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Inhibition of Silencing and Accelerated Aging by Nicotinamide, a Putative Negative Regulator of Yeast Sir2 and Human SIRT1*

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The *Saccharomyces cerevisiae* Sir2 protein is an NAD^+-dependent histone deacetylase that plays a critical role in transcriptional silencing, genome stability, and longevity. A human homologue of Sir2, SIRT1, regulates the activity of the p53 tumor suppressor and inhibits apoptosis. The Sir2 deacetylation reaction generates two products: *O*-acetyl-ADP-ribose and nicotinamide, a precursor of nicotinic acid and a form of niacin/vitamin B_3. We show here that nicotinamide strongly inhibits yeast silencing, increases rDNA recombination, and shortens replicative life span to that of a *sir2* mutant. Nicotinamide abolishes silencing and leads to an eventual delocalization of Sir2 even in G_1-arrested cells, demonstrating that silent heterochromatin requires continual Sir2 activity. We show that physiological concentrations of nicotinamide noncompetitively inhibit both Sir2 and SIRT1 *in vitro*. The degree of inhibition by nicotinamide (IC_{50} < 50 μM) is equal to or better than the most effective known synthetic inhibitors of this class of proteins. We propose a model whereby nicotinamide inhibits deacetylation by binding to a conserved pocket adjacent to NAD^+, thereby blocking NAD^+ hydrolysis. We discuss the possibility that nicotinamide is a physiologically relevant regulator of Sir2 enzymes.

Transcriptional silencing involves the heritable modification of chromatin at distinct sites in the genome. Silencing is referred to as long range repression as it is promoter nonspecific and often encompasses an entire genomic locus (1, 2). In yeast these silent regions, which are similar to the heterochromatin of higher eukaryotes, are subject to a wide variety of modifications (3). Among the best studied of these modifications is the reversible acetylation of histones (reviewed by Refs. 4 and 5).

There are two types of enzymes that affect the acetylation state of histones: histone acetyltransferases and the opposing histone deacetylases (HDACs).[1] Compared with more transcriptionally active areas of the genome, histones within silent regions of chromatin are known to be hypoacetylated, specifically on the NH_2-terminal tails of core histones H3 and H4 (6). Three classes of histone deacetylases have been described and classified based on homology to yeast proteins. Proteins in class I (Rpd3-like) and class II (Hda1-like) are characterized by their sensitivity to the inhibitor trichostatin A (TSA) (7, 8). Studies using this inhibitor have provided a wealth of information regarding the biochemistry and cellular function of these proteins (reviewed by Ref. 9).

Yeast Sir2 is the founding member of Class III HDACs. Sir2-like deacetylases are not inhibited by TSA and have the unique characteristic of being NAD^+-dependent (10–13). Proteins of this class are found in a wide array of organisms, ranging from bacteria to humans. At least two Sir2 homologues, yeast Hst2 and human SIRT2, are localized to the cytoplasm and human SIRT1, a nuclear protein, has recently been shown to target p53 for deacetylation (11, 13–15). These results indicate that only a subset of the Sir2 family are likely to be histone deacetylases. Although insensitive to TSA, several synthetic small molecule inhibitors of Sir2 have been isolated and have provided novel insights into the biology of these proteins (16, 17).

The term silent information regulator (SIR) was first coined to describe a set of nonessential genes required for repression of yeast mating-type loci (*HML* and *HMR*) (18). Transcriptional silencing in yeast is also observed at telomeres and the ribosomal DNA (rDNA) locus (2, 19). The formation of silent heterochromatin at mating-type loci and the poly($TG_{1–3}$) tracts of yeast telomeres is mediated by a complex of Sir2, Sir3, and Sir4 (20, 21). At the rDNA locus, Sir2 is part of the RENT (regulator of nucleolar silencing and telophase exit) complex, which includes Net1 and Cdc14 (22, 23). Of these proteins, Sir2 is the only factor that is indispensable for silencing at all three silent regions (24–26).

The yeast rDNA locus (*RDN1*) consists of 100–200 tandemly repeated 9-kb units encoding ribosomal RNAs. A major cause of yeast aging has been shown to stem from recombination between these repeats (27–29), which can lead to the excision of an extrachromosomal rDNA circle. Extrachromosomal rDNA circles can accumulate to a DNA content greater than that of the entire yeast genome in old cells and are thought to kill cells by titrating essential transcription and/or replication factors (30). Although Sir2 silences polymerase II-transcribed genes integrated at the rDNA, there is evidence that its primary function at this locus is to suppress rDNA recombination. Deletion of *SIR2* eliminates rDNA silencing and increases the frequency that a marker gene is recombined of the rDNA by 10-fold (31).

Sir2 is a limiting component of yeast longevity. A single extra copy of the *SIR2* gene suppresses recombination and extends life span by 40% (28, 32, 33), whereas deletion of *SIR2* increases extrachromosomal rDNA circle formation and dramat-

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[1] The abbreviations used are: HDAC, histone deacetylase; TSA, trichostatin A; SC, synthetic medium; 5-FOA, 5-fluoroorotic acid; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; GFP, green fluorescent protein.

ically shortens life span (31, 34). Recently, it has been shown that *SIR2* is essential for the increased longevity provided by calorie restriction (32), a regimen that extends the life span of every organism it has been tested on. Moreover, increased dosage of the Sir2 homologue *sir-2.1* has been shown to extend the life span of the nematode *Caenorhabditis elegans* (35), and the nearest human homologue SIRT1 has been shown to inhibit apoptosis through deacetylation of p53 (36, 37). These findings suggest that Sir2 and its homologues have a conserved role in the regulation of survival at both the cellular and organismal levels.

Recently, a great deal of insight has been gained into the biochemistry of Sir2-like deacetylases (reviewed by Ref. 38). *In vitro*, Sir2 has specificity for lysine 16 of histone H4 and lysines 9 and 14 of histone H3 (10, 12, 13). Although TSA-sensitive HDACs catalyze deacetylation without the need of a cofactor, Sir2 requires NAD^+, perhaps allowing for regulation of Sir2 activity through changes in the availability of this co-substrate (10–13). The first step in Sir2-catalyzed deacetylation is the cleavage of the high energy glycosidic bond that joins the ADP-ribose moiety of NAD^+ to nicotinamide. Upon cleavage, Sir2 then catalyzes the transfer of an acetyl group to ADP-ribose (10, 11, 15, 39). The product of this transfer reaction is *O*-acetyl-ADP-ribose, a novel metabolite that has recently been shown to cause a delay/block in the cell cycle and oocyte maturation of embryos (40).

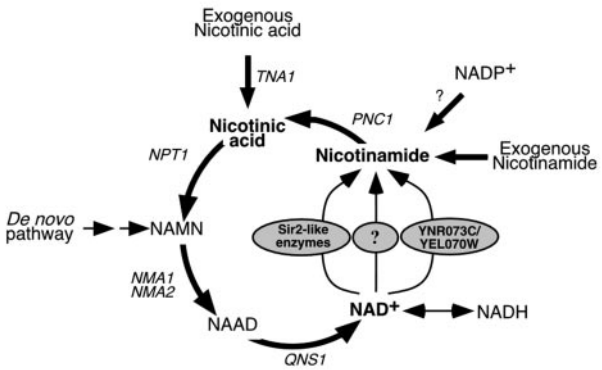

FIG. 1. **The NAD^+ salvage pathway.** Nicotinamide generated by Sir2 is converted into nicotinic acid by Pnc1 and subsequently back into NAD^+ in three steps. *YNR073C* and *YEL070W* are putative NAD^+ glycohydrolases. *Question marks* represent enzymes present in bacteria without obvious homologs in yeast. Abbreviations: *NAD^+*, nicotinamide adenine dinucleotide; *NaMN*, nicotinic acid mononucleotide; *NaAD*, desamido-NAD^+; *$NADP^+$*, nicotinamide adenine dinucleotide phosphate.

The other product of deacetylation is nicotinamide, a form of vitamin B_3 (41). High doses of nicotinamide and its acid derivative, nicotinic acid, are often used interchangeably to self-treat a number of conditions including anxiety, osteoarthritis, and psychosis. Furthermore, nicotinamide is currently in clinical trials as a therapy for cancer and type I diabetes (42). The long term safety of the high doses used in these treatments has been questioned (43), and the effects of these compounds at the molecular level are even less clear. Interestingly, nicotinamide has recently been shown to inhibit yeast Hst2 *in vitro* (44), although the effects of this compound at the organismal level or on other yeast Sir2 family members have not been investigated.

In most organisms, there are two pathways of NAD^+ biosynthesis. NAD^+ may be synthesized *de novo* from tryptophan or recycled in four steps from nicotinamide via the NAD^+ salvage pathway (45) (Fig. 1). In the salvage pathway, nicotinamide produced from NAD^+ cleavage is converted to nicotinic acid by Pnc1, a nicotinamidase (46). Nicotinic acid is subsequently converted into nicotinic acid mononucleotide by a phosphoribosyltransferase encoded by *NPT1*. We recently demonstrated that increased dosage of NAD^+ salvage pathway genes increases silencing at the rDNA locus, telomeres, and mating-type loci. We also showed that a single extra copy of the *NPT1* gene extends life span by 60% without increasing total steady-state NAD^+ levels or NAD^+/NADH ratios (33). This suggests that Sir2 may be regulated either by nuclear specific changes in NAD^+ availability, flux through the salvage pathway, or by levels of an inhibitory molecule (33).

With regards to the later hypothesis, we wished to examine whether Sir2 enzymes might be negatively regulated by nicotinamide, a product of the deacetylation reaction. Here, we show that nicotinamide strongly inhibits silencing at yeast telomeres, rDNA, and mating-type loci, whereas the related

TABLE I
Yeast strains used in this study

Strain	Genotype
W303AR5	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*ADE2, RAD5*
YDS878	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*ADE2, RAD5, sir2*:*TRP1*
YDS1572	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*ADE2, RAD5, LEU2*/*SIR2*
YDS1595	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RAD5*
YDS1596	W303 *MAT***a**, *ADE2, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RAD5*
YDS1097	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN, RAD5, GFP-Sir4*::*URA3*
YDS1099	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN, RAD5, GFP-Sir3*::*LEU2*
YDS1109	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN, RAD5, GFP-Sir3*::*LEU2, sir2*:*TRP1*
YSB0163	W303 *MATα*, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–1, his3–11, 15, MATα hmrWT*::*TRP1, HMR*::*URA3*::*ADE2*
YDS1183	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*ADE2, RAD5, SIR2-HA*::*URA3*
YDS1782	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*URA3, sir2*::*TRP*
YDS1078	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*ADE2, RAD5, Sir2-GFP*::*LEU2*
YDS1784	W303 *MAT***a**, *ade2–1, leu2–3, 112, can1–100, trp1–1, ura3–52, his3–11, 15, RDN1*::*ADE2, RAD5, Sir2-GFP, leu2*::*URA3*, *Δhml*::*LEU2*
PSY316AT	*MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1, 01 can1–100 ADE2-TEL V-R*
YDS1594	PSY316 *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R, sir2*:*TRP1*
YDS970	PSY316 *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R, HMR*::*GFP*
YDS1005	PSY316 *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R, HMR*::*GFP*
YDS1499	PSY316 *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R, HMR*::*GFP, sir4*:*HIS3*
YDS1690	PSY316 *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R, HMR*::*GFP*, *Δhml*::*LEU2*
YDS1652	PSY316AT, *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R RDN1*::*URA3*
YDS1795	PSY316AT, *MATα, ura3–53 leu2–3, 112 his3-Δ200 ade2–1,01 can1–100 ADE2-TEL V-R, pSP400-URA3*
JS209	*MATα, his3Δ200, leu2*Δ1, *met15Δ200, trp1*Δ63, *ura3–167*
JS241	JS209 MATα, *his3Δ200, leu2*Δ1, *met15Δ200, trp1Δ63, ura3–167, Ty1-MET15*
JS237	JS209 MATα, *his3Δ200, leu2*Δ1, *met15Δ200, trp1Δ63, ura3–167, RDN1*::*Ty1-MET15*
JS218	JS237 MATα, *his3Δ200, leu2*Δ1, *met15Δ200, trp1Δ63, ura3–167, RDN1*::*Tyl-MET15, sir2*::*HIS3*
YDS1583	JS237 MATα, *his3Δ200, leu2*Δ1, *met15Δ200, trp1Δ63, ura3–167, RDN1*::*Ty1-MET15, LEU2*/*SIR2*

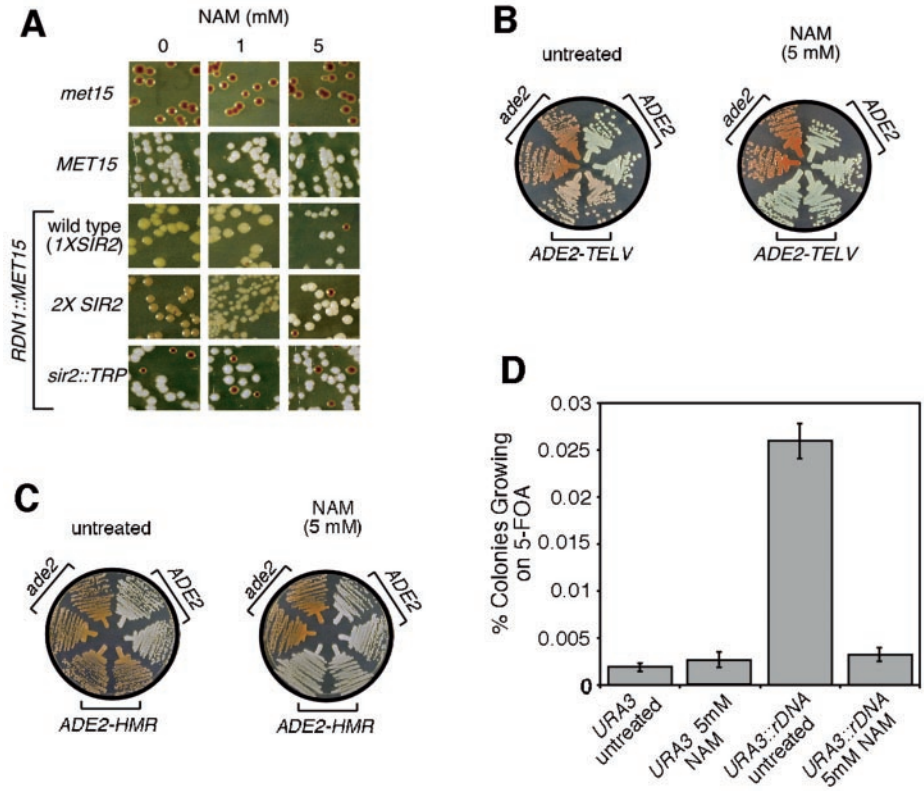

FIG. 2. **Nicotinamide inhibits rDNA, telomeric, and mating-type locus silencing.** *A*, silencing at the rDNA locus was assayed by streaking isogenic derivatives of JS237 (*RDN1*::*MET15*) on rich medium containing 0.07% $PbNO_3$ and 0, 1, or 5 mM nicotinamide (*NAM*). Silencing of the *MET15* marker is indicated by the accumulation of a *brown* pigment. *Single dark brown* colonies in *RDN1*::*MET15* strains represent marker loss events. Relevant genotypes: *met15*Δ (JS209), *MET15* (JS241), *RDN1*::*MET15* (JS237), *sir2*::*TRP1* (JS218), 2x*SIR2* (YDS1583). *B*, strains with an *ADE2* marker at the telomere were streaked onto SC medium containing limiting amounts of adenine and either 0 or 5 mM nicotinamide. Silencing of the *ADE2* marker results in the accumulation of a *red* pigment. Relevant genotypes: PSY316 ADE2-TELV (PSY316AT), W303-1A *ADE2* (YDS1596), and W303-1A *ade2* (YDS1595). *C*, strains with an *ADE2* marker integrated at the *HMR* locus were streaked onto SC medium containing limiting amounts of adenine and either 0 or 5 mM nicotinamide. Relevant genotypes: *HMR*::*ADE2* (YSB0163), W303-1A *ADE2* (YDS1596), and W303-1A *ade2* (YDS1595). *D*, strains were plated on either SC or SC medium containing 5-FOA, each with or without 5 mM nicotinamide. Colonies that arose on 5-FOA after 48 h were scored and expressed as a percentage of colonies on SC plates. Values represent the average of three independent experiments (± S.D.). Strains: PSY316AT *URA3* (YDS1795), PSY316 *URA3*::*RDN1* (YDS1652).

nicotinic acid has no effect. Nicotinamide also increases recombination at the rDNA locus and shortens yeast life span to that of a *sir2* mutant. We use this inhibitor to show that maintenance of silenced chromatin and the localization of Sir2/3/4 to telomeres require the continual activity of Sir2, even in nondividing cells. Physiological concentrations of nicotinamide inhibit Sir2 and human SIRT1 noncompetitively *in vitro*, raising the possibility that nuclear nicotinamide negatively regulates Sir2 activity *in vivo*. Our findings also suggest that the medicinal use of nicotinamide should be given careful consideration.

EXPERIMENTAL PROCEDURES

Yeast Assays—All yeast strains used in this study are listed in Table I. Cells were grown at 30 °C on YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose, w/v) unless otherwise stated. The extent of silencing at the ribosomal DNA locus was determined by growing *RDN1*::*MET15* strains on Pb^{2+}-containing medium (0.3% peptone, 0.5% yeast extract, 4% glucose, 0.02% (w/v) ammonium acetate, 0.07% $Pb(NO_3)_2$, and 2% agar). *ADE2*-based telomeric and *HM* locus silencing assays were performed as described previously (32). To monitor silencing of the *RDN1*::*URA3* marker, log phase cultures were preincubated in YPD with or without 5 mM nicotinamide for 2 h. Following this, cells were spread onto either synthetic complete (SC) medium (1.67% yeast nitrogen base, 2% glucose, 40 mg/liter each of histidine, uridine, tryptophan, adenine, and leucine) or SC medium containing 0.4 mg/ml 5-fluoroorotic acid (5-FOA) each with or without 5 mM nicotinamide. Colonies were counted after 48 h using Bio-Rad Quantity One software.

Ribosomal DNA recombination frequencies were determined as described (44). Replicative life span determination was performed by micromanipulation as described (25). A minimum of 40 cells were examined per experiment and each experiment was performed at least twice independently. Statistical significance of life span differences was determined using the Wilcoxon rank sum test. Differences are stated to be significant when the confidence is higher than 95%.

GFP fluorescence in strain YDS1005 was quantified by fluorescence-activated cell sorting (FACS) using a FACSCalibur flow cytometer (BD Biosciences) as described (45). For G_1-arrest experiments, cells were treated with 10 μg/ml α-factor mating pheromone (Sigma) for 2 h. DNA content was determined by FACS analysis of fixed cells stained with propidium iodide (Sigma) as described (47). Typically 20,000 cells were analyzed per sample. Data acquisition and analysis were performed using CELLQuest software (BD Biosciences).

Protein Determination and Fluorescence Microscopy—Western blots were performed using standard techniques. The HA epitope tag was detected using monoclonal antibody HA.11 (CRP, Richmond, CA). Actin was detected with monoclonal antibody MAB1501R (Chemicon, Temecula, CA). GFP fluorescence was visualized in live cells grown to log phase in SC medium. For arrest experiments, strain YDS1078 was first deleted for *LEU2* using a *URA3*-based disruption plasmid. The resulting strain was deleted for *HML* using a *LEU2*-based plasmid and the disruption was confirmed by Southern blot. Disrupted cells were grown to early log phase and treated with 10 μg/ml α-factor for 2 h after which time the culture was split and treated with either 0 or 5 mM nicotinamide. Images were captured using a Nikon Eclipse E600 microscope and analyzed with Scion Image software.

In Vitro Deacetylation Assays—Recombinant glutathione *S*-transferase-tagged yeast Sir2p (gift of D. Moazed) and recombinant human SIRT1 (48) were assayed for deacetylase activity using the HDAC fluorescent activity assay/drug discovery kit (AK-500, BIOMOL Research Laboratories). This assay system allows detection of a fluorescent signal upon deacetylation of a histone substrate when treated with

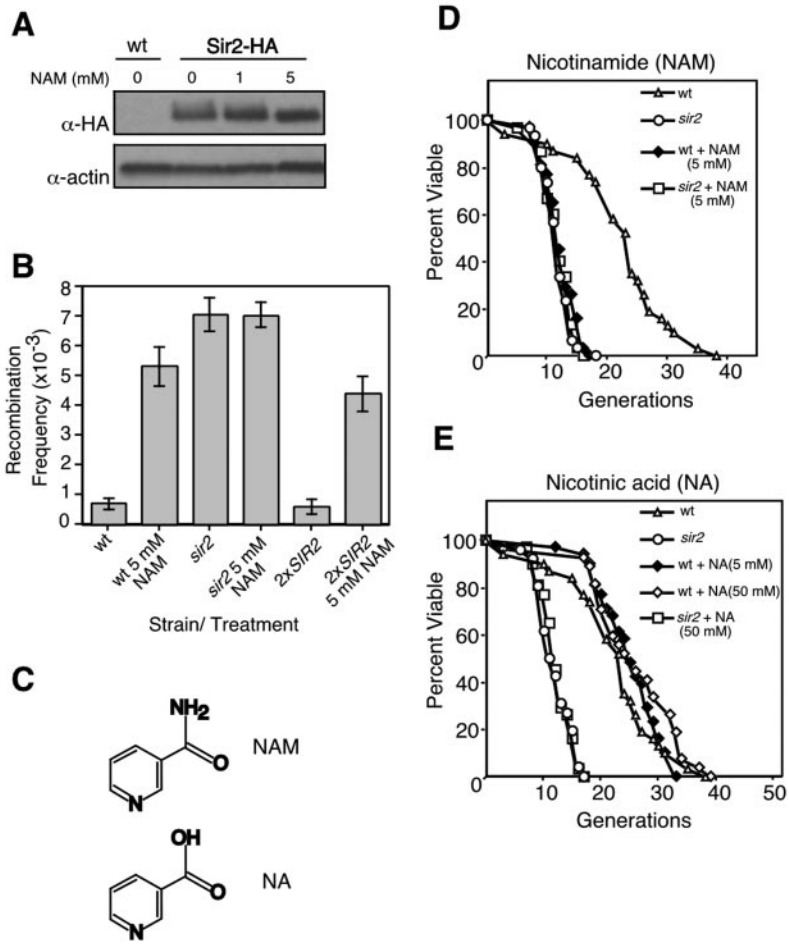

FIG. 3. **Nicotinamide increases rDNA recombination and shortens yeast life span but does not decrease Sir2 protein levels.** *A*, a HA_3 sequence was inserted in-frame with the 3′ end of the native *SIR2* open reading frame in W303AR5 (YDS1183). Cells were grown in YPD medium at 30 °C in the presence of 0, 1, or 5 mM nicotinamide. Protein extracts were analyzed by Western blot to detect the HA-tagged Sir2 using α-HA antibody. Actin levels served as a loading control. The difference between levels of Sir2 in strains grown in 0 and 5 mM nicotinamide was <6%. *B*, strains were assayed for rDNA stability by examining the rate of loss of an *ADE2* marker integrated at the rDNA locus. Cells were pretreated with 5 mM or no nicotinamide for 2 h and plated on 2% glucose YPD medium with or without 5 mM nicotinamide (*NAM*) and the frequency of half-sectored colonies, reflecting a marker loss event at the first cell division, was measured. More than 10,000 colonies were examined for each strain and each experiment was performed in triplicate. Average recombination frequencies (± S.D.) per cell division are shown. Relevant strains: W303-1A *RDN1*::*ADE2* (W303AR5) and W303AR5 derivatives 2x*SIR2* (YDS1572) and *sir2*::*TRP1* (YDS878). *C*, comparison of structures for nicotinamide (*NAM*) and nicotinic acid (*NA*). *D* and *E*, life spans were determined by scoring the number of daughter cells produced by each mother cell before cessation of cell division (65, 66). Cells were pre-grown for a minimum of 48 h on complete glucose medium. *D*, mortality curves for wild-type (PSY316AT) and *sir2*::*TRP1* (YDS1594) strains in 0 or 5 mM nicotinamide. Average life spans were: wild-type (*wt*), 22.4, 12.1; and *sir2*, 12.1, 11.7, respectively. *E*, mortality curves for wild-type and *sir2* strains from *C*, in the presence of 0, 5, or 50 mM nicotinic acid (*NA*). Average life spans were: wild-type, 22.4, 26, and 25; and *sir2,* 12.1, 12.2.

developer. Fluorescence was measured on a fluorometric reader (Cytofluor II 400 series PerSeptive Biosystems) with excitation set at 360 nm and emission detection set at 460 nm. Reactions consisted of either 5 μg of glutathione *S*-transferase-Sir2 or 2.5 μg of SIRT1, incubated with 250 μM acetylated histone substrate, 1 mM dithiothreitol, and a range of NAD^+ concentrations as described. Reactions with the yeast and human proteins were carried out at 30 and 37 °C, respectively, for 30 min.

For inhibitor assays, reactions were performed in the presence of 200 μM NAD^+ and either nicotinamide (0, 50, 150, or 300 μM) (Sigma) or 50 μM of the following compounds: nicotinic acid (Sigma), sirtinol, M15 (Chembridge), splitomicin (47), or TSA (BIOMOL).

RESULTS

Nicotinamide Abolishes Silencing at the rDNA, Telomeres, and Mating-type Loci—To examine whether nicotinamide could affect Sir2 activity *in vivo*, we examined strains with either *ADE2* or *MET15* integrated at the rDNA locus (*RDN1*). Silencing of *ADE2* results in the accumulation of a red pigment on plates with limiting adenine, whereas silencing of *MET15* leads to production of a brown pigment on Pb^{2+}-containing medium. We used two marker genes to ensure that the effects we observed were not simply because of changes in adenine or methionine biosynthesis. Strains with a single extra copy of *SIR2* (*2xSIR2*) or lacking *SIR2* (*sir2*::*TRP1*) were included as controls for increased silencing and lack of silencing, respectively. As shown in Fig. 2*A*, we observed a dramatic reduction in silencing when cells were grown in the presence of 5 mM nicotinamide. Silencing of an *ADE2* marker at this locus was similarly attenuated by addition of nicotinamide (data not shown).

To test whether this effect was specific to the rDNA or influenced all heterochromatic regions, we also examined silencing at telomeres and the mating-type locus. To monitor telomeric silencing, we used a strain in which the *ADE2* gene was integrated at the subtelomeric (Y′) region of the right arm of chromosome V (24). On plates with limiting adenine, colonies have red/white sectors because of variegated expression of the *ADE2* marker. In the presence of 5 mM nicotinamide colonies were white indicating a loss of silencing at this locus (Fig. 2*B*).

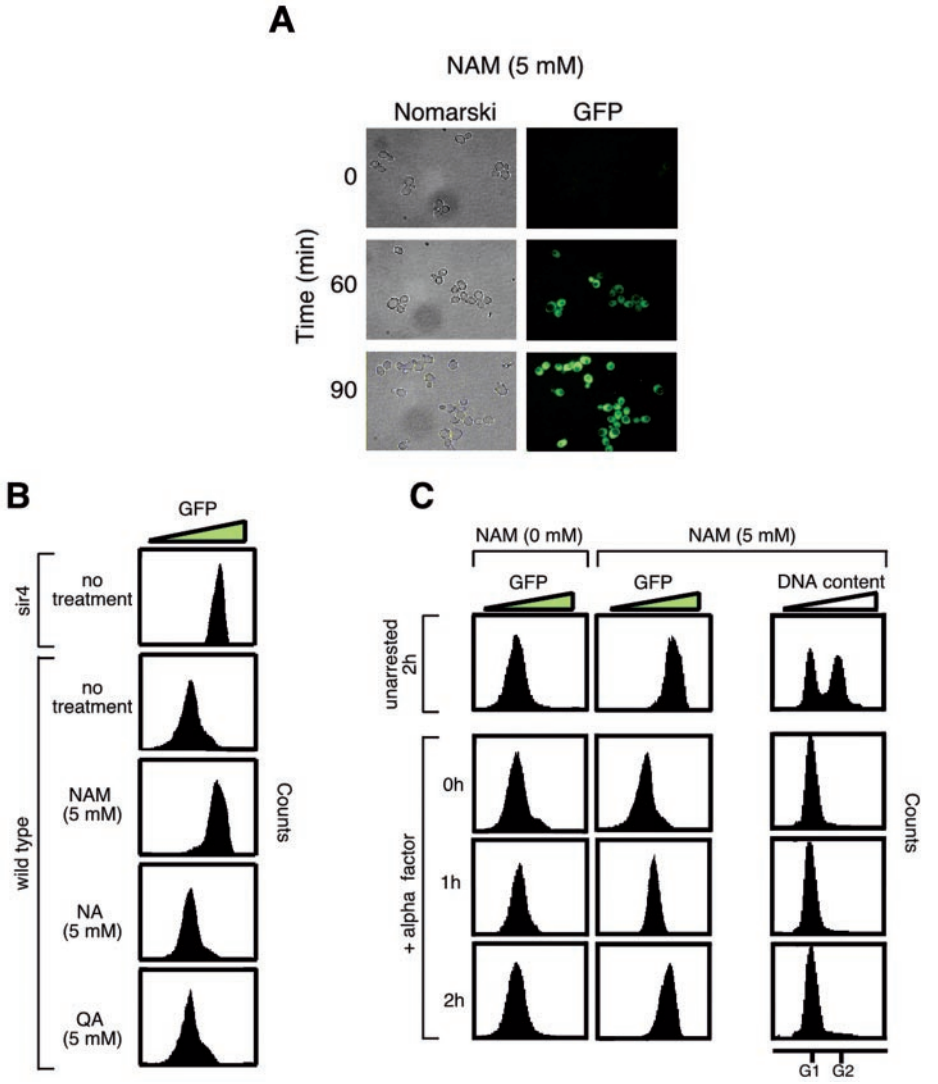

FIG. 4. **Nicotinamide derepresses the silent mating-type locus (*HMR*) in the both cycling and G_1 arrested cells.** *A*, cells containing an *ADH* driven *GFP* transcript inserted at the *HMR* locus (YDS970) were grown in YPD medium at 30 °C to mid-log phase and treated with 5 mM nicotinamide (*NAM*) for the indicated times. Cells were photographed live. *B*, strains YDS970 or the isogenic *sir4*Δ mutant (YDS1499) were treated with 5 mM nicotinamide, 5 mM nicotinic acid (*NA*), or 5 mM quinolinic acid (*QA*) for 2 h. Cells were analyzed by FACS to determine the extent of *ADH-GFP* expression. *C*, a MATa derivative of strain YDS970 (YDS1005) was deleted for *HML* and treated with 10 μg/ml α-factor for 2 h. Cells were then grown in the presence of 5 mM nicotinamide for the indicated times and examined by FACS as above. Cell cycle progression was monitored at each time point by FACS analysis of propidium iodide-stained cells.

To monitor silencing of mating-type genes, a strain with an *ADE2* marker integrated in the *HMR* locus was treated with 0 or 5 mM nicotinamide. Similar to the effect at the rDNA and telomeres, treatment with nicotinamide led to a dramatic loss of repression at this locus (Fig. 2*C*).

We wished to obtain a more quantitative measure of the extent of desilencing induced by nicotinamide. To do this, we utilized a strain containing a *URA3* marker integrated at the *RDN1* locus and a wild-type strain with an endogenous nonsilenced *URA3* gene. An equivalent number of cells of each strain were plated for single colonies onto SC medium or SC medium containing 5-FOA, in the presence or absence of nicotinamide. As shown in Fig. 2*D*, in the presence of 5 mM nicotinamide the number of colonies able to grow on 5-FOA decreased ~8-fold to a level similar to that of Ura+ cells. This increase in 5-FOA sensitivity is indicative of increased *URA3* expression resulting from a severe abrogation of silencing.

Nicotinic acid, another intermediate in the NAD^+ salvage pathway, is structurally similar to nicotinamide (see Fig. 3*C*). Nicotinic acid is taken up efficiently by yeast cells and a specific transporter for this compound, Tna1, was recently identified (49, 50). In each of the above assays, we examined the effect of 5 mM nicotinic acid on Sir2-dependent silencing and in each case found that nicotinic acid had no effect (data not shown).

Nicotinamide Increases rDNA Recombination and Shortens Yeast Life Span—Although the most likely explanation for the above observations was that Sir2 is catalytically inactivated by nicotinamide, it was plausible that Sir2 was down-regulated in the presence of this compound. To test this, we compared Sir2 protein levels in the presence and absence of nicotinamide after 2 h, a time at which silencing is almost entirely lost (see below). As shown in Fig. 3*A*, we observed no significant difference in Sir2 levels in the presence of 0, 1, and 5 mM nicotinamide. Next, we wished to address whether the loss of silencing was because of inhibition of Sir2 activity, in which case nicotinamide-treated cells should mimic a *sir2*Δ strain. One of the best characterized phenotypes of a *sir2* mutant is an increased frequency of rDNA recombination. Therefore, the loss of an *ADE2* marker integrated at the rDNA locus was scored for wild-type, 2x*SIR2,* and *sir2* strains, in the presence and absence of nicotinamide. As shown in Fig. 3*B*, treatment of wild-type and 2x*SIR2* cells with nicotinamide increased the frequency of marker loss 7-fold, similar to that of a *sir2* mutant. Importantly, treatment of the *sir2* strain did not further increase recombination, arguing that the observed marker loss was because of inhibition of Sir2.

Recombination of the rDNA locus has been shown to be a major cause of yeast replicative aging (27, 28). We therefore examined the effect of nicotinamide on yeast life span. Cells were grown for 2 days on fresh YPD medium to ensure that they were not calorie restricted prior to the assay. Daughter cells that emerged from previously unbudded (virgin) mother cells were micro-manipulated away and scored. Fig. 3*D* shows representative life span curves of both wild-type (*triangles*) and

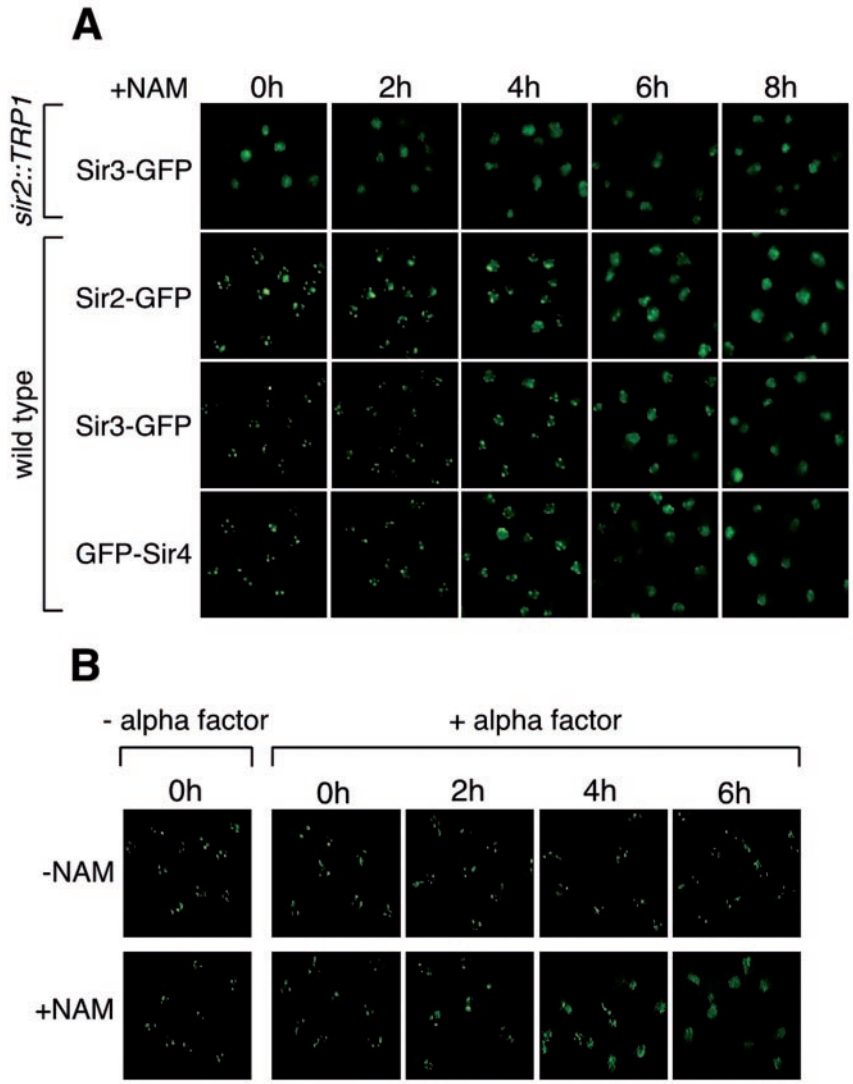

FIG. 5. **Localization of Sir2-GFP in the presence of nicotinamide (*NAM*).** *A*, wild-type strains containing *SIR2-GFP* (YDS1078), *SIR3-GFP* (YDS1099), or *GFP-SIR4* (YDS1097), and an isogenic *sir2* derivative expressing *SIR3-GFP* (YDS1109), were grown for the indicated times in the presence of 5 mM nicotinamide. GFP fluorescence was detected in live cells. *B*, the *SIR2-GFP* strain was deleted for *HML*(YDS1784) arrested in G_1 by 10 μg/ml α-factor and treated with 0 or 5 mM nicotinamide. Times indicated are post-nicotinamide treatment.

the short-lived *sir2* mutant (*circles*). Cells grown on medium containing 5 mM nicotinamide (*closed diamonds*) exhibited an average life span ~45% that of wild-type, equivalent to that of the *sir2* mutant. Treatment of the *sir2* strain with nicotinamide did not further shorten life span (*squares*). Consistent with the silencing data, we observed no detrimental effect on replicative life span in the presence of nicotinic acid (5 or 50 mM) (Fig. 3*E*, *closed* and *open diamonds*, respectively).

Nicotinamide Inhibits Silencing in Nondividing Cells—Experiments with a temperature-sensitive *sir3* allele have indicated that the presence of the Sir complex is required continually throughout the cell cycle to maintain a silenced state (51). If disrupted, re-establishment of silencing requires passage through S phase (51), although the trigger does not appear to be DNA replication (52, 53). We have shown that nicotinamide inhibits silencing in cycling cells and we wondered whether this related to its effect on the maintenance or on the establishment of silent chromatin. To test this, we used a strain containing a GFP reporter integrated at the *HMR* locus, allowing us to quantify the effects of nicotinamide on silencing in single cells. We first validated the system in cycling cells. As shown in Fig. 4*A*, GFP was not expressed in untreated cells because of the high degree of silencing at this locus. However, after 45 min in 5 mM nicotinamide we observed a dramatic increase in the level of expression, which became even more pronounced after 90 min (Fig. 4*A*, *second* and *third panels*, respectively).

To gain a more quantitative measure of silencing, cells were analyzed by FACS. The *top two panels* of Fig. 4*B* show the GFP expression profiles of asynchronous cultures of *sir4* and wild-type strains. Deletion of *SIR4* disrupts the telomeric and mating-type loci SIR complexes, leading to a redistribution of Sir2 away from these sites to the rDNA locus. Thus, the profile of the *sir4* strain represents complete derepression of the *HMR* locus. Fig. 4*B* shows that growth of wild-type cells in 5 mM nicotinamide lead to a derepression of this locus (*third panel*), similar to that observed in a *sir4* mutant. Cells treated with 5 mM nicotinic acid or the structurally related quinolinic acid (a substrate in the *de novo* NAD^+ synthesis pathway) showed no increase in GFP expression (Fig. 4*B*, *bottom two panels*), demonstrating that the desilencing effect is specific to nicotinamide.

Using this assay system, we were able to monitor the effects of nicotinamide on heterochromatin in G_1-arrested cells. A *MAT***a** strain containing the *GFP* transgene was deleted for the *HML* locus to ensure that the cells did not escape G_1-arrest because of the co-expression of **a** and α genes. After arrest in G_1 by treatment with α factor, cells were exposed to 5 mM nicotinamide and examined by FACS every 30 min. Fig. 4*C* shows the expression profiles of α factor-treated cells, in the presence and absence of this compound. In the presence of nicotinamide, arrested cells showed a loss of silencing similar to that seen in cycling cells. Measurement of DNA content by FACS confirmed

that the cells remained in G_1 for the duration of the experiment (Fig. 4*C*, *right column*). These results demonstrate that exogenous nicotinamide derepresses silent chromatin even in nondividing cells and suggests that heterochromatin is an unstable structure in the absence of Sir2 deacetylation.

Nicotinamide Causes Cell-cycle Independent Delocalization of Sir2—We next examined whether the above desilencing was associated with delocalization of Sir2. In untreated cells, Sir proteins are known to localize to distinct foci near the nuclear periphery, each focal point representing a cluster of several telomeres (54). In a *sir2* strain, Sir3 is released from telomeres and shows diffuse nuclear localization (Fig. 5*A*, *top panel*). Log-phase cultures were grown in the presence or absence of 5 mM nicotinamide for 8 h, during which time the localization of Sir2-GFP was monitored by fluorescence microscopy. Following treatment with nicotinamide, we observed no significant change in the Sir2-GFP localization pattern at time points up to 2 h, a time at which cells showed nearly maximal derepression of *HMR* (Fig. 5*A*, *second row*). At time points greater than 2 h, however, Sir2 foci became more diffuse and by 8 h no foci were apparent. We also examined the effects of nicotinamide on two other members of the Sir silencing complex, Sir3 and Sir4. The lower *two panels* of Fig. 5*A* show the localization pattern of Sir3-GFP and GFP-Sir4 at various time points post-treatment with nicotinamide. As was the case for Sir2, the pattern of fluorescence of these two proteins became diffuse after extended periods in nicotinamide.

Next, we wanted to determine whether the nicotinamide-induced delocalization of Sir2 could occur in nondividing cells, or whether it required a cell cycle-specific event. We deleted the *HML* locus in the strain carrying the *SIR2-GFP* fusion and arrested cells in G_1 with α factor as above. Treatment of arrested cells with 5 mM nicotinamide resulted in the diffusion of Sir2-GFP foci, with kinetics similar to those of cycling cells (Fig. 5*B*). Together these results indicate that Sir2 activity is required to maintain its localization, even in nondividing cells, although delocalization does not occur immediately upon Sir2 inhibition.

Nicotinamide Is a Strong Noncompetitive Inhibitor of Both Yeast Sir2 and Human SIRT1 in Vitro—To further explore the effects of nicotinamide on Sir2-dependent activities, and to gain more insight into the mechanism of desilencing, we directly measured Sir2 activity *in vitro* in the presence of varying amounts of this compound. We utilized a novel class III HDAC activity assay that generates a fluorescent signal upon deacetylation of a histone substrate. When incubated with acetylated substrate and NAD^+, recombinant glutathione *S*-transferase-tagged Sir2 gives a strong fluorescent signal 10-fold greater than no enzyme and no NAD^+ controls (data not shown). Using this assay, we tested the ability of nicotinamide to inhibit deacetylation in the presence of varying concentrations of NAD^+. A double reciprocal Lineweaver-Burk plot of the data (Fig. 6*A*) shows that nicotinamide is a strong noncompetitive inhibitor of this reaction. We next tested whether the inhibitory effects of nicotinamide could be extended to the Sir2 homologues of higher eukaryotes. Specifically, we examined whether nicotinamide could also inhibit human SIRT1 *in vitro*. Using recombinant SIRT1, we monitored deacetylation of substrate in the presence of varying amounts of nicotinamide and NAD^+. Similar to Sir2, a Lineweaver-Burk plot of the data shows that nicotinamide inhibits SIRT1 in a noncompetitive manner (Fig. 6*B*). These results imply that nicotinamide does not inhibit deacetylation by competing with NAD^+ for binding to Sir2 or SIRT1, but suggest that nicotinamide and NAD^+ might bind the enzymes simultaneously.

Recently two groups have identified synthetic compounds

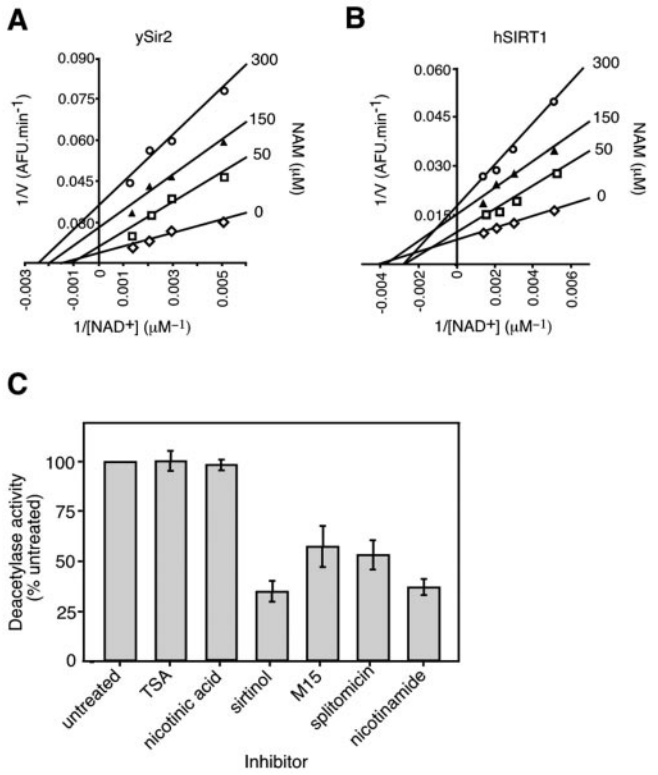

FIG. 6. **Nicotinamide is a noncompetitive inhibitor of yeast Sir2 and human SIRT1 *in vitro*.** *A*, recombinant glutathione *S*-transferase-tagged Sir2 was incubated with acetylated substrate for 30 min at 30 °C in the presence of 1 mM dithiothreitol, 200, 350, 500, or 750 μM NAD^+, and the indicated concentrations of nicotinamide. Reactions were terminated by the addition of developer and samples were analyzed by fluorometry (excitation set at 360 nm and emission at 460 nm). Experiments were performed in triplicate. Data are shown as a Lineweaver-Burk double-reciprocal plot of arbitrary fluorescence units (*AFU*) min^{-1} *versus* 1/[NAD^+] (μM). *B*, experiments were performed as in *A*, except that recombinant human SIRT1 was used and reactions were carried out at 37 °C. *C*, deacetylation reactions were performed in triplicate with 2.5 μg of SIRT1, 1 mM dithiothreitol, 200 μM NAD^+, and 50 μM nicotinic acid, sirtinol, M15, splitomicin, nicotinamide, or matched controls. Reactions were carried out at 37 °C for 30 min and fluorescence was measured as in *A*.

that inhibit Sir2-like proteins using high-throughput phenotypic screens of small molecule libraries (16, 17). None of these compounds, however, has been examined for its ability to inhibit SIRT1 activity. To compare the efficacy of inhibition of these compounds to that of nicotinamide we measured recombinant SIRT1 activity in the presence of 50 μM of each of these inhibitors. We also included nicotinic acid and the class I/II HDAC inhibitor TSA for comparison. In support of our *in vivo* results, nicotinic acid had no effect on the activity of SIRT1 *in vitro*, whereas nicotinamide inhibited SIRT1 with an $IC_{50} < 50$ μM, a value that was equal to, or lower than, that of all the other inhibitors tested (Fig. 6*C*).

DISCUSSION

We have shown that nicotinamide, a product of the Sir2 deacetylation reaction, is a strong inhibitor of Sir2 activity both *in vivo* and *in vitro*. Addition of exogenous nicotinamide to yeast cells derepresses all three silent loci, increases recombination at the rDNA locus, and shortens yeast life span to that of a *sir2* mutant. We have recently shown that strains carrying extra copies of NAD^+ salvage pathway genes show increased silencing and are long-lived, yet they do not have increased total steady-state NAD^+ or NADH levels (33). In addition, we and others have shown that two NAD^+ salvage pathway enzymes, Npt1 and Nma2, are predominately nuclear (33, 50). Based on these find-

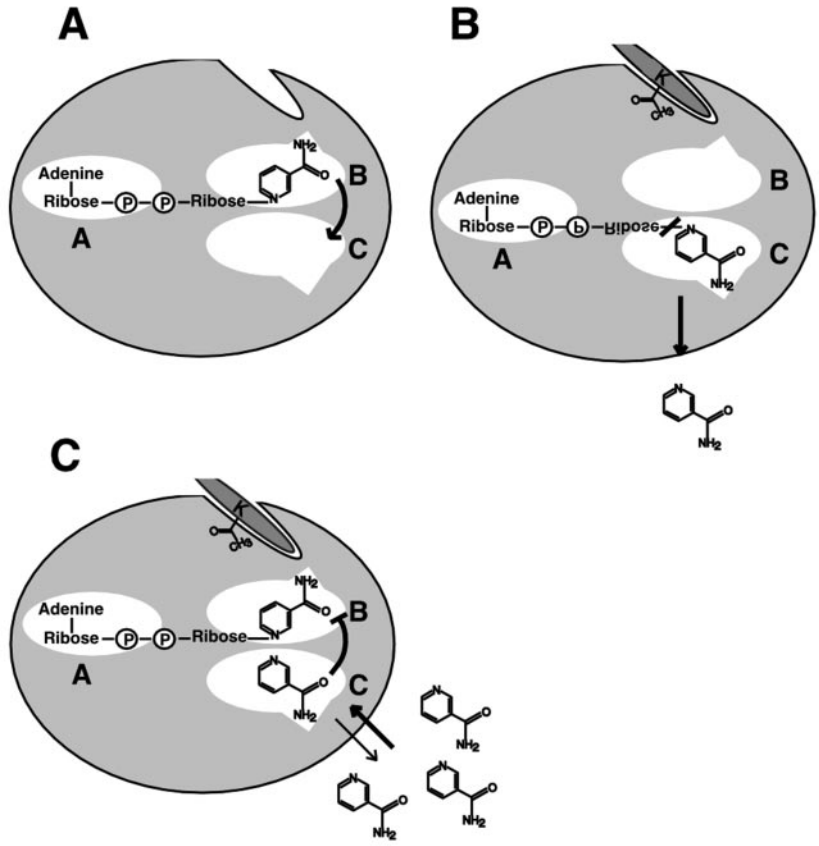

FIG. 7. **Model for noncompetitive inhibition of Sirs by nicotinamide.** *A*, the Sir2 family of deacetylases contains an NAD^+ binding pocket divided into three distinct regions. The adenine-ribose moiety of NAD^+ binds the A site whereas, in the absence of acetylated substrate, the nicotinamide moiety is bound to the B site. *B*, in the presence of an acetyllysine (*K*) a rotation around a phosphodiester bond of the pyrophosphate moiety would position the nicotinamide group near the C site where hydrolysis and subsequent nicotinamide release may take place. *C*, when present at elevated levels, free nicotinamide may bind to the C site preventing the conformational change and hydrolysis of NAD^+.

ings, we hypothesized that increased longevity is mediated by nuclear-specific increases in NAD^+ availability or increased flux through the salvage pathway. The latter model implies that there may be continual cleavage of NAD^+ by Sir2 family members. Consistent with this, we have shown with nicotinamide that Sir2 activity is required constitutively for the maintenance of heterochromatin. This is also consistent with the recent finding of Bedelov *et al.* (17) that the *MAT*α gene at the silenced *HML* locus is expressed in G_1 cells treated with splitomycin.

We find that nicotinamide disrupts Sir2 localization, consistent with recent studies showing that catalytically inactive Sir2 mutants do not associate with telomeric chromatin (55, 56). Taking advantage of the fact that nicotinamide can rapidly inhibit Sir2, we have shown that Sir2 telomeric foci remain for up to 2 h after addition of this compound and that their eventual delocalization occurs even in nondividing cells. These findings demonstrate that continual Sir2 activity is required for its localization to telomeres, and suggest that nicotinamide interferes with the maintenance of Sir2 localization, not just its establishment during the cell cycle.

We have shown that nicotinamide strongly inhibits the deacetylase activity of both yeast Sir2 and the human homologue, SIRT1 *in vitro*. The fact that nicotinamide acts noncompetitively to inhibit Sir2 enzymes, suggests that this compound does not compete with NAD^+ for binding. A similar result has recently been obtained for yeast Hst2, a cytoplasmic Sir2 homologue (44). Based on the reaction mechanism for Sir2 deacetylation and the crystal structure of an archeal Sir2 homologue, we propose the following model for Sir2 regulation by nicotinamide. Sir2-catalyzed deacetylation consists of two hydrolysis steps that are thought to be coupled. Cleavage of the glycosidic bond connecting nicotinamide to the ADP-ribose moiety of NAD^+ is followed by cleavage of the C-N bond between an acetyl group and lysine. A recent structural analysis indicates that the NAD^+ binding pocket of Sir2 enzymes contains three spatially distinct sites (A, B, and C), the later two of which are thought to be directly involved in catalysis (57) (Fig. 7*A*). In the presence of an acetyllysine, NAD^+ bound to the B site can undergo a conformational change bringing the nicotinamide group in proximity to the C site, where it may be cleaved (Fig. 7*B*). The ADP-ribose product of this reaction may then return to the B site where deacetylation of the acetyllysine occurs. We propose that nicotinamide binds to and blocks the internal C site, preventing the conformational change and subsequent cleavage of NAD^+ (Fig. 7*C*).

We have shown that the potency of nicotinamide rivals that of the most effective synthetic Sir2 inhibitors identified thus far. The fact that SIRT1 is inhibited by such low concentrations of nicotinamide *in vitro* raises the possibility that this mode of inhibition may be physiologically relevant. Levels of nicotinamide in mammalian tissues have been reported to lie in the range of 11–400 μM (41, 58–60). More recently, levels of nicotinamide in cerebrospinal fluid were determined with high accuracy to be 54.2 μM (61), a value that is similar to the IC_{50} for nicotinamide reported here. We propose that fluctuations in cellular nicotinamide levels may directly control the activity of Sir2 proteins *in vivo*. These fluctuations may in turn be regulated by enzymes involved in nicotinamide metabolism, including Pnc1.

The yeast *PNC1* gene encodes a nicotinamidase that is situated in a key position to regulate NAD^+-dependent deacetylases. By converting nicotinamide into nicotinic acid as part of the NAD^+ salvage pathway, Pnc1 may reduce levels of this inhibitor and simultaneously increase the availability of NAD^+ to Sir2 (see Fig. 1). Interestingly, *PNC1* is one of the most highly induced genes in response to stress and conditions that resemble calorie restriction (62, 63), both of which have been shown to extend replicative life span (64, 32). This raises the possibility that high levels of Pnc1 induce silencing under conditions of stress or nutrient limitation, by removing the inhib-

itory effects of nicotinamide and increasing NAD^+ production. Our previous finding that a single extra copy of *PNC1* increases Sir2-dependent silencing (33) adds further support to this model. It will be interesting to determine whether intracellular nicotinamide levels vary in response to such conditions.

Nicotinamide and nicotinic acid are used at high doses (up to 10 g/day) to self-treat a wide variety of ailments (43). Both are considered forms of vitamin B_3 and are often used interchangeably, although nicotinamide has become preferred in many cases because of an apparent lack of side effects. We have shown that these two related compounds have drastically different effects at the molecular level. In addition, nicotinamide is currently in trials as a therapy to prevent cancer recurrence and insulin-dependent (type I) diabetes (42). Our results clearly demonstrate that nicotinamide can inhibit Sir2 enzymes, even in noncycling cells, and raise the concern that there may be deleterious consequences of long term nicotinamide therapy in humans.

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