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Deacetylase Enzymes: Biological Functions and the Use of Small-Molecule Inhibitors

Review

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Introduction

The reversible acetylation of histones plays a critical role in transcriptional regulation in eukaryotic cells, and there is mounting evidence that acetylation of non-histone substrates is important for other cellular processes (reviewed in [1]). Two families of deacetylase enzymes have been identified: the histone deacetylases, or HDACs, and the Sir2 (silent information regulator)-like family of NAD-dependent deacetylases, or sirtuins (reviewed in [2]). Both families have been evolutionarily conserved from prokaryotes to humans, and both consist of several different proteins with nonredundant cellular functions, many of which involve transcriptional regulation. Several processes have evolved to control the activity of deacetylases; such processes range from the recruitment of these proteins by specific transcription factors to the more general inactivation of the deacetylases by sequestration. In the case of HDACs, small-molecule inhibitors have proven to be crucial for the identification and subsequent characterization of these enzymes. Structural differences among the members of this family suggest that it will be possible to develop specific inhibitors for each of these proteins, which would be useful for elucidating their individual cellular functions. In the case of the sirtuins, no functional characterization of any of the human proteins has been reported. Thus, the recent identification of a sirtuin deacetylase inhibitor [3] may provide a very powerful tool for studying the biology of these enzymes.

Regulation of Cellular Processes by Acetylation

Eukaryotic DNA is packaged into nucleosomes, which consist of 146 base pairs of DNA wound around a core of eight histone proteins. These nucleosomes pack together to create higher-order structures to form chromatin. X-ray crystallographic structural studies suggest that by protruding from the core and interacting with adjacent nucleosomes, the N-terminal tails of the histones mediate the formation of condensed chromatin structures. Acetylation of the lysine residues in the histone tails negates their positive charge, which could result in the destabilization of both the inter-nucleosomal interactions and the association of the histones with the negatively charged phosphate backbone of DNA. Thus, acetylation would result in the unwinding of the nucleosomal array, which is consistent with the fact that hyperacetylated chromatin is associated with active gene expression, presumably because of its increased accessibility to transcription factors. The reversible acetylation and deacetylation of histones is regulated by histone acetyltransferase and histone deacetylase enzymes (reviewed in [4]).

Although most research on reversible acetylation in the cell has focused on transcriptional regulation, the dynamic acetylation and deacetylation of non-histone proteins and small molecules is critical for many other cellular processes. There is increasing evidence that the function of several other proteins is also regulated by their acetylation state. For example, the DNA binding activity of p53, E2F1, EKLF, and GATA1 is enhanced by the acetylation of a lysine residue, and acetylation of α-tubulin appears to stabilize microtubules. Thus, acetylation of lysine residues and the negation of the positive charges associated with these side chains may be a general mechanism for altering protein structures or protein-protein interactions, much like phosphorylation (reviewed in 1). In addition to proteins, small organic molecules in the cell are also dynamically acetylated and deacetylated. Notably, polyamines such as spermidine and spermine appear to function in DNA condensation processes as free amines and are inactivated by acetylation of these amine groups [5]. Hence, while it is clear that histone acetyltransferases and histone deacetylases are critical for the regulation of transcription, these proteins, or unidentified proteins, undoubtedly function in other cellular processes as well.

The HDAC and Sirtuin Families of Deacetylases

There have been two families of deacetylases identified in eukaryotes, the histone deacetylases, or HDACs, and the Sir2-like deacetylases, or sirtuins. The HDAC enzymes possess a highly conserved catalytic domain of approximately 390 amino acids and appear to deacetylate their substrates by activating a water molecule with a divalent zinc cation coupled to a histidine-aspartate charge-relay system [6]. The HDAC family members can be divided into two classes based on their similarity to yeast histone deacetylases Rpd3 (class I) and Hda1 (class II) [7]. Four class I (HDAC1, 2, 3, and 8) and five class II (HDAC4, 5, 6, 7, and 9) HDACs have been identified and partially characterized in humans [2, 8], and there are potentially more deacetylases in this family according to the genome sequence [9]. Recently, a second family of histone deacetylases, the sirtuins, was identified. This group of proteins is related to the yeast transcriptional repressor Sir2, and its members can be divided into five classes based on their primary structure [10]. The sirtuin deacetylases contain a conserved 275 amino acid catalytic domain, which is unrelated to that of the HDACs, and the sirtuins operate by a very different mechanism that requires NAD as a substrate. Despite these structural and mechanistic differences, proteins from both families have been shown to silence transcription at specific promoters or chromosomal domains by

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localized histone deacetylation. However, it is likely that members of the two families function in other cellular processes with non-histone substrates.

HDAC Family Members

HDAC1 was the first histone deacetylase to be identified and characterized. HDAC1 was isolated by affinity chromatography with a derivative of a natural product, trapoxin A, which was discovered in a phenotypic screen involving the change in the spindle-like morphology of v-sis-transformed NIH 3T3 cells to the flattened morphology of fibroblasts [11]. Subsequently, trapoxin A was shown to inhibit the histone deacetylase activity [12, 13] that had been detected in nuclear extracts by Allfrey, now more than forty years ago (reviewed in [14]). By affinity chromatography, trapoxin A was found to bind to a previously unidentified protein, and subsequent characterization of this protein led to the determination that it was responsible for the activity observed by Allfrey [15]. This discovery of HDAC1 opened up many investigations of histone deacetylase function and quickly led to the observation that HDACs both deacetylate histones in vitro and silence gene expression in vivo [15, 16].

Sequence analysis of HDAC1 revealed that it was the human homolog of the yeast protein Rpd3 (reduced potassium dependency), a known transcriptional regulator. Subsequent fractionation of the histone deacetylase activity in yeast yielded at least two distinct protein complexes, one of which contained Rpd3, and the other of which contained a highly related protein, Hda1. Hda1 contains the conserved HDAC catalytic domain and possesses deacetylase activity, but it is significantly larger in size than Rpd3. The eight cloned human HDACs clearly fall into two separate classes by primary structure and size: those with homology to Rpd3, or the class I HDACs (HDAC1, 2, 3, and 8), and those with greater similarity to Hda1, or the class II HDACs (HDAC4, 5, 6, 7, and 9) (reviewed in [2]). All of these HDACs contain the conserved catalytic domain and possess in vitro histone deacetylase activity, but the class II proteins are two to three times larger in size than the class I proteins. Furthermore, there are certain conserved sequence motifs in the catalytic domain that differ between the two classes [7].

These two classes of HDACs appear to have diverged early in evolution and to have been conserved in a wide variety of eukaryotes. A comparison of the human and yeast HDAC sequences with those of the eubacterium *Bacillus subtilis* acetoin utilization protein (AcuC) and the proteobacterium *Mycoplana ramosa* acetylspermine deacetylase (ASD) reveals that these proteins contain features present primarily in class I and class II HDACs, respectively. Both enzymes are postulated to catalyze deacetylation of their substrates [17–19], further strengthening the evolutionary and functional link between these proteins and HDACs. This suggests that ASD and AcuC may have diverged from a common ancestor and subsequently evolved to give rise to the two classes of eukaryotic HDACs that comprise the HDAC family [7]. The two classes of HDACs have been maintained in other higher eukaryotes as well, including *C. elegans* and *Drosophila*. The evolutionary conservation of these proteins from insects to human suggests that these enzymes perform critical nonredundant functions.

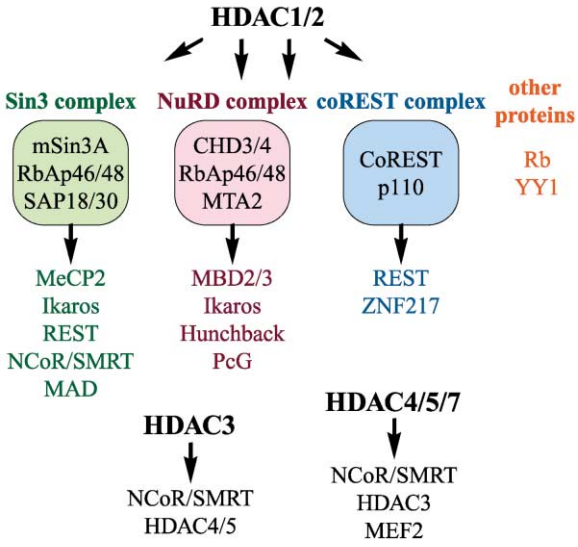

Figure 1. Protein Complexes of HDAC Deacetylases

HDAC1 and 2 are found in three main complexes, the Sin3, NuRD, and CoREST complexes, which are recruited to promoters or DNA binding domains by various proteins. HDAC1 and 2 can also be recruited directly by certain transcription factors. HDAC3, 4, 5, and 7 directly associate with the nuclear corepressors NCoR and SMRT, and with each other. HDAC4, 5, and 7 can also interact directly with members of the MEF2 family of transcription factors.

Protein Complexes of HDACs

HDAC1 and HDAC2 are the best characterized of the HDAC proteins and are generally found in stable, multicomponent complexes of proteins, which are then recruited by DNA binding proteins (Figure 1). Three complexes containing HDAC1 and HDAC2 have been characterized thus far: the Sin3, NuRD, and CoREST complexes. A "core complex," consisting of HDAC1/2 and the histone binding proteins RbAp46/48, has been described; this complex stably associates in vivo and in vitro [20]. The Sin3 complex consists of this core complex, along with SAP18 and SAP30, which appear to aid in stabilizing the protein associations, and mSin3A, which serves as a scaffold for the assembly of the complex and its interaction with various DNA binding proteins (reviewed in [21]). The NuRD complex (nucleosomal remodeling and deacetylation) also contains the core complex, as well as MTA2, which is related to a protein (MTA1) found to be overexpressed in metastatic tumor cells, and CHD3 and CHD4 (also called Mi-2α and Mi-2β), which possess the DNA helicase/ATPase domains found in the SWI/SNF family of chromatin remodeling proteins (reviewed in [22]). The coupling of chromatin remodeling and deacetylase activity may play an important role in the deacetylation of chromatinized histones in the cell; although histones can be deacetylated by HDAC1 in the absence of ATP, ATP hydrolysis, and presumably chromatin remodeling, is required for the deacetylation of oligonucleosomes in vitro [23, 24]. Unlike the other two complexes, the CoREST complex

contains HDAC1 and HDAC2 but, surprisingly, does not appear to include RbAp46 or RbAp48. It also contains a protein homologous to MTA1 and MTA2, termed CoREST, which is competent to recruit HDAC1/2 and silence expression from a reporter gene. CoREST contains two 50 amino acid SANT domains, which resemble the DNA binding domains of Myb-related DNA binding proteins. MTA1 and MTA2 also contain SANT domains, as do several other proteins involved in transcriptional regulation. Such proteins include SWI3, ADA3, NCoR and TFIIIB, for which SANT is named [25]. These SANT domains may mediate critical protein-protein interactions; indeed, the N-terminal SANT domain of CoREST is required for the recruitment of HDAC1 to promoters [26]. Another member of this complex is p110, which contains an FAD binding domain and is homologous to polyamine oxidases, although enzymatic activity has not yet been demonstrated for this protein [26, 27]. However, treatment of cells with [^{3}H]-riboflavin, a precursor to FAD, does result in the purification of radioactivity with the CoREST complex, suggesting that FAD does indeed bind to p110 [27]. Interestingly, the sirtuin family of deacetylases requires NAD as a substrate in order to deacetylate lysine residues (see below). Thus, there is a provocative link between redox cofactors and histone deacetylase activity, and these cofactors may play critical roles in transcriptional regulation.

The other HDACs have also been found in complexes with HDACs or with corepressor proteins. HDAC4 associates with RbAp48, whereas HDAC5 does not seem to associate with this protein. HDAC3 associates with HDAC4 and HDAC5, and it might associate with HDAC7, given that this protein is highly homologous to HDAC4 and 5 [7]. Furthermore, all four of these HDACs have been shown to associate directly with the corepressors NCoR and SMRT [28–32]. Additionally, HDAC4, 5, and 7 contain common N-terminal extensions, which interact directly with the MADS box domain of the MEF (myocyte enhancing factor) family of transcription factors [33–35].

Regulation of Transcriptional Silencing by HDACs

In order for transcription to be silenced, HDACs must be targeted to the promoter regions of genes because they cannot directly interact with DNA or histone proteins. Four mechanisms for regulating HDAC activity in the cell have been described (reviewed in [36, 37]). The first three mechanisms involve recruiting HDACs to genes via interactions with specific DNA binding proteins, whereas the fourth mechanism involves a higher-order regulatory system in which HDACs are inactivated by sequestration. In the simplest case, HDACs can directly interact with a DNA binding protein that specifically associates with a set of promoters. For example, HDAC1 appears to interact directly with the transcription factor YY1 [38], whereas HDAC4, 5, 7, and 9 bind to the MEF family of transcription factors. The second mechanism is similar, but in this case a complex of HDAC proteins can be recruited by a DNA binding protein, via interactions between this protein and other members of the complex. For example, the Sin3 complex is recruited by nonliganded nuclear hormone receptors. When the hormone ligand binds, the receptor alters its conformation such that it exchanges the HDAC complex for a histone acetyltransferase complex. Thus, the genes regulated by these nuclear hormone receptors shift from a silent state to one of active expression upon the addition of a small-molecule ligand (reviewed in [4]). Alternatively, the NuRD complex is recruited by the polycomb and hunchback repressors, which are involved in silencing gene expression from clusters of homeotic genes. Given these observations, it is hypothesized that the Sin3 complex is involved in silencing specific genes, whereas NuRD silences large chromosomal domains by a mechanism that may require its chromatin remodeling activity (reviewed in [22]). However, there are cases in which transcription factors recruit multiple HDAC complexes. For example, Ikaros, a protein involved in lymphoid cell development and T cell activation, can bind to both Sin3 and NuRD (reviewed in [22]), whereas REST, which silences neuronal-specific genes in non-neuronal cells (reviewed in 39), can bind to both the Sin3 and CoREST complexes [40–43]. Additionally, both the Sin3 and NuRD complexes have been shown to associate with different members of the methylated DNA binding protein family (reviewed in [22]).

A third mechanism for regulating HDAC activity involves blocking the binding of an HDAC to a transcription factor. Such a mechanism regulates the interaction between HDAC4 and MEF2 [44]. HDAC4 and the histone acetyltransferase p300 compete for the same binding site on MEF2; thus, altering the binding partner allows MEF2 to switch from silencing to activating gene expression. The MEF2 binding site on HDAC4 overlaps with a calmodulin binding site, and in the presence of calcium, HDAC4 binds to calmodulin, loses its interaction with MEF2, and thereby allows MEF2 to recruit p300 (Figure 2). Interestingly, the N-terminal domain of HDAC4/5/7 can slightly inhibit MEF2 transcription in the absence of the deacetylase catalytic domain [33, 35, 44, 45]; this may be due to its ability to block p300 binding. A protein with homology to the N-terminal domains of HDAC4 and 5 but without any catalytic domain has been identified. It is possible that this protein functions as a dominant-negative version of HDAC4 and 5 by blocking its binding to protein partners [46].

The fourth mechanism for regulating HDAC activity involves sequestration of HDACs in an extranuclear compartment. This method of regulation thus operates at a higher level than the previously described mechanisms. This process has been characterized for the class II HDACs 4, 5, and 7 (Figure 2). These HDACs can be phosphorylated at serine residues at three 14-3-3 binding sites in their N-terminal domains. This phosphorylation both disrupts the HDAC-MEF interaction [47] and allows binding of 14-3-3, which occludes an importin binding site and results in the shuttling of the HDACs out of the nucleus into the cytoplasm, where they are presumably held in an inactive state with respect to transcriptional regulation [48]. After the release of the HDAC proteins, MEF can recruit a histone acetyltransferase complex and activate the transcription of genes required for muscle differentiation. Thus, upon induction of differentiation of myoblasts into myotubes by serum starvation, HDAC4, 5, and 7 shuttle out of the nucleus [33, 49], and histones in the promoters of the MEF-

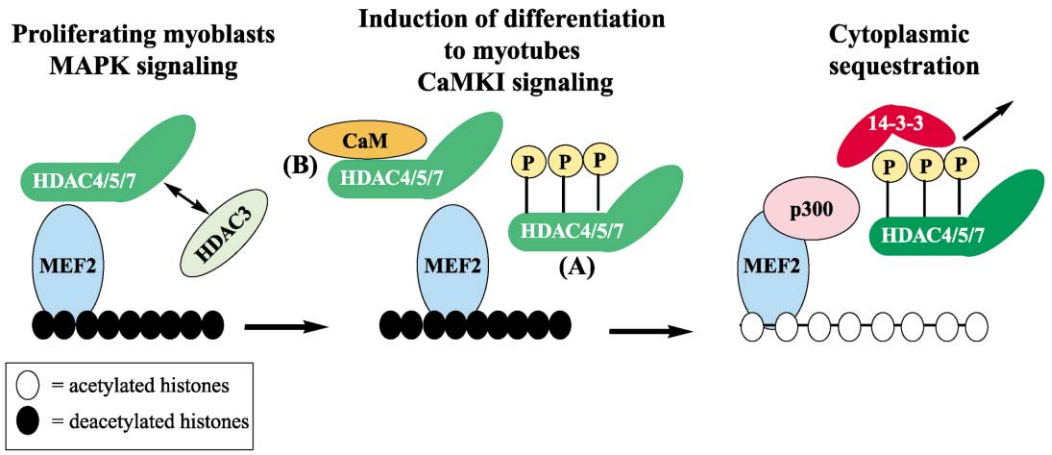

Figure 2. Model for Regulation of HDAC4, 5, and 7 Transcriptional Silencing Activity

In proliferating myoblasts, the MAPK signaling pathway is activated, and HDAC4, 5, and 7 are localized to the nucleus, where they interact with HDAC3 and inhibit MEF2 transcriptional activity. Upon the induction of differentiation to myotubes, the HDACs dissociate from MEF2, either because of phosphorylation by CaMKI signaling (A) or because of the binding of calmodulin (CaM) (B). HDAC is sequestered in the cytoplasm by 14-3-3 proteins, and MEF2 recruits the histone acetyltransferase p300 to activate gene expression.

regulated genes become acetylated [50], which is correlated with the expression of these genes.

This shuttling process appears to be regulated in part by CaMKI (Ca^{2+}/calmodulin-dependent kinase I) because overexpression of CaMKI causes cytoplasmic localization of HDAC4/5 and CaMKI can phosphorylate HDAC5 in vitro. Mutation of the 14-3-3 binding sites in HDAC5 prevents dissociation of HDAC5 and MEF upon CaMKI overexpression [47] and blocks myogenesis [49]. Interestingly, a second type of phosphorylation seems to be required to shuttle HDAC4 into the nucleus. It has recently been shown that HDAC4 is associated with ERK1/2 kinases and can be phosphorylated by them in vitro. Activation of the Ras-MAPK (mitogen-activated protein kinase) signal transduction pathway causes an increased nuclear localization of HDAC4 [51]. Notably, ERK1 and 2 have been implicated in the repression of myoblast proliferation by growth factors [52], and thus the CaMKI and MAPK signaling pathways may control muscle differentiation via their competing effects on HDAC localization.

Mechanism of Deacetylation by HDACs

In 1999, the crystal structure of a bacterial HDAC homolog, HDLP (histone-deacetylase-like protein), bound to an HDAC inhibitor, trichostatin A (TSA), was reported [6]. The catalytic domain of HDLP is very closely related to those of both classes of HDACs, and thus the mechanism of deacetylation is presumably conserved as well. HDLP has a single domain structure related to the open α/β class of folds. It contains a central eight-stranded parallel β sheet, with four α helices packed on either face. Eight additional α helices and large loops in the β sheet further extend the structure and result in the formation of a deep, narrow pocket with an adjacent internal cavity. TSA binds to this pocket, which thus represents the active site of the enzyme. HDLP requires Zn^{2+} for activity; this cation is positioned near the bottom of the pocket and is coordinated by several histidine and aspartate residues (Figure 3). The channel leading to the active site is surrounded by hydrophobic residues, which is presumably where the aliphatic chain of the acetyl-lysine residue is nestled.

The cocrystallization of TSA with HDLP allows for an analysis of the structural properties of these HDAC inhibitors. TSA contains a cap group, an aliphatic chain, and a terminal hydroxamic acid functional group (Figure

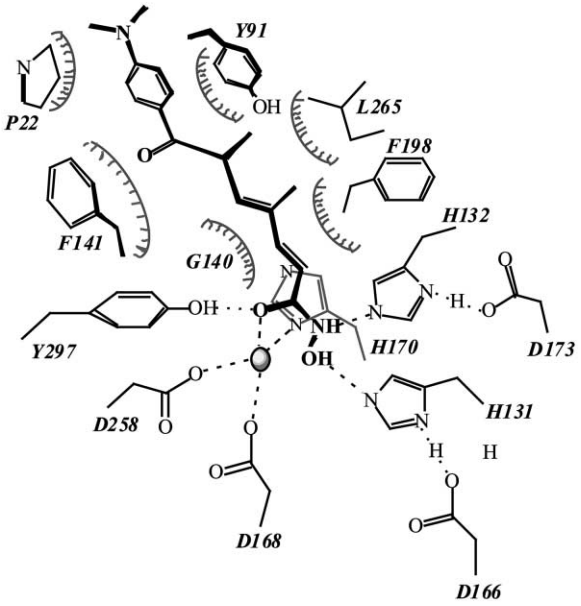

Figure 3. Structure of HDLP Active Site Bound to Trichostatin A

The hydroxamic acid functional group of TSA coordinates the zinc cation and makes hydrogen bonds (shown as dashed lines) to several amino acids in the active site. The aliphatic chain of TSA makes van der Waals contacts (thatched semi-circles) with the residues lining the channel, and the cap group of TSA interacts with proline 22 and tyrosine 91 of HDLP. Notably, Y91 is the only residue whose conformation is altered upon TSA binding [6].

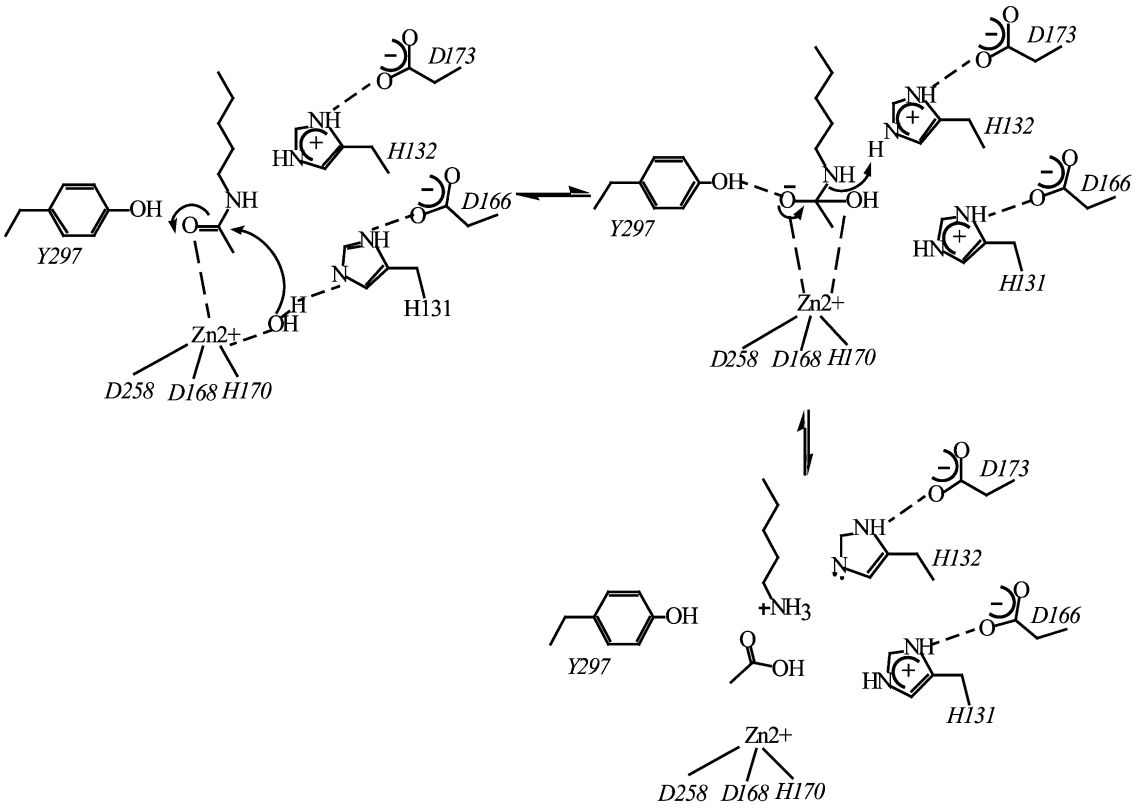

Figure 4. Proposed Mechanism for HDAC Deacetylation

The HDLP-mediated deacetylation of an acetylated lysine residue is depicted above. A bound water molecule is activated by both the zinc cation and a histidine-aspartate charged relay system to attack the amide bond. The oxy-anion intermediate is stabilized by the zinc cation and an adjacent tyrosine residue. As this intermediate collapses, the carbon-nitrogen bond breaks, and the nitrogen accepts a proton from an activated histidine residue [6].

3). The hydroxamic acid coordinates the zinc cation in a bidentate fashion and hydrogen bonds with some of the active site residues. Fitting snugly into the channel, the aliphatic chain makes several van der Waals contacts with the channel residues. The cap group contacts residues on the rim of the pocket and possibly mimicks the amino acids adjacent to the acetylated lysine residue in the histone. The binding of TSA causes a conformational change in a tyrosine residue on this rim (tyrosine 91) and thereby allows tighter packing of the cap group.

The proposed mechanism for the deacetylation reaction is similar to that seen in metallo- and serine proteases (Figure 4). The carbonyl oxygen of the N-acetyl amide bond is thought to coordinate to the zinc cation and to thereby position it closely to a bound water molecule and activate it for a nucleophilic attack by the water.

The nucleophilicity of the water molecule, in turn, could be enhanced by an interaction with the negatively charged histidine-aspartate pair, and it is proposed to be oriented by coordination to the zinc ion. Attack of the water molecule on the carbonyl carbon would produce an oxy-anion intermediate, which is possibly stabilized by the zinc ion and by hydrogen bonding to a nearby tyrosine. The collapse of this intermediate would result in cleavage of the carbon-nitrogen bond, with the nitrogen accepting a proton from the histidine residue, and would thereby produce the observed acetate and lysine products. Note that this mechanism requires that the zinc ion function in coordinating both the water molecule and the carbonyl oxygen. The geometry of electrostatic interactions in this system makes it unlikely that the zinc can coordinate both molecules, and indeed, most metal-dependent proteases use the metal ion either to activate the water or to stabilize the oxy-anion intermediate (G.L. Verdine, personal communication). Thus, it is likely that zinc ion either coordinates the water molecule or stabilizes the oxy-anion intermediate, whereas the other process is carried out by interactions with the amino acid residues in the active site of the enzyme.

Structural Differences of the Human HDACs

The catalytic domains of the nine known human HDACs are very well conserved, but there are certain differences that may allow for the production of specific inhibitors. Most of the residues that are seen in the HDLP structure to interact directly with TSA are completely conserved among all of the HDACs, but there is less conservation in the surrounding residues, with significant differences apparent between the class I and class II HDACs (Figure 5). Notably, there is a striking divergence in the region of tyrosine 91 of HDLP, and this tyrosine residue itself is very poorly conserved among the human HDACs. This is particularly interesting in that tyrosine 91 is positioned on the rim of the channel and interacts directly with the

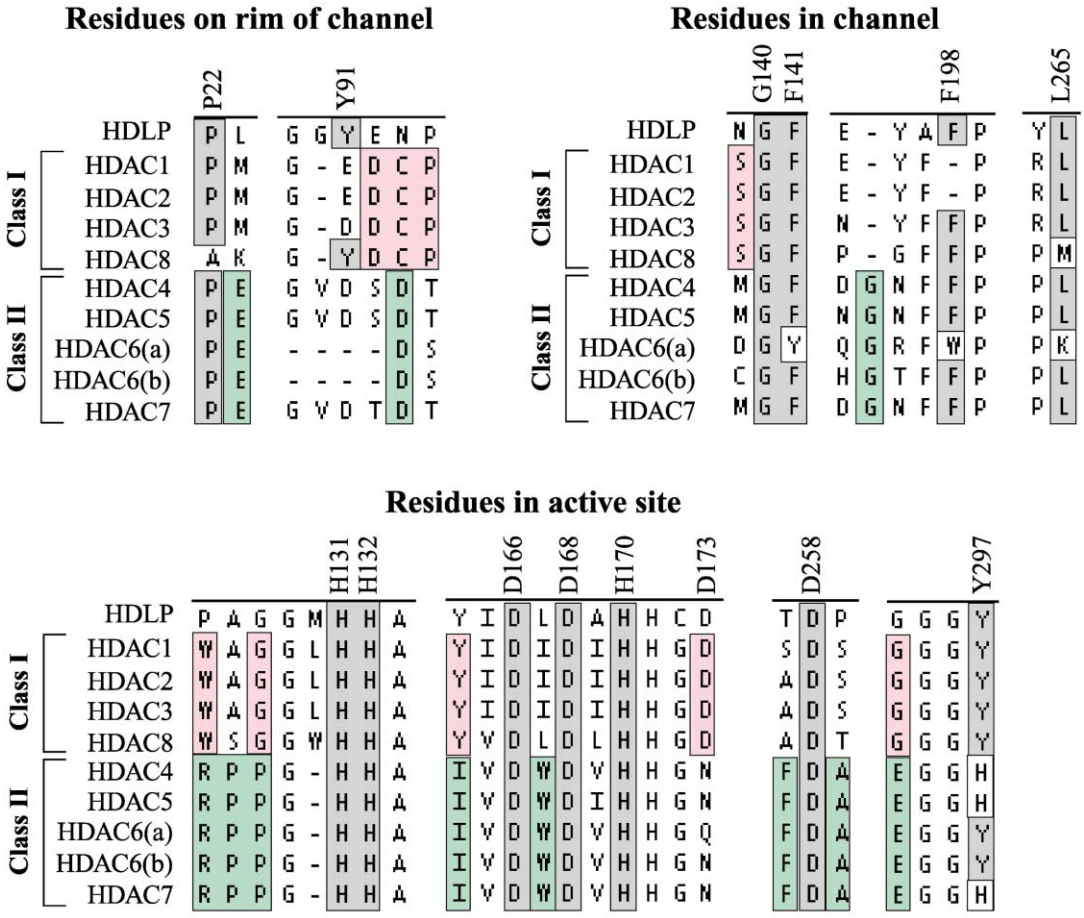

Figure 5. Partial Sequence Comparison of the HDAC Family Members

The catalytic domain of HDLP was aligned with those of HDACs 1–8, and the regions surrounding the active site were analyzed. The residues of HDLP directly interacting with TSA are labeled and are colored in gray when they are conserved among the other HDACs. Adjacent residues that differ between the class I and class II HDACs are boxed. Note that there are several sequence differences in these areas among the eight HDACs, particularly in the vicinity of tyrosine 91 of HDLP (GenBank Accession Numbers: HDAC1 Q13547, HDAC2 Q92769, HDAC3 NP_003874, HDAC4 AAD29046, HDAC5 AAD29047, HDAC6 AAD29048, HDAC7 NP_056216, and HDAC8 AAF73428; not shown: HDAC9 AAK66821).

cap group of TSA, and it is the only residue that shifts its conformation upon TSA binding [6]. Thus, the considerable diversity in the region of the protein suggests that it will be possible to develop specific inhibitors by altering the structure of this cap group.

Synthetic HDAC Inhibitors

Several non-natural inhibitors of histone deacetylases have been synthesized, and analysis of these will facilitate the discovery of specific inhibitors. The basic structure of these small molecules mimics that of TSA in that they possess a cap group, an aliphatic chain, and a functional group that would chelate the metal cation in the active site (Figure 6). The experimentally determined optimal length for the aliphatic chain is five to six carbon residues, with inhibitory activity decreasing rapidly for longer and shorter chains [53]. There are several possible functional groups, hydroxamic acids [53–56] and phenylene diamines [57], which presumably reversibly coordinate the zinc cation, as well as epoxides, which appear to form covalent bonds with active site residues [54] that are irreversible under native conditions.

Two types of cap groups are found in natural HDAC inhibitors, either a small planar group (as in trichostatin

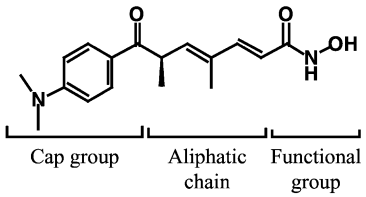

Figure 6. Structural Properties of HDAC Inhibitors

Trichostatin A contains a hydroxamic acid functional group that chelates the zinc cation in HDAC and makes hydrogen bonds to various amino acids in the active site. The aliphatic chain mimics that of lysine and forms van der Waals interactions with the residues lining the channel. Note that the optimal chain length is 5–6 carbon residues. Finally, the cap group interacts with the residues around the rim of the channel.

A) or a cyclic tetrapeptide group (as in trapoxin A). Interestingly, the type of cap group alters the ability of the compound to inhibit HDAC activity in vitro. Molecules with a small cap group are not active if the functional group is a carboxylic acid [53], whereas molecules with a cyclic tetrapeptide cap group and a carboxylic acid functional group are potent inhibitors [54].

Generation of Specific HDAC Inhibitors

Specific HDAC inhibitors would be extremely valuable, both as tools for probing the biological functions of these different enzymes and for therapeutic purposes, such as inhibiting a specific HDAC that is associated with a particular disease. The structural differences in the active sites of the HDACs is encouraging in this regard. Indeed, alterations in the structure of the cap group have led to specific inhibitors of HDACs in two cases. First, N-alkylations of the indole residue in the cap group of apicidin has led to the development of apicidin derivatives that are approximately 20-fold more potent as inhibitors of malarial HDACs than is human HDAC1 [58]. Secondly, HDAC6 is not inhibited by any of the cyclic tetrapeptide inhibitors, even when a hydroxamic acid is used as a functional group [54]. Notably, HDAC6 has the greatest divergence from the other HDACs in the rim region surrounding tyrosine 91 in HDLP. Thus, it should be possible to generate specific inhibitors of the HDAC family of histone deacetylases by creating biased combinatorial libraries containing an aliphatic chain and terminal functional group attached to diverse capping groups.

Sirtuin Family Members

Members of the sirtuin family of histone deacetylases possess several conserved sequence motifs. The catalytic domain consists of approximately 275 amino acids and contains two sets of CXXC motifs, which may function as zinc finger domains [59], as well as one or more hydrophobic regions that may function as leucine zippers [60]. Both of these types of domains could mediate protein-protein or protein-DNA interactions, although there is no evidence for the latter activity in any of the sirtuins. There are several other short conserved sequences in this domain as well, and mutation of some of these have been shown to abrogate catalytic activity [59, 61].

Several sirtuins have been identified in prokaryotes and eukaryotes, and these can be divided into five classes based on sequence homology. *S. cerevisiae* has five sirtuins, including the founding member, Sir2, and four homologs, Hst1–4 (homolog of Sir two). Four sirtuins have been identified thus far in *C. elegans*, and five in *Drosophila*. Seven sirtuins (SIRT1-7) have been identified in humans. Several bacterial species, including *E. coli*, *Streptomyces*, and *Bacillus subtilis*, have sirtuins as well. All five yeast sirtuins are class I proteins, as are human SIRT1, 2 and 3. SIRT4 is a class II sirtuin, SIRT5 is in class III, and SIRT6 and SIRT7 are class IV proteins. There are no class I or IV sirtuins in prokaryotes, and thus these sirtuins may represent the most recently evolved members of this family, whereas most bacterial sirtuins are of the class III variety [10].

Protein Complexes and Cellular Functions of Sirtuins

Yeast Sir2 is the best-characterized sirtuin protein. It was originally identified in a screen for mutants that disrupted the transcriptional silencing of the *HM* loci in yeast [62, 63], and it has since been shown to be involved in silencing gene expression at telomeres and rDNA locus as well (Figure 7). Sir2 is found in two major complexes, one of which is responsible for silencing expression from the telomeric and *HM* loci (Sir2, Sir3, and Sir4), and a second that suppresses genetic recombination by forming a closed chromatin structure at the rDNA locus (Sir2, Net1, Cdc14) (reviewed in [64]).

When tethered to telomeric loci, Sir2 performs several functions aside from silencing gene expression. Telomeric Sir2 is required for the maintenance of genomic integrity and may play a role in the DNA damage response. Deletions of Sir3 or Sir4 result in a shortening of the telomeres and loss of chromosomes during mitosis. Furthermore, overexpression of Sir4 results in an increase in telomere length, suggesting that the Sir2/3/4 complex plays a direct role in stabilizing telomeres in yeast [65]. Additionally, a fraction of the telomeric Sir2/3/4 complex is bound there by the Ku70/80 heterodimer (Figure 7). Upon double-stranded DNA break formation, Ku relocates to the damaged sites in a Mec1-dependent manner and subsequently recruits telomeric Sir3, Sir4, and possibly Sir2 to these sites. Indeed, DNA damage has been shown to derepress telomeric genes, presumably by the movement of the Sir2/3/4 complex from the telomeres to damaged regions of the genome [66–69]. Thus, the telomere may serve as a resevoir for Sir2 proteins that can be mobilized to sites of DNA damage when necessary.

At the rDNA locus, yeast Sir2 inhibits genetic recombination and thereby modulates life span. The rDNA locus consists of 100 to 200 copies of genes encoding rRNA and thus is prone to high recombination frequencies. The frequency of these recombination events is reduced by the formation of a hypoacetylated, condensed chromatin structure by Sir2. Furthermore, nucleolar Sir2 also binds to Pch2, a protein required for meiotic pachytene checkpoint, which inhibits recombination at the rDNA locus during meiosis [70]. Additionally, nucleolar Sir2 also appears to be in a complex with Cdc14, a phosphatase that is required for passage through telophase (Figure 7) [71] and which must be released from the nucleolus at the end of mitosis in order for the cell cycle to proceed. Interestingly, Sir2 is also released from the rDNA locus at mitosis, but the function of this is unknown [72]. Sir2 will also silence reporter genes with weak promoters that have been inserted into the rDNA locus [73, 74].

Sir2 has been linked to aging in yeast, and this is thought to be mediated by its NAD dependence and its repression of rDNA recombination (Figure 8, reviewed in [75]). As previously discussed, acetylation and chromatin decondensation at the rDNA locus leads to increased recombination rates in this region. Because of the large arrays of repeated DNA sequences, this allows the rDNA repeats to be spliced out, producing extrachromosomal circles (ERCs). ERCs accumulate in older yeast cells and have been found to decrease yeast life

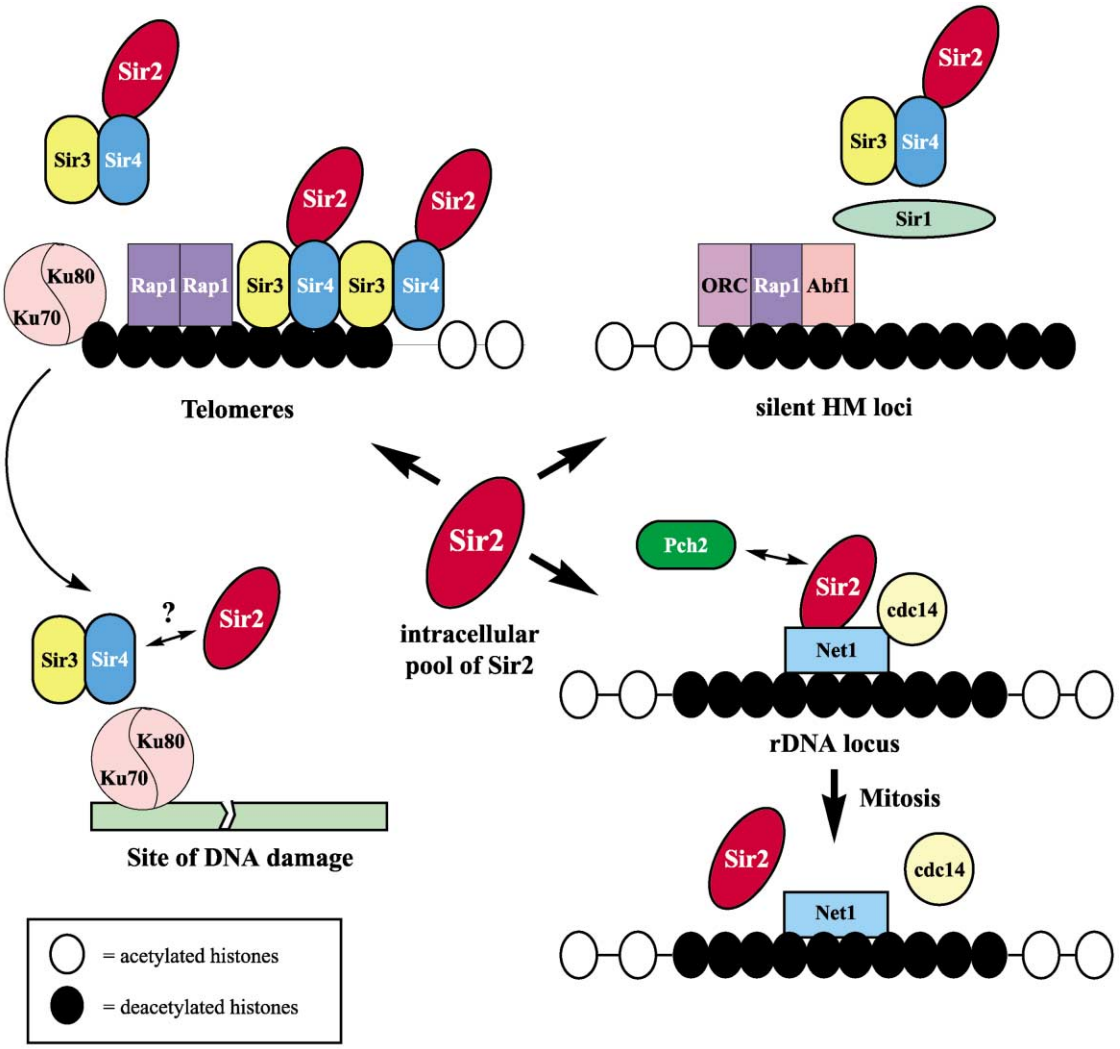

Figure 7. Complexes of Sir2

Various DNA binding proteins recruit the intracellular pool of Sir2 to the telomeric and silent mating loci (*HMRE* is shown here) as a complex with Sir3 and Sir4. Upon DNA damage, the Ku heterodimer relocates from the telomeres to the damaged regions and recruits Sir3/4 and, presumably, Sir2. At the rDNA locus, Sir2 is associated with Net1, Pch2, and Cdc14. At the end of mitosis, Sir2 and Cdc14 are released from the nucleolus.

span when artificially introduced. Loss of function mutations or deletions of Sir2 causes an increase in ERC formation as well as a decrease in life span, whereas the addition of an extra copy of Sir2 results in a substantial increase in life span. Notably, a deletion of a protein responsible for recombination at the rDNA locus will rescue the decreased life span and increased frequency of ERCs found in Sir2 mutants, suggesting that increased recombination is indeed the main deleterious factor [76].

It has also been shown that starvation or glucose deprivation leads to an increased life span for a variety of organisms. Indeed, mutation of a glucose-sensing pathway in yeast causes an increase in life span, and this is not further enhanced by overexpression of Sir2, suggesting that they function on the same pathway [77]. Since NAD levels are closely linked to metabolic activity in the cell, it is hypothesized that Sir2 is regulated by the metabolic rate. Thus, increases in glucose intake lead to increased metabolism, which results in a reduction of NAD to NADH. This decrease in the amount of available NAD decreases Sir2 activity and increases recombination rates and the formation of toxic ERCs, which accumulate in the nucleus of the mother cell and lead to a shortened life span (Figure 8). The critical role of NAD levels in this process is evident from the fact that mutations in an NAD biosynthetic pathway in yeast lead to a decrease in life span that is not rescued by mutation in the glucose-sensing pathways or Sir2 [77]. Notably, the accumulation of ERCs is not apparent during the aging of other metazoan cells, although there is evidence for a decreased expression of neuronal rRNA due to a loss of rDNA genes during aging in mammals (reviewed in [78]). However, in these cases, loss of Sir2 activity could lead to altered expression of sets of genes that are critical for the maintenance of the cell, which

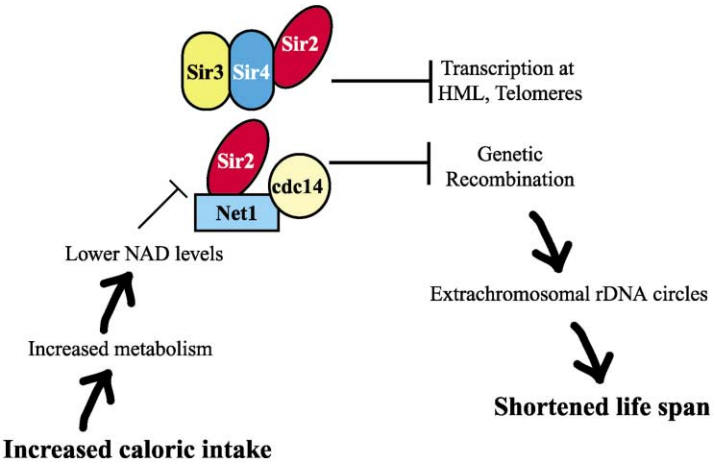

Figure 8. Model of the Regulation of Life Span by Sir2

Nucleolar Sir2 inhibits recombination at the rDNA locus and thereby inhibits the formation of toxic extrachromasomal rDNA circles. Increased metabolic levels due to increased caloric intake have also been shown to decrease life span, and it is thought that this is mediated by the effects of NAD levels on Sir2 activity.

could lead to the onset of senescence and death (reviewed in [75]).

The other yeast sirtuins also appear to have roles in regulating transcription, modulating cell cycle progression, maintaining genomic integrity, and DNA damage repair. Hst1 is the closest Sir2 homolog in yeast and is recruited by the DNA binding protein Sum1 to promoters of meiosis-specific genes to repress expression of these genes during vegetative growth [79]. Hst2 is a cytosolic protein and therefore probably is not directly involved in transcriptional regulation unless it can be shuttled into the nucleus under certain conditions. It cannot restore silencing in a Sir2 deletion strain, but overexpression in a Sir2+ strain enhances rDNA silencing and inhibits telomeric silencing. Since this phenotype is also observed when Sir2 itself is overexpressed, it has been suggested that Hst2 and Sir2 use a common substrate that is depleted by overexpression of either of these proteins. Notably, overexpression of Hst2 containing a point mutation that destroys catalytic activity still disrupts telomeric and rDNA silencing, and thus NAD is not the limiting factor. Furthermore, since Sir4 is delocalized from the telomeres upon overexpression of Sir2, but not Hst2, it does not appear that the core Sir2/3/4 complex is involved [80]. Hst3 and Hst4 are less closely related to Sir2, but a Δ*hst3*Δ*hst4* double deletion is defective in telomeric silencing. Furthermore, these double mutants also show an increased sensitivity to UV damage and have a higher rate of chromosomal missegregation. Additionally, these cells arrest in G2/M. This suggests that Hst3 and Hst4 may function redundantly in pathways required for ensuring genomic integrity and cell cycle checkpoints [60]. Thus, although mutations or deletions of all of the known homologs of Sir2 in yeast effect transcriptional regulation, some appear to have separate primary functions.

No cellular function has been assigned to any of the seven known human sirtuins. SIRT1 is the closest homolog of yeast Sir2, both because of its primary structure and because it contains a nuclear localization sequence [10]. SIRT2 appears to be most closely related to Hst2, and indeed it is also localized to the cytoplasm [81]. In the mouse, mSIRT2 and mSIRT3 were both found to localize in the cytoplasm, but whereas mSIRT2 was uniformly distributed, mSIRT3 was concentrated to areas in the region of the Golgi and ER membranes, consistent with it containing a predicted transmembrane domain [82]. No characterization of SIRT4–7 has been reported, although SIRT5 is predicted to be mitochondrial based on its sequence, whereas SIRT6 and 7 contain nuclear localization sequences [83]. Thus, whereas SIRT1, 6, and 7 may be directly involved in transcriptional regulation, the four other human sirtuins presumably function in other processes.

Partial characterization of the mouse and *Drosophila* sirtuins suggest that these proteins function in transcriptional regulation. Mapping of the binding sites of the *Drosophila* Sir2 homolog by microarray analysis revealed that this protein is associated with promoters of active genes such as α-tubulin and not with the rDNA cluster [84]. Thus, this sirtuin may actually be involved in activating gene expression. Additionally, mouse Sir2a can deacetylate TAF_I68 and thereby repress RNA polymerase I transcription in vitro. Thus, this sirtuin may repress transcription through deacetylation of a nonhistone substrate [85].

Regulation of Transcriptional Silencing by Sir2

Regulation of transcriptional silencing by Sir2 is similar to that observed in the HDAC family of histone deacetylases in that Sir2 must be initially recruited to a specific promoter or to chromosomal domains via DNA binding proteins. However, Sir2 complexes can also spread to adjacent chromosomal domains, which has not yet been observed for the HDACs. Finally, Sir2 is generally regulated in a higher order manner similar to the class II HDACs in that it is inactivated by sequestration within the nucleus.

Telomeric and HM loci silencing appear to proceed by the same mechanism, in which the Sir2/3/4 complex is initially recruited by a DNA binding protein and then spreads to and silences the adjacent chromatin. At telomeres, Rap1 (repressor activator protein) binds to a 300 base pair region containing $C_{1-3}A$ repeats at the ends of telomeres, then recruits the Sir2/3/4 complex via its interactions with Sir3 and Sir4. The Sir2/3/4 complex then spreads an additional 2–4 kb along the adjacent chromatin. This spreading process occurs because of

interactions between Sir3/4 and the tails of histones H3 and H4, as well as interactions between the Sir3/4 proteins in adjacent complexes (reviewed in [85]). There is some evidence that the acetylation state of the chromatin may be involved in the spreading of the Sir2 complex. Chromatin immunoprecipitation experiments have shown that lysine 12 of histone H4 is specifically acetylated in these transcriptionally silent regions, whereas lysines 5, 8, and 16 are not acetylated. If all four lysines are mutated to arginine in vivo, mimicking hypoacetylation, silencing is abrogated [87]. Since hypoacetylation is normally associated with silent chromatin, this observation suggests that acetylation at lysine 12 may be required for the spreading and silencing activity of this deacetylase complex. Notably, Sir3 cannot bind to histone H4 if both lysine 12 and lysine 16 are mutated to glutamine, mimicking acetylation at these sites [88]. This suggests that lysine 12 should be acetylated and lysine 16 should remain unacetylated in order for the Sir2/3/4 complex to spread. Hence, according to this model, a specific pattern of charges on the histone tails may be required for spreading of the Sir 2/3/4 complex.

As is the case with the class II HDACs, Sir2 transcriptional silencing activity is more generally regulated by sequestration. There is a limiting pool of Sir2 in the cell, and this is shifted between the different loci so that it regulates their expression in a concerted manner. It has been hypothesized that Sir2 is stored at the telomere until it is required for silencing at other loci, at which point it moves from the telomeres to these other chromosomal domains. In support of this model, deletion of the C-terminal domain of Sir4 will cause a derepression of telomeric genes and an enhanced repression of rDNA loci genes [89]. Deletion of Rap1 will enhance repression of artificial reporter genes that have been inserted into internal regions of the chromosome that are not usually silenced [90]. This shifting of Sir2 from telomeric loci to the rDNA locus, as well Sir3 and Sir4 recruitment by Ku proteins from the telomeres to sites of DNA damage, has led to the suggestion that the main role of the telomere is to sequester limiting pools of Sir proteins and to thereby regulate gene expression [91].

Mechanism of Deacetylation by the Sirtuins

Recently, Sir2 has been shown to possess NAD-dependent histone deacetylase activity (reviewed in [92; 61, 93, 94]). It seems likely that this HDAC activity is responsible for Sir2's ability to silence gene transcription since the regions regulated by Sir2 are hypoacetylated [87] and since deletion of Sir2 results in a dramatic increase in the acetylation of the histones at these sites, whereas overexpression of Sir2 results in substantial global deacetylation [95]. Sir2 must cleave one molecule of NAD for each molecule of acetyl lysine that is deacetylated, and the kinetics for the hydrolysis of nicotinamide from NAD are very close to those observed for the deacetylation reaction, suggesting that these reactions are tightly linked [96]. Furthermore, Sir2 will not hydrolyze the glycosidic bond of NAD in the absence of acetyl lysine, suggesting either that both substrates must be bound to the active site or that the deacetylation is necessary for NAD hydrolysis. Furthermore, it was found that the acetyl group of lysine is transferred directly to the ACP-ribose molecule formed by the hydrolysis of the nicotinamide, resulting in the production of an O-acetyl-ADP-ribose molecule. The position at which the acetyl group is attached has not been determined yet, but it is anticipated that the hydrolysis of NAD proceeds via an oxocarbenium ion ADP-ribose intermediate, and thus the acetyl group may be transferred to the ribose ring (Figure 9; [96, 97]).

Since the HDACs require only a metal to activate a water molecule to catalyze the deacetylation reaction, it is interesting to speculate as to why the sirtuins require the cleavage of the nicotinamide ribose glycosidic bond of NAD. The energy released by this cleavage may be necessary to surmount an unusually large kinetic barrier in this particular deacetylation reaction, or it may be used to drive another process, such as chromatin remodeling. It is possible that this is simply due to the evolution of Sir2 deacetylase activity from an ancestral protein, in which NAD hydrolysis may have served a different purpose, or it may serve a relevant function in the cell. For example, the 1-O-acetyl ADP ribose molecule may be a signaling molecule for an unidentified receptor, or Sir2 may be sensing the levels of NAD in the cell and regulating transcription accordingly [92]. The unusual mechanism that has been elucidated reveals that NAD serves as a true stoichiometric cofactor rather than as a component of the catalytic machinery or as an allosteric regulator of the enzyme. Thus, levels of NAD in a cell are related in a 1:1 manner to the amount of Sir2-based deacetylation. This mechanism correlates well with the proposed link between Sir2 deacetylase activity and metabolic levels; if Sir2 used NAD only as an element of the catalytic machinery, one molecule of NAD could be used to deacetylate many molecules of the acetyl-lysine substrate. In this case, NAD would not be limiting for the reaction, and the cellular levels of NAD would be less effectively related to the amount of Sir2 activity. However, with NAD actually serving as a substrate, only one acetyl-lysine residue can be deacetylated for each NAD molecule, and hence the amount of Sir2 activity would be tightly linked to the cellular NAD concentrations and thus to the metabolic state of the cell. With this mechanism, Sir2 truly would function as a sensor for NAD levels and metabolic activity.

Crystal Structure of Sir2 Homolog

The crystal structure of a Sir2 homolog from *Archaeoglobus fulgidus*, Sir2-AF1, bound to NAD has recently been solved [98]. This protein cannot deacetylate histones in vitro, but it is competent to deacetylate BSA. Sir2-Af1 consists of a small and large domain, connected by a large loop. The protein was crystallized in two distinct forms, differing mainly in the structure of the loop, suggesting that is it fairly flexible in solution. The loop covers the pocket between the two domains, where the NAD molecule is bound. In one crystal form, the loop is close to the pocket, while in the other it shifts to render the pocket more accessible. Two main structural motifs are found in Sir2-AF1. In the small domain, two pairs of cysteines bind to a zinc ion. Unlike the zinc ion in the HDAC structure, this zinc is too far removed from the

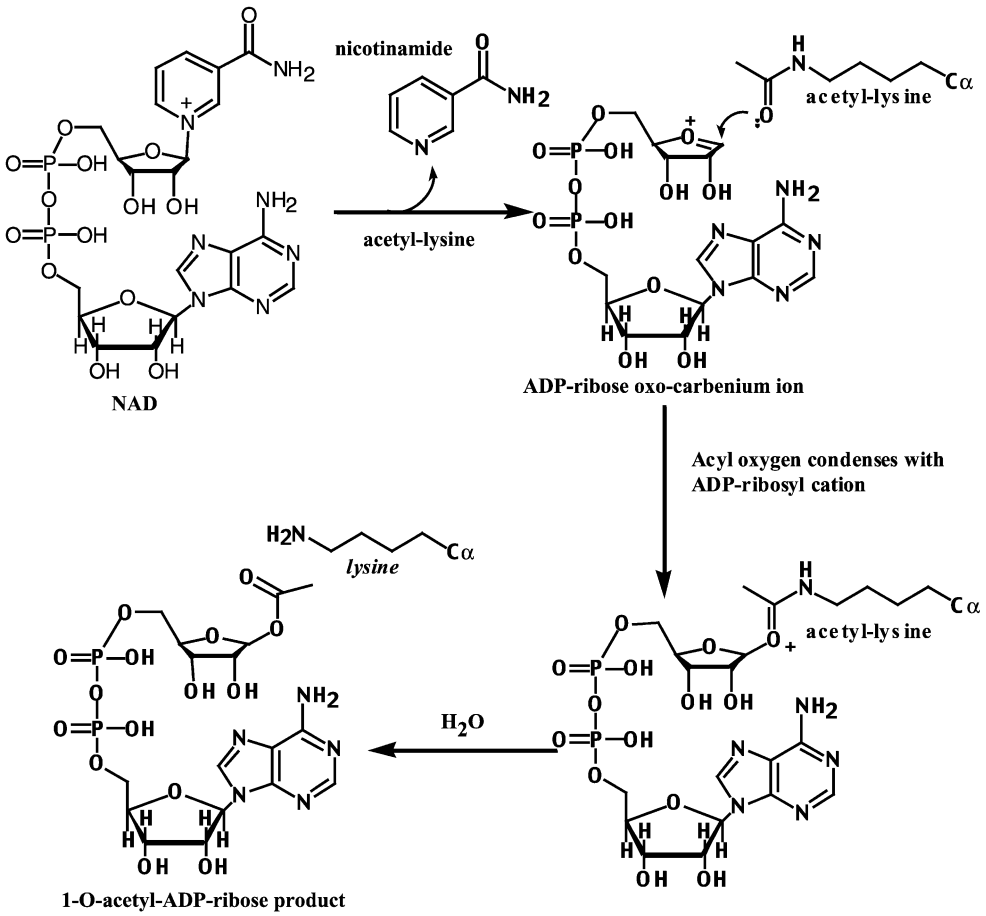

Figure 9. Proposed Mechanism for Sirtuin Deacetylation

During the deacetylation process, the nicotinamide group from NAD is hydrolyzed, presumably forming an oxo-carbenium intermediate. The acetyl group is then transferred to the ADP-ribose molecule. One possible mechanism for this is shown above [96].

presumed active site to participate directly in catalysis, but it seems to be necessary for the structural integrity of the protein since zinc chelators inhibit enzymatic activity. NAD is bound in the pocket between the two domains, at a Rossmann fold in the large domain. It is bound in an extended conformation, typical of that observed in other proteins with Rossman folds, but its orientation is inverted.

The mechanism by which Sir2-AF1 catalyzes deacetylation is not obvious from this structure. The NAD binding pocket contains three sites: site A, which binds the adenine-ribose moiety; site B, which binds the nicotinamide-ribose moiety; and site C, which is deep within the pocket and does not contact the NAD directly in the crystal structure. However, the residues in site C are very well conserved, and mutation of these sites disrupts enzymatic activity, suggesting that this region is involved in the catalytic process. The binding site for the acetyl-lysine is not apparent; it could not access the NAD binding pocket without disrupting the NAD. Thus, it is hypothesized that the NAD molecule changes configuration such that the nicotinamide moves into the C site and thus frees the B site to bind the acetyl-lysine. A serine residue in the C site could act as a nucleophile to cleave the nicotinamide from the NAD. The serine-NAD intermediate could then be cleaved by a water molecule that is bound to the C site. This would produce the postulated oxo-carbenium ion intermediate, which could swing back into the B site and participate in the deacetylation reaction [98].

Small-Molecule Modulators of Sirtuins

As in the case of the HDAC family of deacetylases, the identification of small-molecule inhibitors of the sirtuin family of deacetylases would provide a useful tool with which to dissect their biological function in a variety of systems. This is particularly critical for studying the function of the sirtuins in metazoans since these are completely uncharacterized. Although a nonhydrolyzable NAD analog has been used as an effective inhibitor of sirtuin deacetylase activity [93], such molecules undoubtedly will nonspecifically inhibit other NAD-dependent enzymes. We have successfully identified a class of cell-permeable small-molecule inhibitors of both human and yeast sirtuins [3]. These inhibitors contain a 2-hydroxyl-1-napthol moiety that is sufficient for inhibition, and thus it may be possible to produce specific inhibitors for the seven human sirtuins by constructing analogs based on this structure. These small-molecule sirtuin inhibitors could be used to study the roles of sirtuins

in transcriptional regulation, cell cycle control, DNA damage repair, and developmental biology.

Conclusions and Perspectives

Although acetylation has been studied mainly with respect to its role in transcriptional regulation, there is mounting evidence that it is a general mechanism for controlling a variety of cellular processes. The function of several members of the human HDAC family of deacetylases and all of the human sirtuin proteins has yet to be determined. Elucidation of the biological roles of these enzymes will undoubtedly lead to the refinement of our current models of transcriptional regulation, as well as to the discovery of several new areas of cell biology. Although standard biochemistry and cell biology will be important for studying these proteins, the use of small-molecule modulators will greatly increase the scope and speed of these investigations since they permit fine temporal control and are transferable between several different experimental systems. Specific small-molecule inhibitors of the deacetylase activity of HDACs and sirtuins will be powerful tools for probing the biology of these proteins. Small-molecule inhibitors of other enzymatic activities, such as chromatin remodeling or histone methylation, that are involved in transcription would also help in the elucidation of these basic cellular mechanisms. Furthermore, it should be possible to identify inhibitors of specific protein-protein or protein-DNA interactions involved in transcription as well, and these would help dissect the function of particular pathways. For example, specifically inhibiting the recruitment of either the CoREST or Sin3 complex to the REST transcription factor would allow for the characterization of their individual roles. Furthermore, inhibitors of the DNA binding activity of methylated DNA binding proteins would greatly aid the study of the role of these proteins in cellular differentiation and cancer. A great number of unanswered questions in the field of transcriptional regulation provide exciting and challenging opportunities for the application of chemical genetics.

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Note Added in Proof

While this manuscript was in preparation, several additional relevant articles were published. The synthesis of a library of 7200 structurally diverse potential HDAC inhibitors has been reported (Sternson, S.M., Wong, J.C., Grozinger, C.M., and Schreiber, S.L. [2001]. Org. Lett. *3*, 4239–4242). Human SIRT1 has been reported to deacetylate p53 and thus represents the first characterized human sirtuin (Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. [2001]. Cell *107*, 137–148; Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. [2001]. Cell *107*, 149–159). Finally, the crystal structure of the catalytic domain of human SIRT2 has been reported as well (Finnin, M.S., Donigian, J.R., and Pavletich, N.P. [2001]. Nat. Struct. Biol. *8*, 621–625).