The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase

ARTICLE *in* MOLECULAR CELL · MARCH 2003

Impact Factor: 14.02 · DOI: 10.1016/S1097-2765(03)00038-8 · Source: PubMed

5 AUTHORS, INCLUDING:

Eric Verdin
University of California, San Francisco
236 PUBLICATIONS **20,310** CITATIONS

Molecular Cell, Vol. 11, 437–444, February, 2003, Copyright ©2003 by Cell Press

The Human Sir2 Ortholog, SIRT2, Is an NAD+-Dependent Tubulin Deacetylase

Brian J. North,[1,3] Brett L. Marshall,[3] Margie T. Borra,[4] John M. Denu,[4] and Eric Verdin[2,3,*]
[1]Department of Physiology and
[2]Department of Medicine
[3]Gladstone Institute of Virology and Immunology
University of California, San Francisco
San Francisco, California 94141
[4]Department of Biochemistry and Molecular Biology
Oregon Health & Science University
Portland, Oregon 97201

Summary

The silent information regulator 2 protein (Sir2p) of *Saccharomyces cerevisiae* is an NAD-dependent histone deacetylase that plays a critical role in transcriptional silencing. Here, we report that a human ortholog of Sir2p, sirtuin type 2 (SIRT2), is a predominantly cytoplasmic protein that colocalizes with microtubules. SIRT2 deacetylates lysine-40 of α-tubulin both in vitro and in vivo. Knockdown of SIRT2 via siRNA results in tubulin hyperacetylation. SIRT2 colocalizes and interacts in vivo with HDAC6, another tubulin deacetylase. Enzymatic analysis of recombinant SIRT2 in comparison to a yeast homolog of Sir2 protein (Hst2p) shows a striking preference of SIRT2 for acetylated tubulin peptide as a substrate relative to acetylated histone H3 peptide. These observations establish SIRT2 as a bona fide tubulin deacetylase.

Introduction

Reversible histone acetylation is involved in the regulation of transcriptional silencing and activation (Kuo and Allis, 1998). In addition to histones, a rapidly growing number of nonhistone proteins are acetylated on lysine residues (reviewed in Polevoda and Sherman, 2002). The level of acetylation of histones and other acetylated proteins is controlled by the opposing enzymatic activities of histone deacetylases (HDACs) and histone acetyltransferases. HDACs are separated into three distinct classes based on their homology to yeast transcriptional repressors. Class I and class II deacetylases are homologs of the Rpd3p and Hda1p proteins, respectively. Class III HDACs are defined based on their homology to the yeast transcriptional repressor, Sir2p.

The silent information regulator (SIR) proteins mediate transcriptional silencing at a number of loci, including the mating type locus, telomeres, and the rDNA locus (Gasser and Cockell, 2001). Sir2p encodes an NAD-dependent histone deacetylase and is thought to mediate silencing by regulating histone acetylation (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). The SIR2 gene family is conserved from archaebacteria to eukaryotes (Frye, 2000). In *S. cerevisiae*, this family consists of *Sir2* and four homologs of Sir2 (*Hst1-4*) genes (Brachmann et al., 1995). Humans have seven proteins with homology to Sir2p, which have been named sirtuins (SIRTs) (Frye, 1999, 2000). Both human SIRT1 and mouse Sir2α deacetylate the transcription factor protein p53 and suppress p53-dependent apoptosis in response to DNA damage (Luo et al., 2001; Vaziri et al., 2001). The human SIRT2 protein shows a cytoplasmic distribution (Afshar and Murnane, 1999; Perrod et al., 2001). Both SIRT2 and Hst2p may regulate rDNA and telomeric silencing indirectly from their cytoplasmic location (Perrod et al., 2001), but could also deacetylate cytoplasmic proteins such as α-tubulin within the microtubule network.

The microtubule network is formed by the polymerization of α- and β-tubulin heterodimers and plays an important role in the regulation of cell shape, intracellular transport, cell motility, and cell division (Nogales, 2000). α- and β-tubulin subunits are subject to numerous post-translational modifications, including tyrosination, phosphorylation, polyglutamylation, polyglycylation, and acetylation (MacRae, 1997). Tubulin acetylation occurs on lysine-40 of the α-tubulin subunit (Nogales et al., 1999; Piperno et al., 1987). A variety of drug treatments and physiological signals have been reported to modulate the level of tubulin acetylation, including the anticancer drug paclitaxel (Taxol) (Piperno et al., 1987), Trichostatin A (TSA) (Grozinger et al., 2001), ras activation (Harrison and Turley, 2001), as well as microtubule interaction of MAP1 and 2C, tau (Takemura et al., 1992), and the herpes simplex virus encoded protein VP22 (Elliott and O'Hare, 1998). Similarly, microtubules associated with stable structures, such as cilia, contain relatively hyperacetylated α-tubulin (Poole et al., 2001). However, a lack of knowledge on the enzymes responsible for the reversible acetylation and deacetylation of tubulin has precluded a thorough analysis of the biological role of tubulin acetylation in microtubule functions.

Results

SIRT2 Is a Cytoplasmic Protein that Colocalizes with the Microtubule Network

Using indirect immunofluorescence and a specific antiserum for endogenous SIRT2, we determined that hSIRT2 was predominantly cytoplasmic (Figure 1A). Merging of the pictures obtained with the SIRT2 and α-tubulin antisera showed strong colocalization between SIRT2 and tubulin (Figure 1A). Fractionation of 293T cells into nuclear and cytoplasmic fractions followed by Western blotting analysis showed that SIRT2 was localized predominantly in the cytoplasm but was also present to a lesser degree in the nucleus (Figure 1B). Probing the same fractions for known cytoplasmic (p65) and nuclear (lamin A) proteins confirmed the purity of our fractions (Figure 1B).

Tubulin Deacetylation by Human SIRT2 In Vivo

The localization of SIRT2 along the microtubule network and the previous observation that α-tubulin is acet-

*Correspondence: everdin@gladstone.ucsf.edu

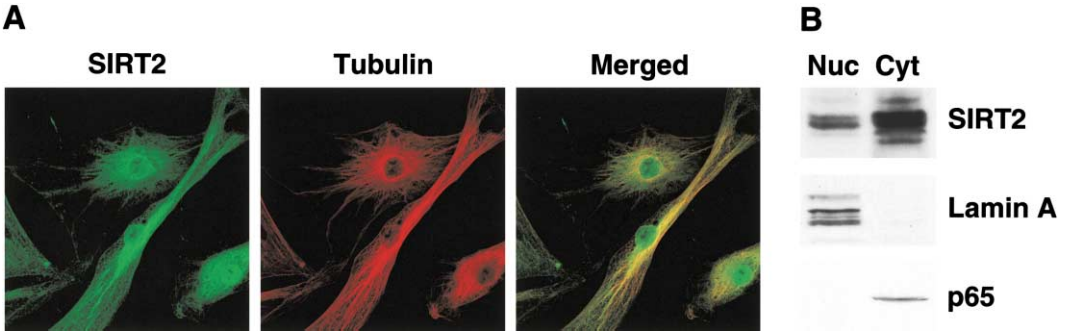

Figure 1. SIRT2 Is Predominantly Cytoplasmic and Colocalizes with the Microtubule Network

(A) Confocal microscopy analysis of SIRT2 and tubulin in human fibroblast. Cells were stained for SIRT2 (green) and tubulin (red) to visualize the microtubule network.

(B) Nuclear and cytoplasmic extracts from 293T cells were analyzed by Western blotting with specific antisera for SIRT2, lamin A, and p65.

ylated, enticed us to test whether SIRT2 can deacetylate tubulin. HeLa cells transfected with GFP-SIRT2 (which remains catalytically active as a deacetylase on a histone substrate; data not shown) were stained with an antiserum specific for α-tubulin acetylated at lysine-40 (Piperno et al., 1987). Cells expressing GFP-SIRT2 had markedly less acetylated tubulin than neighboring untransfected cells (Figure 2A). Cells transfected with an expression vector for GFP alone showed no change in the level of acetylated tubulin (Figure 2B). Staining with another α-tubulin antibody that recognizes α-tubulin independently of its acetylation state showed no change in the microtubule network, quantitatively or qualitatively, in response to GFP-SIRT2 expression (Figure 2C).

To verify that the deacetylase activity of SIRT2 was necessary for α-tubulin deacetylation, we transfected GFP-SIRT2 expression vectors containing catalytically inactive mutations into HeLa cells. We replaced asparagine-168 with alanine (N168A) and histidine-187 with tyrosine (H187Y) in GFP-SIRT2. Fusion proteins with

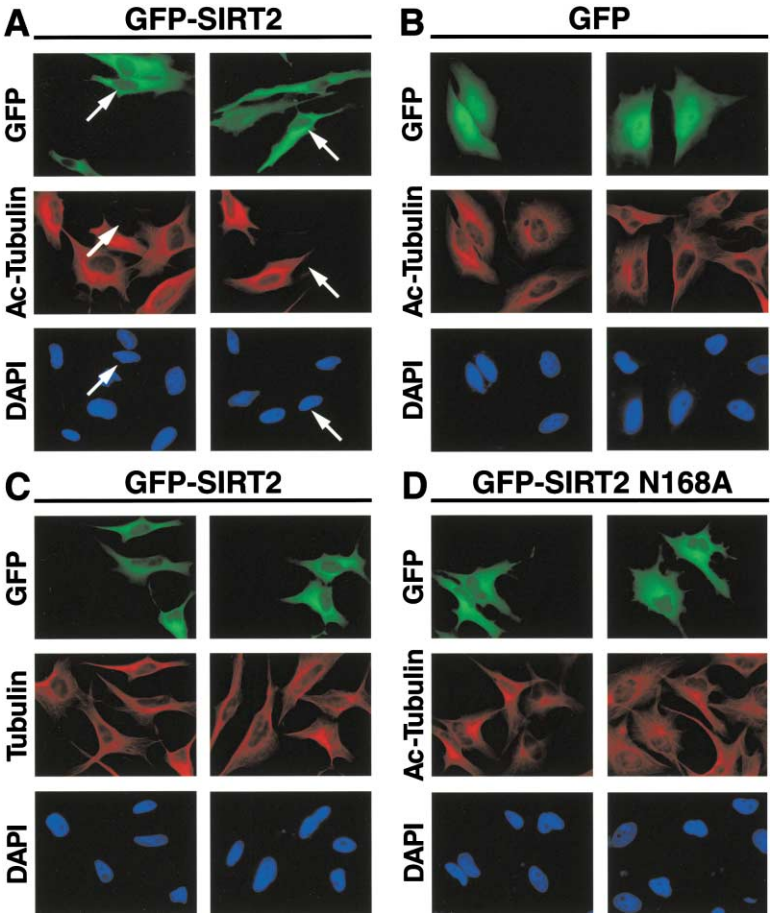

Figure 2. In Vivo Tubulin Deacetylation by GFP-SIRT2

(A) HeLa cells transfected with GFP-SIRT2 were treated with 400 nM TSA for 12 hr and subsequently stained for acetylated tubulin. DNA was stained with DAPI. Each frame was visualized for GFP (green), acetylated tubulin (red), and DNA (blue). White arrows identify cells transfected with GFP-SIRT2.

(B) HeLa cells transfected with GFP and treated as in (A) were stained for acetylated tubulin (red).

(C) HeLa cells transfected with GFP-SIRT2 and treated as in (A) were stained for total tubulin (red) to examine the microtubule network.

(D) HeLa cells transfected with the N168A point mutant of GFP-SIRT2 and treated as in (A) were stained for acetylated tubulin (red).

these substitutions were inactive on a histone substrate, whereas the wild-type showed robust activity after immunoprecipitation with antiserum for GFP (data not shown). The catalytically inactive mutant N168A did not modify the level of acetylation of α-tubulin (Figure 2D). Similar results were obtained with the H187Y mutant (data not shown). These results indicate that expression of wild-type SIRT2 in vivo leads to the deacetylation of lysine-40 on α-tubulin, mediated by the deacetylase activity of SIRT2.

Human SIRT2 Deacetylates Tubulin In Vitro

To test the ability of SIRT2 to deacetylate α-tubulin in vitro, we transfected 293T cells with SIRT2-FLAG and immunoprecipitated the FLAG-tagged protein (Figure 3A). The immunoprecipitated material was separated into two fractions. The first fraction was used to measure HDAC activity using a peptide derived from histone H4 (amino acids 1–23) acetylated in vitro. The second fraction was used for a tubulin deacetylation activity assay using total cellular lysates from untransfected 293T cells (Figure 3A). The extent of α-tubulin deacetylation was determined by Western blotting with antisera specific for acetylated α-tubulin and for total α-tubulin. Incubation of cellular lysate with the immunoprecipitated SIRT2-FLAG protein deacetylated tubulin in a NAD-dependent manner (Figure 3B, lanes 2 and 3). The catalytically inactive mutant N168A did not deacetylate α-tubulin (data not shown). The SIRT2-mediated deacetylation of tubulin was completely inhibited by nicotinamide, a specific inhibitor for class III HDACs (Luo et al., 2001), while TSA, a specific inhibitor of class I and class II HDACs, had no effect (Figure 3B, lane 5). Utilizing the same immunoprecipitated material in an HDAC activity assay on a histone H4 peptide, we observed a 90% reduction in SIRT2 activity in the presence of nicotinamide (Figure 3B, lane 4), and no inhibition of SIRT2 activity by TSA (Figure 3B, lane 5).

To determine whether α-tubulin deacetylation activity is restricted to SIRT2, we expressed all seven human SIRT proteins each with a C-terminal FLAG tag in 293T cells. Only SIRT2 deacetylated tubulin in vitro, whereas the remaining SIRT proteins contained no significant tubulin deacetylase activity (Figure 3C). This is in contrast with the activity of the same immunoprecipitated proteins on a histone H4 peptide, where SIRT1, 2, 3, and 5 showed significant deacetylase activity (Figure 3C, deacetylase activity). SIRT4, 6, and 7 had low to undetectable HDAC activity on the histone H4 peptide (Figure 3C). These results demonstrate that SIRT2 is the only class III HDAC capable of deacetylating α-tubulin.

These results confirm that the tubulin deacetylase within the immunoprecipitated SIRT2 material after overexpression can be solely attributed to SIRT2.

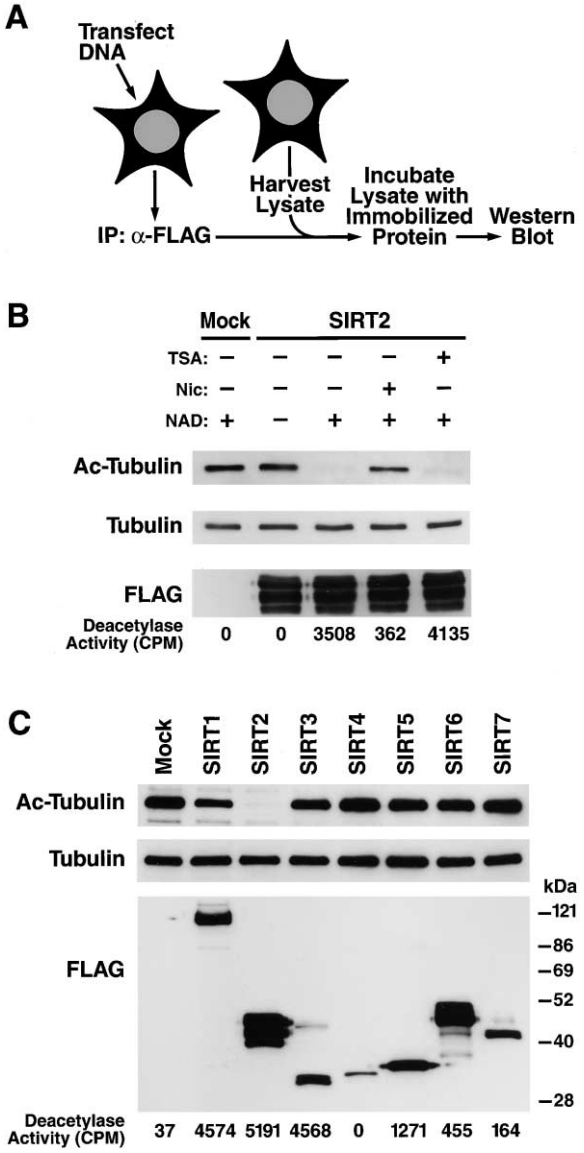

Figure 3. SIRT2 Deacetylates Tubulin In Vitro

(A) Schematic diagram of in vitro tubulin deacetylation assay.

(B) One-half of the immunoprecipitated protein corresponding to SIRT2-FLAG was incubated with cellular lysate with or without 1 mM NAD in vitro. Specified reactions were incubated with 5 mM nicotinamide or 400 nM TSA. The reaction products were separated by SDS-PAGE and visualized by Western blotting with specific antisera for acetylated tubulin, tubulin, and FLAG. The second half of the immunoprecipitated material was subjected to HDAC activity assay with an acetylated [^{3}H] acetylated histone H4 peptide.

(C) The same assays as in (B) were conducted using the seven class III HDACs, SIRT1-7-FLAG.

Enzymatic Kinetics of Human SIRT2 and Yeast Hst2

To test whether purified human recombinant SIRT2 has NAD-dependent deacetylase activity, we incubated recombinant SIRT2 purified from *E. coli* with increasing concentrations of NAD and a histone H4 peptide. A dose-dependent increase in HDAC activity was observed with increasing concentrations of NAD (Figure 4A). A further increase in NAD concentration to 10 mM resulted in a reduction in deacetylase activity (Figure 4A).

To further analyze the deacetylation of tubulin by SIRT2, a detailed enzymatic analysis was performed. For comparison, the highly active yeast histone deacetylase Hst2p was analyzed alongside the human enzyme. Hst2p exhibits strong selectivity for peptides corresponding to histone H3 acetylated at lysine-14 (Landry et al., 2000; Tanner et al., 2000). We utilized a synthetic

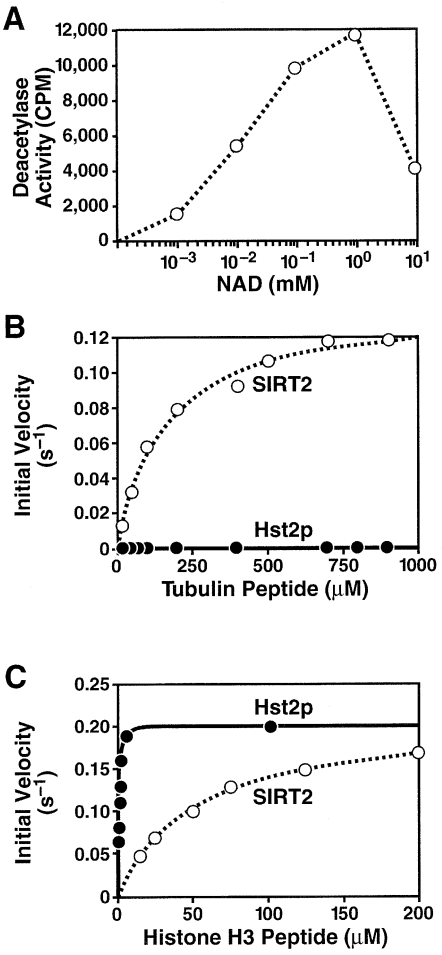

Figure 4. Tubulin Is a Preferred Substrate for SIRT2 in Comparison to Hst2p

(A) The enzymatic activity of recombinant 6-His-SIRT2 on a [^{3}H] acetylated histone H4 peptide was measured in the presence of increasing concentrations of NAD (0, 0.001, 0.01, 0.1, 1.0, and 10 mM). Released acetate was extracted and measured by scintillation counting.

(B) Initial velocities measured at varying concentrations of an acetylated tubulin peptide for SIRT2 (open circles) and for Hst2p (closed circles) with concentrations and conditions described in Experimental Procedures. The curve with SIRT2 represents the average rates from three different experiments. The Hst2p curve is a representative data set from one of three separate experiments. The indicated NAD concentrations are saturating with respect to each enzyme.

(C) Initial velocities for each enzyme measured at varying concentrations of acetylated H3 peptide for SIRT2 (open circles) and for Hst2p (closed circles) with concentrations and conditions described in Experimental Procedures. The indicated NAD concentrations are saturating with respect to each enzyme.

acetylated α-tubulin peptide (amino acids 36–44) acetylated at lysine-40 and a synthetic histone H3 peptide (amino acids 1–20) acetylated at lysine-14 to measure initial velocities of Hst2p and SIRT2 at various acetylated tubulin or histone H3 concentrations. The resulting saturation curves were fitted to the Michaelis-Menten equation, yielding the kinetic parameters k_{cat}, K_m, and *V/K*. The most physiologically relevant constant is *V/K*, as this second-order constant defines the rate of the reaction when substrate concentrations are not at saturating levels and reflects both substrate binding and catalysis. Because cellular enzymatic reactions rarely occur under maximal velocity conditions (i.e., saturating substrate levels), dramatic differences in the *V/K* value of the enzyme will likely reflect the most relevant in vivo consequences.

SIRT2 exhibited a striking preference for acetylated tubulin peptide as a substrate relative to yeast Hst2p (Figure 4B). This difference was ~60-fold and was reflected in both the k_{cat} and *V/K* values, which were 0.144 ± 0.005 s^{-1} and 894 ± 100 $M^{-1}s^{-1}$ for SIRT2 and 0.00254 ± 0.0003 s^{-1} and 14.9 ± 5.4 $M^{-1}s^{-1}$ for Hst2p, respectively. In contrast, Hst2p demonstrated a ~200-fold stronger preference for H3 peptide relative to SIRT2 (Figure 4C). These differences were reflected in the *V/K* and K_m values, which were 3930 ± 261 $M^{-1}s^{-1}$ and 54.2 ± 3.6 μM for SIRT2, and 717,900 ± 35,900 $M^{-1}s^{-1}$ and 0.280 ± 0.014 μM for Hst2p, respectively. The k_{cat} values (~0.2 s^{-1}) were similar between the two enzymes.

These results indicate that the Sir2-related deacetylases display remarkable differences in substrate specificity and that human SIRT2 has a marked preference for the acetylated tubulin peptide.

Knockdown of SIRT2 and HDAC6 Expression Leads to Tubulin Hyperacetylation

A recent report has indicated that HDAC6, a class II HDAC, functions as a microtubule-associated deacetylase (Hubbert et al., 2002). To evaluate the relative role of both HDAC6 and SIRT2 in the deacetylation of tubulin in vivo, we used specific siRNAs to knockdown the expression of either gene. An siRNA specific for SIRT2 was transfected into 293T leading to a marked inhibition of SIRT2 expression (Figure 5A). Treatment of cells with this siRNA led to an increase in the level of acetylated α-tubulin when compared to mock-transfected cells or to cells transfected with an unrelated siRNA (Figure 5A, lanes 1–3). Likewise, knockdown of HDAC6 by siRNA resulted in an increase in acetylated α-tubulin (Figure 5A, lane 4). Transfection of siRNAs for both SIRT2 and HDAC6 led to the same degree of tubulin hyperacetylation (data not shown). These results demonstrate that both SIRT2 and HDAC6 function in the regulation of α-tubulin acetylation.

SIRT2 and HDAC6 Colocalize and Coimmunoprecipitate

Considering that both SIRT2 and HDAC6 colocalize with microtubules (Figure 1B, data not shown) (Hubbert et al., 2002), we wanted to determine whether both proteins might interact. First, we transiently cotransfected 293T cells with a FLAG epitope-tagged HDAC6 (HDAC6-FLAG) and an HA epitope-tagged SIRT2 (SIRT2-HA) and immunoprecipitated the HDAC6-FLAG protein. We observed that when both SIRT2-HA and HDAC6-FLAG were coexpressed, SIRT2-HA coimmunoprecipitated with HDAC6-FLAG (Figure 5B, lane 4). This coimmunoprecipitation was independent of an intact microtubule network since it also occurred in cells treated with colchicine (Figure 5B, lane 6). Importantly, tubulin did not coimmunoprecipitate with either protein (Figure 5B). In a similar reverse experiment, immunoprecipitation of SIRT2-HA with an anti-HA antiserum led to the coimmu-

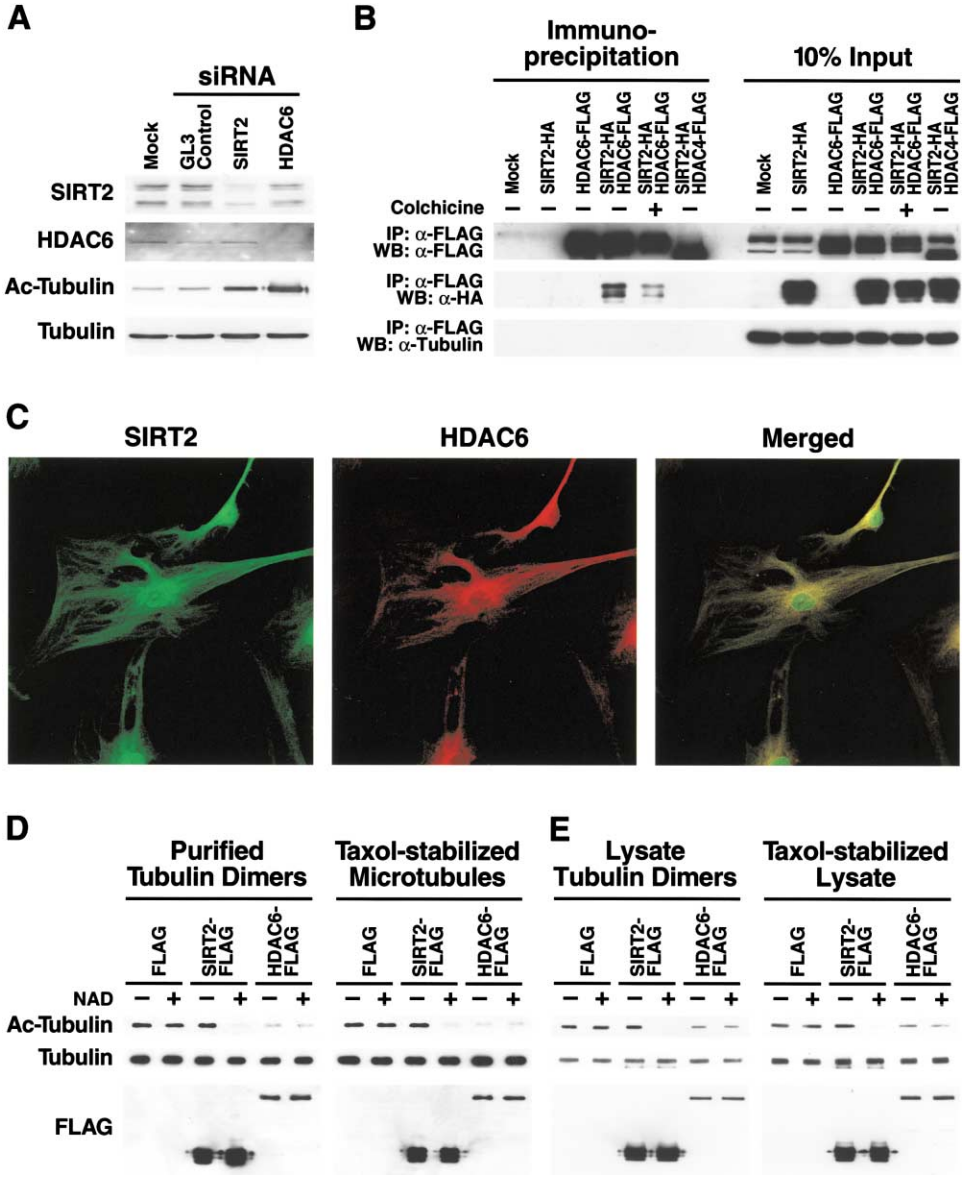

Figure 5. Deacetylation of Tubulin by SIRT2 and HDAC6

(A) Knockdown of SIRT2 and HDAC6 with siRNA. 293T cells were mock transfected or transfected with siRNA duplexes for GL3 luciferase (control), SIRT2 (260-278), and HDAC6 (211-229) three times over 5 days and collected 48 hr post final transfection. Cellular lysates were probed by Western blotting with specific antisera for acetylated α-tubulin, tubulin, SIRT2, and HDAC6.

(B) SIRT2 and HDAC6 coimmunoprecipitate. 293T cells were either mock transfected or transfected with SIRT2-HA, HDAC6-FLAG, and HDAC4-FLAG in different combinations. Cellular lysates were immunoprecipitated with anti-FLAG and probed by Western blotting with antisera specific for FLAG and HA. The same immunoprecipitated samples were blotted for α-tubulin. One sample was obtained from cells treated with 25 μM colchicine for 12 hr as indicated. Ten percent of protein input was analyzed by Western blotting with antisera for FLAG or HA.

(C) Confocal microscopy analysis of SIRT2 localization with HDAC6 in human fibroblast. Cells were stained for SIRT2 (green) and HDAC6 (red).

(D) 293T cells were transfected with FLAG, SIRT2-FLAG, or HDAC6-FLAG. Cellular lysates were harvested and immunoprecipitated with antiserum for FLAG. The immunoprecipitated protein corresponding to FLAG, SIRT2-FLAG, or HDAC6-FLAG was incubated with purified tubulin substrates ±1 mM NAD in vitro. The reaction products were analyzed by Western blotting with specific antisera for acetylated α-tubulin, tubulin, and FLAG.

(E) Same as in (D) except that tubulin from whole-cell lysates were used, either as dimers or as Taxol-stabilized microtubules.

noprecipitation of HDAC6-FLAG (data not shown). Second, we used indirect immunofluorescence and antisera specific for endogenous SIRT2 and HDAC6 in a fibroblast cell line and visualized that both proteins colocalized on a cytoplasmic network reminiscent of the microtubule network (Figure 5C). The colocalization of endogenous SIRT2 and HDAC6 and their ability to coimmunoprecipitate suggest that SIRT2 and HDAC6 interact either directly or indirectly within a multiprotein complex.

To better characterize the substrate specificities of SIRT2 and HDAC6 on tubulin, we compared the deacetylase activities of immunoprecipitated SIRT2-FLAG with HDAC6-FLAG on a variety of tubulin substrates. When using purified tubulin heterodimers (Figure 5D, purified tubulin dimers), or microtubules polymerized from purified tubulin heterodimers by addition of Taxol and GTP (Figure 5D, Taxol-stabilized microtubules) as substrates, both SIRT2 and HDAC6 deacetylated α-tubulin efficiently. The SIRT2 activity was NAD dependent, whereas the HDAC6 activity was constitutive, as predicted. In contrast, when the deacetylation of tubulin heterodimers or microtubules induced with Taxol and GTP from 293T lysates were analyzed as substrates (Figure 5E, lysate tubulin dimers and lysate Taxol stabilized), SIRT2 deacetylated both substrates equally while the activity of HDAC6 on these substrates was barely detectable (Figure 5E). These data suggest that, while SIRT2 can deacetylate tubulin in the context of most substrates, tubulin or microtubules reconstituted from whole-cell lysates are relatively resistant to the deacetylase activity of HDAC6.

Discussion

Despite the identification of tubulin acetylation more than 20 years ago, little is known on the biological role of this modification in microtubule function. By analogy with histone proteins, we can anticipate that the level of tubulin acetylation reflects the competing influences of tubulin acetyltransferase(s) and tubulin deacetylase(s). Here we report the identification of a human tubulin deacetylase, the class III deacetylase SIRT2.

The microtubule localization of SIRT2 suggested that it could function as a tubulin deacetylase. We demonstrate that GFP-SIRT2 deacetylates tubulin in vivo and that knockdown of endogenous SIRT2 by siRNA causes tubulin hyperacetylation.

Despite the fact that SIRT2 may be part of a multiprotein complex containing HDAC6, a previously recognized tubulin deacetylase, three observations indicate that SIRT2 is a bona fide tubulin deacetylase. First, the tubulin deacetylase activity of SIRT2 is not modified by TSA, a specific inhibitor of HDAC6. Second, the tubulin deacetylase activity associated with SIRT2 is inhibited by nicotinamide, an inhibitor of class III HDACs (Luo et al., 2001). Third, a recombinant form of SIRT2 purified after expression in *E. coli* shows specific tubulin deacetylase activity.

Human SIRT2 is most closely related to yeast Hst2p, one of five Sir2-like proteins in *S. cerevisiae*, which is also localized to the cytoplasm (Perrod et al., 2001). The substrate specificities of these two closely related proteins have diverged significantly. Although our results suggest that SIRT2 has maintained an ability to deacetylate histone H3 peptide acetylated on lysine-14, Hst2p cannot efficiently deacetylate an acetylated tubulin peptide. Our results are consistent with the notion that SIRT2 has evolved away from its close relatives, Hst2p, and the other human SIRTs, to specifically deacetylate α-tubulin.

The recent identification that HDAC6 is localized along the microtubule and is involved in the regulation of α-tubulin acetylation provides an explanation for the TSA-induced hyperacetylation of α-tubulin (Hubbert et al., 2002). Interestingly, endogenous SIRT2 and HDAC6 colocalize along the microtubule network, and both proteins coimmunoprecipitate after overexpression. These observations suggest that both proteins are part of a single multiprotein complex when coexpressed in cells. Interestingly, HDAC6 and SIRT2 differ in their ability to deacetylate tubulin substrates. Both enzymes deacetylate tubulin heterodimers and microtubules composed of purified tubulin proteins. In contrast, only SIRT2, and not HDAC6, deacetylates tubulin (dimers or microtubules) assembled from cell lysates. This observation could indicate the presence of a selective HDAC6 inhibitor in cellular lysate microtubules. In future experiments, it will be important to establish the relative role of each protein in tubulin deacetylation, the nature of the multiprotein complex harboring both proteins, the physiological role of the NAD-dependency of SIRT2, and the possible regulation of HDAC6 activity by cytoplasmic protein(s). However, our observation that inhibition of either HDAC6 or SIRT2 alone (via siRNA) is sufficient to induce hyperacetylation of tubulin suggest that both proteins function interdependently in tubulin deacetylation. The mechanism underlying this interdependency remains to be elucidated.

The identification of enzymes involved in tubulin deacetylation provides us with novel tools to study the biology of tubulin acetylation. Future experiments will address the role of tubulin acetylation in microtubule-associated functions such as the regulation of cell shape, intracellular transport, cell motility, and cell division.

Experimental Procedures

Tissue Culture

293T and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Bio-products, Woodland, CA) in the presence of penicillin, streptomycin, and 2 mM L-Glutamine (GIBCO Invitrogen Corp., Carlsbad, CA). The human fibroblast cell line (GM03318) was obtained from NIGMS Human Genetic Mutant Cell Repository and grown in Minimum Essential Media (MEM; GIBCO Invitrogen Corp) supplemented with 20% fetal bovine serum in the presence of penicillin, streptomycin, and L-Glutamine.

Plasmids and Mutagenesis

Human SIRT1, SIRT2, and SIRT3 constructs were a kind gift from Roy A. Frye. Human SIRT4-7 were cloned from testis and spleen cDNA libraries (Clontech, Mountain View, CA) into pcDNA3.1(+) vector (Invitrogen) by standard PCR-based strategies and confirmed by sequencing. All SIRT and HDAC cDNAs were subcloned to generate C-terminal FLAG-tagged fusions in a derivative of the pcDNA3.1(+) backbone (FLAG vector), and wild-type human SIRT2 was cloned into pEGFP-C1 vector (Clontech) and in a derivative of the pcDNA3.1(+) backbone (HA vector) by standard PCR-based strategies. Site-directed mutagenesis for SIRT2 constructs was performed with QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Purification of Recombinant SIRT2

DH5αF'IQ bacteria (GIBCO Invitrogen Corp.) were transformed with pHEX vector containing the human SIRT2 cDNA and induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for 2 hr. The resulting 6×His-tagged protein was purified under native conditions at 4°C by Ni-NTA (Qiagen, Valencia, CA), HiPrep 26/10

Desalting, and Sepharose Q chromatographies (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Generation of Human SIRT2 Antibody

SIRT2 antibody used in immunoblotting and microscopy experiments was generated by injection of purified recombinant SIRT2 into chickens (Aves Labs, Inc., Tigard, OR) and affinity purified using recombinant full-length SIRT2.

Transient Transfections and Immunoprecipitations

293T cells were transfected by the calcium phosphate DNA precipitation method and lysed 48 hr after transfection in lysis buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 0.5% NP-40, 150 mM NaCl) in the presence of protease inhibitor cocktail (Complete; Roche Molecular Biochemicals, Indianapolis, IN). FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 agarose affinity gel (Sigma, St. Louis, MO), for 2 hr at 4°C from 1 mg of total cell lysate measured by the *Dc* Protein Assay Kit (Bio-Rad, Hercules, CA). Immunoprecipitated material was washed three times for 15 min each in low-stringency lysis buffer.

Nuclear and Cytoplasmic Extracts

293T cells were transfected as described above and subjected to nuclear and cytoplasmic extraction as described (Dignam et al., 1983) modified by the addition of 1.0% NP-40 to buffer C.

Histone Deacetylase Assay

Immunoprecipitated material was washed in SIRT2 deacetylase buffer (50 mM Tris-HCl [pH 9.0], 4 mM $MgCl_2$, and 0.2 mM DTT). Both immunoprecipitated material and recombinant SIRT2 were resuspended in 100 μl SIRT2 deacetylase buffer containing NAD (Sigma) and [^{3}H] acetylated histone H4 peptide (amino acids 1–23) (Emiliani et al., 1998). TSA (WACO BioProducts, Richmond, VA) in DMSO and nicotinamide (Sigma) in ddH_2O was added to reactions at the indicated concentration with all components of the reaction in the absence of NAD for 10 min at room temperature. The enzymatic reactions were started by adding NAD and were incubated for 2 hr at room temperature. Reactions were stopped by adding 25 μl 0.1 M HCl and 0.16 M acetic acid. Released acetate was extracted in ethyl acetate and counted in scintillation fluid.

Western Blotting

Samples were separated on 10% SDS-polyacrylamide gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Inc.). Membranes were blocked with 5% blocking reagent (Bio-Rad) in TBS-Tween (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and were probed with anti-acetylated tubulin 6-11B-1, anti-tubulin B-5-1-2, or anti-FLAG M2 (Sigma), each diluted 1:2000; anti-HA, anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-lamin A (Cell Signaling Technology, Inc., Beverly, MA), each diluted 1:1000; or anti-SIRT2 diluted 1:250, or anti-HDAC6 (Santa Cruz Biotechnology) diluted 1:500. Secondary detection was performed using horseradish peroxidase-coupled sheep anti-mouse IgG (Amersham Pharmacia Biotech, Inc.), goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL), or goat anti-chicken IgY (Aves Labs, Inc.) diluted 1:5000 and ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.).

Immunofluorescence Microscopy

HeLa cells grown on coverslips were transfected with Lipofectamine (GIBCO InVitrogen Corp.). Transfected cells were incubated for 12 hr with 400 nM TSA (WACO BioProducts) 24 hr after transfection. Cells on coverslips were washed twice in PBS for 10 min, fixed in 4% paraformaldehyde (EMS, Ft. Washington, PA) for 10 min, followed by permeabilization in 0.5% Triton-X-100 in PBS for 10 min. After three washes for 10 min each in PBS, cells were incubated in 10% BSA for 10 min and then incubated for 1 hr with anti-acetylated tubulin 6-11B-1, anti-tubulin B-5-1-2 diluted 1:1000 in PBS + 0.1% Tween-20. Cells were washed three times 10 min in PBS + 0.1% Tween-20, followed by incubation with goat anti-mouse IgG (Fc specific) TRITC-conjugated secondary antibody (Sigma) diluted 1:100 in PBS + 0.1% Tween-20. Cells were then incubated in 20 μg/ml DAPI for 5 min, washed three times for 10 min each in PBS and once briefly in ddH_2O, and mounted on slides with Gel Mount (Biomeda Corp., Foster City, CA). For endogenous SIRT2 and HDAC6 immunofluorescence microscopy, human fibroblast cells were grown on coverslips and processed as described above. Anti-SIRT2 and anti-HDAC6 (Santa Cruz Biotechnology) primary antibodies were diluted at 1:10, and SIRT2 was visualized with FITC-conjugated goat anti-chicken IgY (Aves Labs, Inc.), and HDAC6 was visualized with goat anti-mouse IgG (Fc specific) TRITC-conjugated secondary antibodies. Slides were visualized on a Nikon E600 microscope system equipped with a SPOT 2 Digital Camera. Confocal images were acquired by laser-scanning confocal microscopy with an Olympus BX60 microscope equipped with a Radiance 2000 confocal setup (Bio-Rad).

In Vitro Tubulin Deacetylation Assay

For SIRT experiments (Figure 3), immunoprecipitates were resuspended in 100 μl of SIRT deacetylase buffer containing 50 μg of total cellular lysate from untransfected 293T cells and 1 mM NAD. Reactions containing 400 nM TSA or 5 mM nicotinamide (Sigma) were preincubated as described for histone deacetylase assay and incubated for 2 hr at room temperature with constant agitation after addition of NAD. Reactions were stopped by adding 50 μl of 6× SDS-PAGE buffer. Beads were pelleted by centrifugation at 14,000 rpm for 10 min, and 10 μl of each supernatant was separated on 10% SDS-PAGE gels and Western blotted as described above. For HDAC6 and SIRT2 experiments (Figure 5), assays were performed as described above in HDAC deacetylase buffer (10 mM Tris-HCl, pH 8.0, and 10 mM NaCl) for both HDAC6 and SIRT2, and were incubated with the following substrates. For purified tubulin heterodimers, tubulin (Pure, Cytoskeleton, Denver, CO) was either left as heterodimers or polymerized in PEM buffer (80 mM PIPES [pH 6.8], 1 mM $MgCl_2$, 1 mM EGTA) in the presence of 20 μM Taxol (Sigma) and 1 mM GTP (Sigma) for 30 min at 37°C, and 293T total cellular lysates harvested by sonication in PEM buffer were either left as heterodimers or polymerized in the presence of 20 μM Taxol (Sigma) and 1 mM GTP (Sigma) as described above. 25 μg of substrate was added to each reaction and incubated for 2 hr at 37°C in the presence or absence of 1 mM NAD and stopped as described above.

Tubulin and Histone H3 Peptide Kinetics with Hst2p and SIRT2

Increasing concentrations of tubulin peptide MPSD(AcK)TIGG (20–900 μM) and 800 μM of NAD were reacted in the presence of 0.8–1 μM recombinant SIRT2 in 50 mM Tris, [pH 7.5], 1 mM DTT, and 10% methanol at 37°C. For Hst2p reactions, tubulin peptides and 500 μM NAD were reacted in the presence of 9–19 μM recombinant Hst2p under the same conditions. Reactions were quenched with TFA to a final concentration of 1%. Time points were chosen to include initial velocity conditions. Samples were subjected to the High Performance Liquid Chromatography with a Beckman C18 analytical column. Upon injection, the system was run isocratically with solvent A (0.05% TFA in H_2O), followed by a gradient of 0%–10% solvent B (0.02% TFA in CH_3CN) for 4 min, and followed by a gradient of 10%–23% solvent B for 23 min. Deacetylated and acetylated peptides were eluted at 16% and 18% CH_3CN, respectively. The elution of substrates and products was monitored by measuring the absorbance at 214 nM, and corresponding peaks were integrated using the Beckman System Gold Nouveau software. The amount of product was quantified by calculating the percentage of the deacetylated tubulin peptide from the total tubulin peptide based upon their integration values. Graphs of rate versus tubulin peptide concentrations were fitted to the Michaelis-Menten equation to obtain the kinetic parameters K_m, k_{cat}, and V/K.

The monoacetylated histone H3 peptide ARTKQTARKSTGG(AcK)APRKQL (AcLys-14 H3) was utilized as substrate for both Hst2p and SIRT2. H3 peptide was [^{3}H] labeled using the histone acetyltransferase PCAF and purified as described (Tanner et al., 2000). Rate measurements utilized a charcoal binding assay where 70 μl of HDAC reactions were quenched in 10 μl of charcoal slurry (1:3 charcoal volume to glycine buffer volume) containing 2 M glycine, pH 10.0. Reaction times were chosen (usually 2–5 min) such that steady-state initial velocities were maintained. Samples were immediately heated for ≥20 min to liberate free acetate from Ac-ADP ribose before centrifugation. The supernatant was treated with 10 μl

of charcoal slurry before determining the total free acetate liberated (by liquid scintillation counting). Data were converted to initial rates and fitted to the Michaelis-Menten equation $v_0 = ([E]_0 k_{cat} \bullet [S]/(K_m + [S])$. Control experiments indicated that [^{3}H]-Ac-Lys H3 peptide was not hydrolyzed nonenzymatically. Also, addition of activated charcoal (at all pH values and temperatures examined) immediately stopped the enzymatic reaction. Heating at high pH was only necessary to liberate acetate from the Ac-ADP ribose.

siRNA Assay

A 19 nucleotide double-stranded siRNA was generated against HDAC6 using nucleotides 211–229 and SIRT2 using nucleotides 260–278 (Dharmacon Research). 293T cells were transfected by electroporation on a Gene Pulser II system (Bio-Rad) using 200V and 950 μF in a 4 mm gap electroporation cuvette with siRNA at a final concentration of 3.2 μM. Cells were transfected three times over a period of 5 days and harvested 48 hr after final transfection. Cell lysates were separated on 10% SDS-PAGE gels and analyzed by Western blotting as described above.

Acknowledgments

We thank John C.W. Carroll and Stephen Gonzales for graphics, Heather Gravois for manuscript preparation, Stephen Ordway and Gary Howard for editorial assistance, Mike Langer for technical assistance, and Roy A. Frye for human SIRT1, SIRT2, and SIRT3 constructs. This work was supported by the American Cancer Society grant # RSG-01-029-01-CNE (J.M.D.) and by The J. David Gladstone Institutes (E.V.).

Received: May 7, 2002
Revised: December 26, 2002

References

Afshar, G., and Murnane, J.P. (1999). Characterization of a human gene with sequence homology to Saccharomyces cerevisiae SIR2. Gene *234*, 161–168.

Brachmann, C.B., Sherman, J.M., Devine, S.E., Cameron, E.E., Pillus, L., and Boeke, J.D. (1995). The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev. *9*, 2888–2902.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. *11*, 1475–1489.

Elliott, G., and O'Hare, P. (1998). Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. J. Virol. *72*, 6448–6455.

Emiliani, S., Fischle, W., Van Lint, C., Al-Abed, Y., and Verdin, E. (1998). Characterization of a human RPD3 ortholog, HDAC3. Proc. Natl. Acad. Sci. USA *95*, 2795–2800.

Frye, R.A. (1999). Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem. Biophys. Res. Commun. *260*, 273–279.

Frye, R.A. (2000). Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. *273*, 793–798.

Gasser, S.M., and Cockell, M.M. (2001). The molecular biology of the SIR proteins. Gene *279*, 1–16.

Grozinger, C.M., Chao, E.D., Blackwell, H.E., Moazed, D., and Schreiber, S.L. (2001). Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J. Biol. Chem. *276*, 38837–38843.

Harrison, R.E., and Turley, E.A. (2001). Active erk regulates microtubule stability in H-ras-transformed cells. Neoplasia *3*, 385–394.

Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X.F., and Yao, T.P. (2002). HDAC6 is a microtubule-associated deacetylase. Nature *417*, 455–458.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature *403*, 795–800.

Kuo, M.H., and Allis, C.D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays *20*, 615–626.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. USA *97*, 5807–5811.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137–148.

MacRae, T.H. (1997). Tubulin post-translational modifications-enzymes and their mechanisms of action. Eur. J. Biochem. *244*, 265–278.

Nogales, E. (2000). Structural insights into microtubule function. Annu. Rev. Biochem. *69*, 277–302.

Nogales, E., Whittaker, M., Milligan, R.A., and Downing, K.H. (1999). High-resolution model of the microtubule. Cell *96*, 79–88.

Perrod, S., Cockell, M.M., Laroche, T., Renauld, H., Ducrest, A.L., Bonnard, C., and Gasser, S.M. (2001). A cytosolic NAD-dependent deacetylase, Hst2p, can modulate nucleolar and telomeric silencing in yeast. EMBO J. *20*, 197–209.

Piperno, G., LeDizet, M., and Chang, X.J. (1987). Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. J. Cell Biol. *104*, 289–302.

Polevoda, B., and Sherman, F. (2002). The diversity of acetylated proteins. Genome Biol. *3*, 1–6.

Poole, C.A., Zhang, Z.J., and Ross, J.M. (2001). The differential distribution of acetylated and detyrosinated alpha-tubulin in the microtubular cytoskeleton and primary cilia of hyaline cartilage chondrocytes. J. Anat. *199*, 393–405.

Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C., et al. (2000). A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA *97*, 6658–6663.

Takemura, R., Okabe, S., Umeyama, T., Kanai, Y., Cowan, N.J., and Hirokawa, N. (1992). Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. J. Cell Sci. *103*, 953–964.

Tanner, K.G., Landry, J., Sternglanz, R., and Denu, J.M. (2000). Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. Proc. Natl. Acad. Sci. USA *97*, 14178–14182.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149–159.