064**

To identify the domain of p300 required for repression by SIRT1, a series of N- and C-terminal deletion constructs of Gal4-p300 were analyzed by PG5LUC reporter assay in HEK 293 cells cotransfected with SIRT1 or the empty vector control (Fig. 3). For comparison among the different deletion constructs, repression of the full-length Gal4-p300 by SIRT1 was assigned as 100% repression. The N-terminal Gal4-p300 fragment tested (192–1004) was not repressed by SIRT1, demonstrating that SIRT1 repression requires domains outside of...
Full repression by SIRT1 was only observed with the C-terminal fragment (amino acid residues 852–2414), shown in Fig. 3A, right panel. Progressive deletions from the N terminus of the 852–2414 fragment demonstrated loss of SIRT1 repression after deletion of amino acids 852–1064 and 852–1238 (Fig. 3A, right panel). Hence the 852–1064 region defines the boundaries of a domain required for SIRT1 repression of p300.

The 852–1064 region of p300 encompasses the CRD1 transcriptional repression domain, located between amino acids 1004 and 1044 (13, 19). The CRD1 of p300 was identified as a novel transcriptional repression domain that can be derepressed by the cdk inhibitor p21\textsuperscript{CIP/WAF1} in a promoter-dependent manner (19, 23). The minimal functional unit of the CRD1 consists of a tandem, consensus SUMO modification site between 1017 and 1029 (13). SUMO modification of the two SUMO acceptor residues Lys-1020 and Lys-1024 is required for CRD1-dependent repression (13). To examine further the requirement of the CRD1 domain in SIRT1 repression in the context of the full-length Gal4-p300, an internal deletion of the Gal4-p300 CRD1 domain (Δ1004–1045) was tested for PG5LUC reporter activation compared with the full-length p300 (Fig. 2).

**Fig. 2.** The C terminus (1239–2414) of p300 is sufficient for SIRT1 binding. **A** and **B**, HEK 293 cells were transfected with Gal4-p300 deletion constructs and either the empty vector (pcDNA3) or Myc-tagged pcDNA3SIRT1. Cell extracts were immunoprecipitated (IP) with anti-Gal4 antibody (lanes 2–10) or control IgG (lane 1) and analyzed by immunoblotting for anti-Gal4 (top panel) or anti-Myc antibody (middle panel). Whole cell extracts were probed with anti-Myc to indicate the SIRT1 input (bottom panel). **C**, left panel, schematic diagram of the Gal4-p300 expression constructs used in A and B. Right panel, relative binding of SIRT1 to p300 is shown as mean ± S.E. densitometry for n = 2 separate experiments. **WB**, Western blot.
SIRT1 repression of p300-mediated transactivation requires amino acids 852–1064. A and B, HEK 293 cells were transfected with 200 ng of the Gal4-p300 deletion constructs (shown schematically) and either 150 ng of empty vector (pcDNA3) or 200 ng of pcDNA3SIRT1, with 1 μg of the PG5LUC reporter and 50 ng of the RSV-β-gal reporter for normalization. Data are the mean ± S.E. for n = 6. Luciferase activity is relative to Gal4 alone, set at 1. The percentage change in PG5LUC activity by SIRT1 is relative to the activity of each Gal4-p300 construct in the presence of the empty SIRT1 vector, pcDNA3. The percentage change of the full-length Gal4-p300 by SIRT1 was assigned as 100%. HAT, histone acetyltransferase. C, HEK 293T cells were transfected with either 5.25 μg of empty vector (pcDNA3) or 7.5 μg of pcDNA3Myc-SIRT1 with 7.5 μg of different Gal4-p300 constructs as indicated. Cell extracts were analyzed by immunoblotting for anti-Gal4 (top panel) or anti-Myc antibody (middle panel). GDI serves as a protein loading control (bottom panel).
Internal deletion of the 851–1045 region within the context of the full-length p300 reduced SIRT1 repression by 50% (Fig. 3B). To rule out the possibility that SIRT1-mediated p300 repression might be caused by repression of p300 expression by SIRT1, Western blot analysis of Gal4-p300 was performed from the cellular lysates of the HEK 293T cells transfected with different Gal4-p300 constructs with or without the SIRT1 expression plasmid Myc-SIRT1. As shown in Fig. 3C, cotransfection of SIRT1 did not result in alteration of p300 expression in all of the Gal4-p300 constructs tested, suggesting that repression of p300 by SIRT1 is not because of its effect on p300 expression.

SUMO Modification Is Required for SIRT1 Repression of Gal4-p300—We examined further the role of SUMO modification of the p300 CRD1 domain in repression by SIRT1 using the Gal4 reporter system in HEK 293 cells (Fig. 4A). Deletion of the CRD1 motif (Δ1004–1045) within the context of the full-length Gal4-p300 increased Gal4-p300 activity (Fig. 4B), verifying that in our Gal4-p300 reporter system the CRD1 functions as a transcriptional repression domain. The 2-fold increased transcriptional activity of the CRD1 mutant (Δ1004–1045) compared with the full-length p300 was not the result of increased protein expression as determined by Western blot analysis (Fig. 4C). Because the functional unit of the CRD1 is thought to be a tandem SUMO consensus motif, we examined the role of sumoylation in SIRT1 repression of p300 using SSP3. Sumoylation is reversible, and there are several SSPs in mammalian cells (54). SSP3 (SENP2, SMT3IP2, or Axam) (55, 56) has been shown to catalyze deconjugation of SUMO from modified proteins. We reasoned that if SUMO modification of the CRD1 represses p300 transcriptional activity, then specific removal of SUMO from the full-length p300 should lead to activation, whereas the CRD1-deficient p300 should be resistant. We therefore examined the effect of SSP3 or a deconjugase-defective mutant SSP3 (C458A) (13) on Gal4-p300(1–2414) activity compared with the internal CRD1 mutant version (Δ1004–1045) (Fig. 4D).
SSP3 increased the transactivation activity of p300 up to 3-fold. Internal deletion of CRD1(Δ1004–1045) within the full-length p300 abolished SSP3-mediated activation (Fig. 4D), indicating that the CRD1 domain was required for the activity of SSP3. Because sumoylation regulates CRD1-dependent transcriptional repression of p300, we examined the effect of SSP3 on the ability of SIRT1 to repress p300 in HEK 293 cells. SSP3 relieved SIRT1 repression of Gal4-p300 by ~80% (Fig. 4E). The effect of SSP3 was dependent on a catalytically active form of the protease as the C458 mutation abolished the effect. This result suggests that SUMO modification of the full-length p300 is required for SIRT1 repression.

**SIRT1 Repression of p300 Requires Lysine Residues 1020 and 1024 within the Minimal CRD1 Repression Motif (mCRD1)**—Our recent studies identified Lys-1020 and Lys-1024 within the minimal CRD1 repression motif as critical for SIRT1 repression. The minimal CRD1 domain (mCRD1) represented by amino acid residues 1017–1029 of p300 consists of a tandem SUMO acceptor site, with the consensus sequence φKXE (φ = large hydrophobic amino acid, X = any amino acid). The constructs represent an N-terminal region of p300 (amino acids 192–1004) referred to as p300N to which has been fused the mCRD1 (amino acids 1017–1029) and the indicated lysine mutants thereof. HAT, histone acetyltransferase. B, HEK 293 cells were transfected with 200 ng of the listed p300N(1017–1029) constructs and 1 μg of the PG5LUC reporter with 50 ng of the RSV-β-gal reporter for normalization. The luciferase activity is the mean ± S.E. for n = 6 throughout, relative to the Gal4 alone (set at 1). C, HEK 293 cells were transfected with the p300N(1017–1029) constructs listed in A, and Western blot analysis was performed using anti-Gal4 antibody. GDI was the loading control. D, HEK 293 cells were transfected with 200 ng of the listed p300N(1017–1029) constructs and 75 ng of either vector alone or expression constructs for SSP3 or catalytically inactive SSP3(C458A). The Gal4 reporter construct, PG5LUC (1 μg), was normalized with 50 ng of the RSV-β-gal reporter. Data are the mean ± S.E. for n = 6. Luciferase activity is relative to Gal4 alone. -Fold activation is the increase in PG5LUC activity with the SSP3 expression construct compared with the empty SSP3 vector pCMV6-xl4, set at 1. E, HEK 293 cells were transfected with 200 ng of the p300N(1017–1029) constructs together with 200 ng of either empty vector (pcDNA3) or 200 ng of pcDNA3SIRT1, with 1 μg of the PG5LUC reporter and 50 ng of the RSV-β-gal reporter for normalization. Data are the mean ± S.E. for n = 6. Luciferase activity is the -fold change relative to the Gal4 alone compared with the empty pcDNA3 vector. The percentage repression by SIRT1 is relative to the p300N(1007–1029) construct, set as 100%.

**Fig. 5.** The p300 SUMO acceptor residues (Lys-1020 and Lys-1024) are required for SSP3 desumoylase induction and SIRT1 repression. A, the SUMO acceptor residues Lys-1020 and Lys-1024A within the minimal CRD1 repression motif are shown. The minimal CRD1 domain (mCRD1) represented by amino acid residues 1017–1029 of p300 consists of a tandem SUMO acceptor site, with the consensus sequence φKXE (φ = large hydrophobic amino acid, X = any amino acid). The constructs represent an N-terminal region of p300 (amino acids 192–1004) referred to as p300N to which has been fused the mCRD1 (amino acids 1017–1029) and the indicated lysine mutants thereof. HAT, histone acetyltransferase. B, HEK 293 cells were transfected with 200 ng of the listed p300N(1017–1029) constructs and 1 μg of the PG5LUC reporter with 50 ng of the RSV-β-gal reporter for normalization. The luciferase activity is the mean ± S.E. for n = 6 throughout, relative to the Gal4 alone (set at 1). C, HEK 293 cells were transfected with the p300N(1017–1029) constructs listed in A, and Western blot analysis was performed using anti-Gal4 antibody. GDI was the loading control. D, HEK 293 cells were transfected with 200 ng of the listed p300N(1017–1029) constructs and 75 ng of either vector alone or expression constructs for SSP3 or catalytically inactive SSP3(C458A). The Gal4 reporter construct, PG5LUC (1 μg), was normalized with 50 ng of the RSV-β-gal reporter. Data are the mean ± S.E. for n = 6. Luciferase activity is relative to Gal4 alone. -Fold activation is the increase in PG5LUC activity with the SSP3 expression construct compared with the empty SSP3 vector pCMV6-xl4, set at 1. E, HEK 293 cells were transfected with 200 ng of the p300N(1017–1029) constructs together with 200 ng of either empty vector (pcDNA3) or 200 ng of pcDNA3SIRT1, with 1 μg of the PG5LUC reporter and 50 ng of the RSV-β-gal reporter for normalization. Data are the mean ± S.E. for n = 6. Luciferase activity is the -fold change relative to the Gal4 alone compared with the empty pcDNA3 vector. The percentage repression by SIRT1 is relative to the p300N(1007–1029) construct, set as 100%.
FIG. 6. HPLC assay and mass spectrometry of mSIR2α-dependent deacetylation of p300. Aa, chromatographic separation of NAD and NAD-derived metabolites immediately after the addition of murine SIR2α (mSIR2α) enzyme. Ab, HPLC of reaction mixture with mSIR2α and p300 peptide (3 h at 28 °C) showing production of ADPR, AADPR, and nicotinamide (NAM). Ac shows a chromatogram of the reaction mixture of...
1024 as key SUMO acceptor lysines within the CRD1 domain (13). An N-terminal fragment of p300 (192–1004) linked to Gal4 (Gal4-p300N) was fused in-frame to the minimal CRD1 domain (1017–1029) (Fig. 5A). This system can be used to dissect the contribution of the CRD1 domain in the repression of the p300 N-terminal transactivation domain. The Gal4-p300N construct has an intrinsically high level of transcriptional activation because of it lacks the CRD1 repression domain. Appendage of the minimal CRD1(1017–1029) to the Gal4-p300N fragment (192–1004) leads to a reduced level of transactivation which is dependent on the two SUMO acceptor sites, Lys-1020 and Lys-1024. Consistent with our previous findings (13), the p300N construct to which had been appended the wild-type minimal CRD1(1017–1029) motif had a low intrinsic level of transcriptional activity which could be increased by mutation of each single SUMO acceptor site (Lys-1020 and Lys-1024) in isolation (K1020A, K1024A) and more so by double mutation (K1020A/K1024A, K1020R/K1024R) (Fig. 5B). This effect was not the result of an increase in protein expression levels of the Gal4-p300N constructs tested as determined by Western blot analysis (Fig. 5C).

To map further whether the CRD1 SUMO acceptor lysine sites are important in SSSP3-mediated activation, comparison was made with the p300N series of constructs (Fig. 5A). Compared with the wild-type CRD1, the single mutants (K1020A and K1024A) were partially compromised in SSSP3-mediated activation, with the double mutants (K1020A/K1024A and K1020R/K1024R) showing minimal activation compared with the inactive SPP3 (Cys-458) mutant (Fig. 5D). These data suggest that sumoylation represses p300 via the minimal CRD1 SUMO acceptor residues Lys-1020 and Lys-1024. To examine whether the CRD1 SUMO acceptor lysine residues can mediate SIRT1 repression we compared the ability of SIRT1 to repress the p300N series to which was appended either the wild-type minimal CRD1 or the SUMO acceptor lysine mutants thereof, shown in Fig. 5A. Mutation of the SUMO acceptor residues Lys-1020 and Lys-1024 in isolation or in tandem relieved SIRT1 repression of Gal4-p300N (1007–1029), shown in Fig. 5E.

Deacetylation of the p300 CRD1 Residues Lys-1020 and Lys-1024 by Mouse SIRT2α in Vitro—Because SIRT1 could form physical complexes with p300, we sought to determine whether this protein, a known deacetylase, could deacetylate p300 in vitro using bacterially expressed murine SIRT2α (mSIRT2α). mSIRT2α, the murine homolog of human SIRT1, was substituted for SIRT1 because of low yields of the human protein (39). Because SIRT1 repression required residues Lys-1020 and Lys-1024, a 20-residue-long oligopeptide containing the sequences corresponding to residues 1014–1033 of p300 was synthesized. The residues in this oligopeptide corresponding to Lys-1020 and Lys-1024 were synthesized in an acetylated form. After incubation with mSIRT2α in presence of 500 μM NAD, we detected an alteration of this oligopeptide substrate by HPLC (Fig. 6A). The deacetylase activity of mSIRT2α utilizes NAD as cofactor. Immediately after the addition of mSIRT2α and NAD, HPLC (215 nm) of the reaction mixture demonstrated that the p300 oligopeptide gave rise to a single prominent peak corresponding to the deacetylated form of the acetylated peptide (Fig. 6A, DiAcp300). In contrast, incubation of this oligopeptide and mSIRT2α in the presence of 500 μM NAD after a 3-h incubation produces additional peaks resulting from mSIRT2α-catalyzed removal of one (MonoAcp300) or two acetyl groups from the peptide (p300) (Fig. 6, Ad versus Ac). HPLC (260 nm) of the reaction mixture after a 3-h incubation at 28 °C demonstrated production of ADPR, AADPR, and nicotinamide, indicating an mSIRT2α-catalyzed deacetylation reaction involving the p300-diacylated peptide (Fig. 6, Aa versus Ab). Fig. 6B represents the LC elution that was then analyzed in situ by MS/MS, and each panel of Fig. 6C represents a portion of the mass spectrum from the peaks indicated in Fig. 6B. LCMS combined with in situ MS/MS analysis of peptide deacetylation reaction mixtures revealed that mSIRT2α preferentially deacetylated the residue corresponding to Lys-1020 (Fig. 6B, C, b versus c), while having relatively smaller effect on the acetylated Lys-1024 residue.

To verify further whether deacetylation of CRD1 p300 is regulated by SIRT1 within the cells, HEK 293 cells were transfected with Gal4-p300 (192–1004) or Gal4-p300 (192–1044) (Fig. 6D). The cells were then treated with either nicotinamide, TSA, or vehicle control. Immunoprecipitation using anti-Gal4 antibody and subsequently analyzed with Western blot (WB) using anti-Gal4 antibody (bottom panel). The same membrane were then stripped and blotted with anti-acetyllysine antibody (upper panel).

**DISCUSSION**

p300 directly acetylates a number of nonhistone substrates, including transcription factors to regulate transcriptional activity, protein-protein interactions, nuclear transport, and protein turnover (2, 26, 57, 58). Herein, SIRT1 repressed p300 transcriptional activity via the CRD1 domain located between amino acids 1004 and 1044 (13, 19, 23). Two lysine residues (Lys-1020 and Lys-1024) within the CRD1 domain were critical for p300 repression by SIRT1. The Lys-1020 and Lys-1024 unreacted diacylated p300 peptide (DiAcp300) immediately upon addition of mSIRT2α enzyme. Ad. chromatogram of reaction mixture after 3 h of incubation. New peaks correspond to deacetylation products resulting from mSIRT2α-catalyzed removal of one or two acetyl groups from peptide (MonoAcp300 or p300, respectively). B, LC/MS/MS of peptide products and unreacted p300 peptide from reaction mixture of mSIRT2α, NAD−, and p300 peptide. C, each panel corresponds to the MS/MS spectrum for each of the four labeled peaks in the LCMS chromatogram (B) labeled accordingly a, b, c, d. These MS/MS assignments demonstrate that the mSIRT2α enzyme deacetylates at both acetyllysine positions but prefers to deacetylate the N-terminal acetyllysine by severalfold. D, schematic diagram of the Gal4-p300 expression constructs with (192–1044) or without (192–1004) the CRD1 domain. HAT, histone acetyltransferase. E, HEK 293 cells were transfected with Gal4-p300 (192–1004) or Gal4-p300 (192–1044) and were treated with either 5 mM nicotinamide, 100 mM TSA, or vehicle control (dimethyl sulfoxide) for 24 h. Cellular lysates were subjected to immunoprecipitation (IP) using anti-Gal4 antibody and subsequently analyzed with Western blot (WB) using anti-Gal4 antibody (bottom panel). The same membrane were then stripped and blotted with anti-acetyllysine antibody (upper panel).
residues lie within a tandem SUMO consensus site and are sumoylated in vivo (13). SSP3, found in mammalian cells, catalyzes the deconjugation of SUMO-containing species (55, 56). SSP3 derepressed the transcriptional activity of p300, which was reliant on the CRD1 and the SUMO acceptor lysines (Lys-1020/Lys-1024). SSP3 reduced SIRT1 repression of p300. FIG. 7. Microarray analysis of the genes coregulated by SIRT1 and p300. A, p300 wild type or and p300-deficient MEF were infected with either control vector virus (MSCV-IRES-GFP) or SIRT1 expression virus (MSCV-SIRT1-IRES-GFP). The GFP-positive cells isolated by GFP-FACS sorting are shown in the left panel. A phase contrast image of the cells is shown in the right panel. B, SIRT1 expression level in the p300 wild type (WT) or and p300-deficient MEF infected with either control vector or SIRT1 expression virus (MSCV-SIRT1-IRES-GFP) as demonstrated by Western blot analysis using anti-Myc antibody for exogenous Myc-tagged SIRT1. C, summary of genes regulated by SIRT overexpression in a p300-dependent manner. Total RNA was isolated from the cells shown in A and used to probe Affymetrix MU74Av2 arrays (see “Experimental Procedures”). The top 20 probes that showed a log-odds ratio of greater than 0 were then clustered hierarchically. Data from each probe are in the rows, and each experiment is shown as a column. Red and green denote increased and decreased expression levels respectively, with the intensity reflecting the magnitude of change.

TABLE I

| ID    | Symbol     | Name                               | M     | A   | B    |
|-------|------------|------------------------------------|-------|-----|------|
| 93563 | Nid2       | Nidogen 2                          | 6.60E-01 | 6.3 | 1.7492 |
| 103721| Npt1       | Nephronectin                       | 7.22E-01 | 6.4 | 1.5812 |
| 93451 | NA         | NA                                 | 5.57E-01 | 8.4 | 1.3099 |
| 160261| NA         | NA                                 | 5.55E-01 | 10  | 0.9993 |
| 100477| C363002M10Rik | RIKEN cDNA C363002M10 gene        | 5.73E-01 | 7.4 | 0.7528 |
| 95349 | Cxcl1      | Chemokine (C-X-C motif) ligand 1   | 4.45E-01 | 6.9 | 0.7482 |
| 95348 | Cxcl1      | Chemokine (C-X-C motif) ligand 1   | 5.99E-01 | 7.7 | 0.6593 |
| 92332 | Dlx2       | distal-less homeobox 2             | 8.27E-01 | 4.5 | 0.5232 |
| 93574 | Serpinf1   | serine (or cysteine) proteinase inhibitor, clade F, member 1 | 6.15E-01 | 7.7 | 0.4856 |
| 95888 | Lf4l       | Lymphoid nuclear protein related to AF4-like | 4.71E-01 | 5.5 | 0.3472 |
| 102944| Wisp1      | WNT1 inducible signaling pathway protein 1 | 4.39E-01 | 9.1 | 0.2983 |
| 95032 | Frc1       | protein regulator of cytokinesis 1 | 3.54E-01 | 9.9 | 0.2024 |
| 93090 | Fgfr2      | fibroblast growth factor receptor 2 | 3.91E-01 | 8.9 | 0.1936 |
| 99535 | Ccrn4l     | CCR4 carbon catabolite repression 4-like (S. cerevisiae) | 4.15E-01 | 8.7 | 0.1305 |
| 103335| Lgals9     | lectin, galactose binding, soluble 9 | 5.50E-01 | 10  | 0.1074 |
| 94705 | Eya1       | eyes absent 1 homolog (Drosophila) | 3.62E-01 | 6.1 | 0.6593 |
| 102037| Edn1       | endothelin 1                       | 5.67E-01 | 6.2 | 0.0933 |
| 94264 | Raf1       | v-raf-1 leukemia viral oncogene 1  | 3.25E-01 | 8.7 | 0.0748 |
| 161734| Clgn       | calmodulin                        | 4.12E-01 | 8.5 | 0.0165 |
| 94999 | Cubn       | cubulin (intrinsinc factor-cobalamin receptor) | 3.57E-01 | 6.4 | 0.0036 |
requires the SUMO deconjugase activity of SSP3, suggesting that repression of p300 by SIRT1 requires SUMO modification. Collectively these findings suggested that repression of p300 by SIRT1 requires SUMO modification of the CRD1 domain.

The repression of p300 by SIRT1 herein is consistent with previous findings that SIRT1 represses transcription factors acetylated and activated by the p300 histone acetyltransferase domain (p53, RelA/p65, FOXO3/4) (37–43). Acetylation of RelA/p65 by p300, predominantly at lysine 310 and p53 at lysine 382, was diminished by SIRT1 (39, 43). SIRT1 also inhibited p300-dependent acetylation of FOXO3 and FOXO4 (40–42). In addition to antagonism between the opposing enzymatic activities of SIRT1 and p300 at the same target lysine residues of their common functional targets, SIRT1 repression of p300 activity through the CRD1 domain may provide a complementary mechanism of SIRT1-dependent transcriptional repression. Although the CRD1 is a key site for SIRT1 repression, the CRD1 internal deletion mutant of the full-length p300 was not fully repressed, suggesting that additional residues may also participate in SIRT1 repression. p300 contains multiple acetylation sites including the histone acetyltransferase domain itself, which may represent additional sites for SIRT1 regulation (18).

Lysines serve as the attachment sites for several post-translational modifications, including sumoylation, ubiquitination, and acetylation (58). Herein, recombiant mSIRT2 deacetylated the two SUMO acceptor sites (Lys-1020 and Lys-1024) of the CRD1, the same region of p300 required for SIRT1 repression. Treatment of the cells transfected with Gal4-p300 (192–1044) with SIRT1 deacetylase-specific inhibitor nicotinamide resulted in enhanced acetylation of the p300 CRD1 domain. Together with our previous studies demonstrating that lysines 1020 and 1024 are sites of p300 sumoylation (13) the current findings suggest that these residues can serve dual functions. Because acetylation and sumoylation occur at the ε-amino group of the lysine, acetylation may prevent SUMO modification. Potentially, SIRT1 may deacetylate the SUMO acceptor lysine residues within the CRD1 and promote sumoylation of p300.

Several proteins contain lysines within SUMO consensus sites that act as substrates for mutually exclusive modification. The proteins proliferating cell nuclear antigen (PCNA), IκB, and NEMO contain lysine residues within SUMO consensus sites that can be modified by either ubiquitin or SUMO (59–62). One lysine residue within the transcription factor Sp3 can be either acetylated or sumoylated (8, 63). Ubiquitin and SUMO compete for modification of PCNA, an essential processivity factor for DNA replication and repair (59, 61). Ubiquitin is attached either as monoubiquitin or as a polyubiquitin chain to PCNA at Lys-164 (59). SUMO competes for attachment to this lysine residue. SUMO conjugation of IκB inhibits ubiquitination of IκB through a common lysine residue, thereby preventing IκB degradation (62). Consistent with a role for SUMO in stabilizing IκB, SUMO1 overexpression inhibits NF-κB-dependent transcription in mammalian cells (62).

Herein, SUMO modification repressed p300 activity because all mutations that prevented sumoylation or enzymatic SUMO deconjugation increased p300 transcriptional activity. Acetylation may keep the p300 protein active by preventing SUMO modification and subsequent SUMO-mediated HDAC recruitment to the CRD1 (13). Indeed, previous mutagenesis studies of the negative regulatory domain of another transcriptional regulator, Sp3, have identified a single lysine residue (Lys-539) essential for silencing the activation potential of Sp3 (64). Lysine 539 of Sp3, lying within a SUMO consensus motif, is an in vivo target of both acetylation and SUMO conjugation, (8, 63). These data support the possibility that competition between acetylation and SUMO, as has been documented for ubiquitination, may also occur at SUMO consensus sites as a general mechanism for regulating the activation potential of transcriptional regulators.

To identify the molecule genetic signature regulated by SIRT1 in a p300-dependent manner, microarray studies were performed in p300+/– and p300−/− MEF. A subset of genes regulated by SIRT1 in p300-dependent manner was identified, consistent with a role of SIRT1 in regulating cellular differentiation and metabolism. SIRT1 repressed Eya1 expression by more than 3-fold, consistent with the finding that both SIRT1 and Eya1 are important in eye development (65, 66). SIRT1 repressed the expression of cubulin, the intrinsic factor-vitamin B12 receptor, which is essential for the synthesis of folate and purines and in the catabolism of some branched chain amino acids (67). In keeping with the role of Sir2 in caloric restriction and life span extension, p300 and SIRT1 coregulated the expression of Ccrn4, a CCR4 carbon catabolite repression 4-like gene. In addition, SIRT1 and p300 coregulate a subset of genes that are involved in regulation of cellular proliferation, development, and tumorigenesis, including growth factors FGFR2 (68), endothelin1 (69), wnt-1-induced secreted protein 1 (WISP-1) (70), nuclear transcription factor LAF4 (involved in B cell differentiation) (71), microtubule-binding, and bundling protein PRC1 (72, 73). Together, these studies identify the molecular genetic phenotype of SIRT1-regulated genes that are also regulated by p300.

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