

# Mechanisms and Molecular Probes of Sirtuins

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Sirtuins are critical regulators of many cellular processes, including insulin secretion, the cell cycle, and apoptosis. Sirtuins are associated with a variety of age-associated diseases such as type II diabetes, obesity, and Alzheimer's disease. A thorough understanding of sirtuin chemical mechanisms will aid toward developing novel therapeutics that regulate metabolic disorders and combat associated diseases. In this review, we discuss the unique deacetylase mechanism of sirtuins and how this information might be employed to develop inhibitors and other molecular probes for therapeutic and basic research applications. We also cover physiological regulation of sirtuin activity and how these modes of regulation may be exploited to manipulate sirtuin activity in live cells. Development of molecular probes and drugs that specifically target sirtuins will further understanding of sirtuin biology and potentially afford new treatments of several human diseases.

## Introduction

Metabolic dysregulation accounts for a growing number of diseases including type II diabetes, metabolic syndrome, and obesity. Understanding the regulatory mechanisms involved in metabolism will allow development of drugs for treatment of these diseases. Recent identification of sirtuins as novel regulators of many metabolic processes makes sirtuins intriguing drug targets. In addition to their involvement in age-related diseases (Milne and Denu, 2008), sirtuins regulate many metabolic pathways such as insulin secretion and lipid mobilization. Sirtuins are also implicated in control of metabolic protein regulators such as AMP-activated protein kinase (AMPK) and LKB1 (Elliott and Jirousek, 2008; Fulco et al., 2008; Lan et al., 2008).

The human sirtuin family is made up of seven members, Sirt1–7, with each having distinct cellular targets and diverse cellular localizations (Table 1). Most sirtuins possess NAD<sup>+</sup>-dependent protein deacetylase activity. Sirt1 has been the most studied, as it shares the highest homology with the founding member of the Sir2 family from yeast. As many as 30 acetylated proteins have been implicated as Sirt1 substrates, and the list will likely continue to grow. Sirt1 is reported to regulate  $\beta$  cell insulin secretion, mitochondrial biogenesis, and metabolic genes through PGC1 $\alpha$ , and to deacetylate Ku70 (reviewed in Westphal et al., 2007). Sirt1 is localized to either the nucleus or cytoplasm depending on tissue and cell type (Tanno et al., 2007). Sirt1 exhibits variable expression that depends on tissue type, overnight fasting, and caloric restriction (Chen et al., 2008; Cohen et al., 2004). Sirt2 is localized mainly to the cytoplasm, where it is associated with deacetylation of tubulin filaments, HOXA10, and FOXO (Bae et al., 2004; Jing et al., 2007; North et al., 2003; Turdi et al., 2007; Wang et al., 2007). Specific Sirt2 inhibitors discovered in vitro were also shown to ameliorate a model system of Parkinson's disease, suggesting a potential link between specific sirtuin inhibition and Parkinson's treatment (Outeiro et al., 2007). Sirt3 is genetically linked to lifespan in the elderly (Rose et al., 2003). However, the function and localization of this enzyme is a matter of some debate (Hallows et al., 2008). A number of reports indicate that Sirt3, upon proteolytic cleavage in the mitochondria, is an active protein deacetylase against a number of mitochondrial

matrix proteins (Schwer et al., 2002). In stark contrast, one report suggested that full-length Sirt3 exhibits nuclear localization and histone deacetylase activity (Scher et al., 2007). Recently, a report comparing Sirt3<sup>-/-</sup> and Sirt3<sup>+/+</sup> mice provided compelling evidence that endogenous Sirt3 is mitochondrial and is responsible for the majority of protein deacetylation in this organelle (Lombard et al., 2007). Sirt4 is localized to the mitochondria but has exhibited no deacetylase activity to date. However, Sirt4 is reported to ADP-ribosylate and inhibit glutamate dehydrogenase (Haigis et al., 2006). Similarly, Sirt5 is localized to the mitochondria, but cellular targets remain unknown (Michishita et al., 2005). Sirt5 was initially shown to possess weak deacetylase activity and recently was suggested to effect cytochrome c acetylation in vitro (Schlicker et al., 2008), although the mouse knockout of Sirt5 does not affect the bulk acetylation state of mitochondrial proteins (Lombard et al., 2007). Sirt6 is localized to the nucleus, and the loss of Sirt6 leads to a shortened lifespan and premature aging (Mostoslavsky et al., 2006). Sirt6 was initially reported to act as an ADP-ribosyltransferase (Liszt et al., 2005) but histone deacetylase activity was recently demonstrated, implicating Sirt6 in DNA damage repair, chromosome stability, and modulation of telomeric DNA (Michishita et al., 2008). Lastly, Sirt7 is associated with nucleoli, and is implicated in activation of transcription by RNA polymerase I (Ford et al., 2006).

The varied localization and activity of the human sirtuins place them at the center of the many cellular pathways and functions regulated by reversible acetylation. Yet because sirtuins control a number of different pathways and exhibit diverse cellular localization, there is a significant need for homolog-specific and tissue-specific inhibitors, activators, and molecular probes. Specifically, Sirt1 is central to a number of metabolic processes such as insulin secretion and AMPK regulation, and understanding how Sirt1 regulates these cellular processes is critical. Specific inhibitors, activators, and other molecular probes will be valuable tools to understand sirtuin activity and regulation, and to develop pharmaceuticals for treatment and prevention of age-related diseases. Here we review current knowledge of sirtuin chemical and regulatory mechanisms and how this knowledge might be

**Table 1. Summary of the Biology of the Human Sirtuins, Sirt1–7**

Sirtuin	Disease Implication	Localization	Substrates	Interacting Partners	Physiological Summary	References
Sirt1	Metabolic, neurological, cardiovascular, renal, cancer	Nuclear, cytoplasmic	p53, Foxo1, Foxo4, COUP-TF, CTIP2, NFκB, p65, NCoR, Histone H1, Histone H4, Ku70, p300, BCL11A, Tat, PGC1α, MEF2, eNOS, AceCS1, E2F1, Androgen receptor, p73, Smad7, NBS1, Rb, TLE1, IRS2, LXR, SUV39H1, WRN, TORC2	AROS, DBC1	Overexpression is cardioprotective against oxidative stress and heart aging. Increases mitochondrial biogenesis by deacetylation and activation of PGC1α. Overexpression shows both a protective and pro-aging role in neurons. Murine knockout have genomic instability and severe developmental defects.	Hsu et al., 2008; Lagouge et al., 2006; Li et al., 2008; McBurney et al., 2003
Sirt2	Neurological, metabolic, cancer	Cytoplasmic	Tubulin, Foxo, Histone H4, 14-3-3	HOXA10, HDAC6	In cellular and <i>Drosophila</i> model of Parkinson's disease, inhibition of Sirt2 has protective effects.	Outeiro et al., 2007
Sirt3	Metabolic	Mitochondrial	AceCS2	Unknown	Murine knockout displays hyperacetylated mitochondrial proteome.	Lombard et al., 2007
Sirt4	Metabolic	Mitochondrial	GDH, IDE, ANT2, ANT3	Unknown	Murine knockout has increased GDH activity.	Haigis et al., 2006
Sirt5	Neurological	Mitochondrial	Unknown	Unknown	Murine serotonin receptor knockout have increased SIRT5 expression.	Sibille et al., 2007
Sirt6	Cancer	Nuclear	Histone H3	Unknown	Murine knockout have genomic instability displaying premature aging and predisposition to cancer.	Mostoslavsky et al., 2006
Sirt7	Cardiovascular	Nuclear	RNA Pol I, p53	Unknown	Murine knockout have decreased lifespan with inflammatory cardiac hypertrophy.	Vakhrusheva et al., 2008

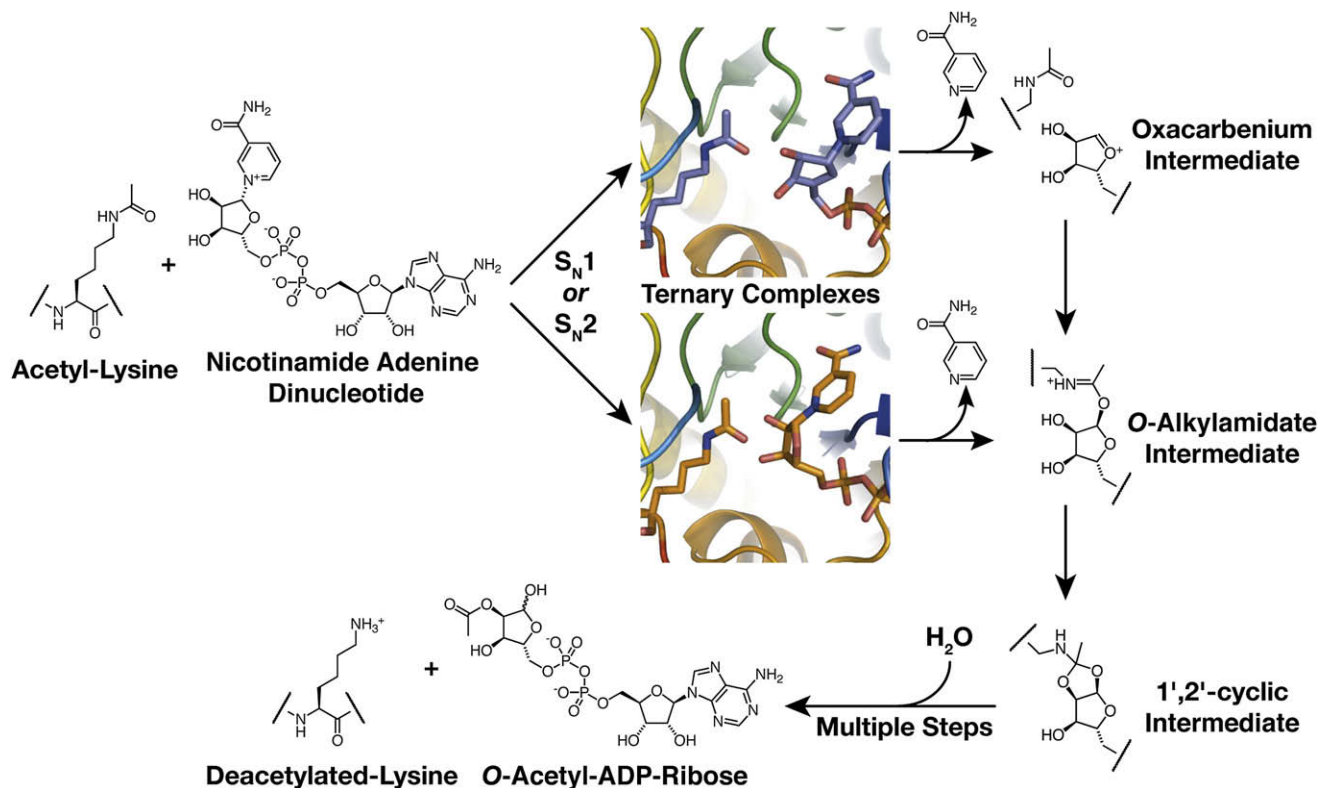
This table was adapted from Lavu et al., 2008.

exploited to develop novel molecular probes to further understanding of sirtuin biology.

### Sirt2 Chemical Mechanisms Deacetylation Mechanism

Detailed comprehension of sirtuin-catalyzed chemical mechanisms is vital to design drugs and other molecular probes that target sirtuin enzymatic activity. Sirtuins exhibiting protein deacetylation activity catalyze NAD<sup>+</sup>-dependent deacetylation of acetyl-lysine residues to form nicotinamide, deacetylated lysine, and 2'-O-acetyl-ADP-ribose (OAADPr) (Jackson and Denu, 2002; Sauve et al., 2001) (Figure 1). Sirtuins accomplish this reaction through a bi-ter kinetic mechanism in which acetyl-lysine binds prior to NAD<sup>+</sup>. Nicotinamide is the first product released, followed by random release of deacetylated lysine and OAADPr (Borra et al., 2004). Crystallographic evidence suggests that bound acetyl-lysine forces the nicotinamide ring of NAD<sup>+</sup> deep within a conserved hydrophobic pocket (Avalos et al., 2004;

Zhao et al., 2004). In the first chemical step after both substrates are bound, the acetyl oxygen undergoes nucleophilic attack of the 1'-carbon of the nicotinamide ribose to form the α-1'-O-alkylamidate intermediate (Figure 1). Evidence supporting this intermediate comes from several fronts. First, <sup>18</sup>O-labeling studies indicate that the acetyl oxygen is directly transferred to the 1'-hydroxyl of OAADPr (Sauve et al., 2001; Smith and Denu, 2006). Second, in a process known as nicotinamide exchange, sirtuins catalyze incorporation of exogenously added [<sup>14</sup>C]nicotinamide to form [<sup>14</sup>C]NAD<sup>+</sup> in the presence of an acetyl-lysine substrate and unlabeled NAD<sup>+</sup> (Jackson et al., 2003; Landry et al., 2000; Sauve and Schramm, 2003). Importantly, the acetyl-lysine substrate is indispensable for nicotinamide-ribose bond cleavage in both nicotinamide exchange and deacetylation reactions. Substitution of 2'-deoxy-2'-fluoro-NAD<sup>+</sup> for NAD<sup>+</sup> arrested deacetylation but nicotinamide exchange was minimally affected, indicating that acetyl-lysine attack proceeds at the 1'-carbon of the nicotinamide ribose (Jackson et al., 2003). Third,



**Figure 1. The Proposed Mechanism of Sir2 Protein Deacetylases**

replacement of acetyl-lysine with thioacetyl-lysine allowed mass spectral as well as crystallographic detection of the corresponding  $\alpha$ -1'-S-alkylamidate intermediate (Hawse et al., 2008; Smith and Denu, 2007b).

For acetyl-lysine attack to form the O-alkylamidate intermediate, both S<sub>N</sub>1 and S<sub>N</sub>2 mechanisms have been proposed (Figure 1). A crystal structure of a yeast Sir2 homolog, Hst2, revealed a ternary complex with an acetyl-lysine peptide and a nonhydrolyzable NAD<sup>+</sup> analog (carba-NAD<sup>+</sup>) in a conformation that precluded direct attack of the acetyl oxygen at the 1'-carbon of the nicotinamide ribose (Figure 1) (Zhao et al., 2004). This observed conformation appeared to support an S<sub>N</sub>1 mechanism in which a distinct oxocarbenium intermediate is formed prior to acetyl oxygen attack. An alternative structure of a bacterial Sir2 homolog, Sir2Tm, revealed the first ternary complex structure with native substrates acetyl-lysine and NAD<sup>+</sup> bound in the active site. A strikingly different NAD<sup>+</sup> conformation was observed in which the acetyl oxygen was placed 3.2 Å from the 1'-carbon on the  $\alpha$  face of the nicotinamide ribose (Figure 1) (Hoff et al., 2006). This NAD<sup>+</sup> conformation satisfied the positioning requirements for an S<sub>N</sub>2 mechanism. However, a more recent structure of an acetyl-lysine peptide and the NAD<sup>+</sup> analog, DADMe-NAD<sup>+</sup>, bound to SirTm led the authors to propose a nucleophilic displacement by electrophile migration mechanism (a type of S<sub>N</sub>1 mechanism) in which nicotinamide and acetyl-lysine remain stationary and an oxocarbenium intermediate migrates from nicotinamide to the acetyl oxygen (Hawse et al., 2008).

Recently, kinetic linear free-energy evidence consistent with an S<sub>N</sub>2 mechanism was obtained utilizing a series of acetyl-lysine analog peptides that varied greatly in the nucleophilicity of the acetyl oxygen (Smith and Denu, 2007c). Acetyl-lysine peptide displayed the fastest rate of nicotinamide formation (i.e., NAD<sup>+</sup> cleavage) (6.7 s<sup>-1</sup>), whereas the slowest analog trifluoroacetyl-lysine peptide displayed a five order of magnitude slower rate (1.1 × 10<sup>-5</sup> s<sup>-1</sup>). Utilizing Hst2 as a representative sirtuin, the plot of log nicotinamide formation rate versus the Taft constant,  $\sigma^*$  (Hansch and Leo, 1979), revealed a linear relationship with a steep negative slope ( $\rho^* = -1.9$ ). Importantly, most of the acetyl-lysine analog peptides exhibited binding constants that were equal to or lower than that of acetyl-lysine peptide, indicating the diminished rate of nicotinamide-ribose bond cleavage was due to chemistry and not initial substrate binding. These data were most consistent with an S<sub>N</sub>2 mechanism in which the greater electron-withdrawing analogs exhibited decreased oxygen nucleophilicity and therefore slower nicotinamide formation rates, but other mechanisms could not be ruled out. More recently, ab initio QM/MM calculations by Hu et al. support an S<sub>N</sub>2 mechanism with a highly dissociative transition state (P. Hu, S. Wang, and Y. Zhang, personal communication). In the calculated transition state, the bond orders between the acetyl oxygen and the 1'-carbon, and the 1'-carbon and nicotinamide were 0.10 and 0.14, indicating significant nucleophilic participation of acetyl-lysine in nicotinamide-ribose bond cleavage. In addition, although formation of a distinct oxocarbenium intermediate was not observed, the calculated transition state

did possess significant oxocarbenium character in line with other  $\text{NAD}^+$  cleavage mechanisms (Berti et al., 1997; Parikh and Schramm, 2004; Rising and Schramm, 1997; Scheuring et al., 1998; Scheuring and Schramm, 1997a, 1997b; Yang et al., 2007).

Whereas much of the evidence points to an  $\text{S}_{\text{N}}2$  mechanism with a highly dissociative oxocarbenium transition state, determination of kinetic isotope effects for the Sir2 reaction would provide more definitive evidence whether or not acetyl-lysine attack occurs in the same transition state as nicotinamide formation. The  $^{15}\text{N}$  isotope effect in N1 of nicotinamide as a function of acetyl analog nucleophilicity would be particularly informative. Other important isotope effects would be the  $^{13}\text{C}$  effect at the 1'-carbon of the nicotinamide ribose and the  $^{18}\text{O}$  effect at the acetyl oxygen. With primary isotope effects for all of the atoms involved directly in the reaction, it should be possible to definitively establish the nature of the transition state for acetyl-lysine attack.

After *O*-alkylamidate formation, the 2'-hydroxyl of the nicotinamide ribose is activated by an active-site histidine (His-135 in Hst2) for attack of the *O*-alkylamidate carbon to form the 1',2'-cyclic intermediate (Sauve et al., 2006; Smith and Denu, 2006) (Figure 1). To complete the reaction, several steps involving elimination of deacetylated lysine followed by water addition result in formation of 2'-OAADPr. Following release from the sirtuin active site, OAADPr undergoes nonenzymatic interconversion between 2'-OAADPr and 3'-OAADPr (Jackson and Denu, 2002; Sauve et al., 2001). Although direct evidence for intermediates after the *O*-alkylamidate remains elusive, several lines of evidence are consistent with a two-step chemical mechanism in which nicotinamide formation is followed by acetyl transfer. First, mutation of the histidine general base to alanine hindered deacetylation, whereas nicotinamide exchange was less affected (Jackson et al., 2003), indicating nicotinamide-ribosyl bond cleavage occurs in a distinct step prior to acetyl transfer. Second, rapid-quenching kinetic analysis utilizing Hst2 revealed a rate of nicotinamide formation of  $8 \text{ s}^{-1}$ , whereas acetyl transfer to the 2'-hydroxyl occurred at a rate of  $2 \text{ s}^{-1}$  (Borra et al., 2004). Product release limits overall turnover at a rate ( $k_{\text{cat}}$ ) of  $0.2 \text{ s}^{-1}$  for Hst2 (Smith and Denu, 2006).

#### Comparison to Class I/III/IV Histone Deacetylases

The mechanism of Sir2 deacetylases (class III HDACs) stands in stark contrast to that of class I/III/IV HDACs that utilize an active-site zinc to direct hydrolysis of acetyl-lysine residues. The distinction between the two mechanisms is best exemplified by comparison of the different roles played by the acetyl group. In sirtuins, the acetyl oxygen is the nucleophile, whereas in class I/III/IV HDACs the acetyl carbon is the electrophile. These mechanistic differences were recently highlighted by comparison of the rates of deacylation of six acetyl-lysine analog peptides (Smith and Denu, 2007a). It was hypothesized that greater electron-withdrawing acetyl analogs (e.g., trifluoroacetyl) would increase the electrophilicity of the acetyl carbon and therefore the deacylation rate by class I/III/IV deacetylases. Conversely, these same electron-withdrawing analogs would decrease the nucleophilicity of the acetyl oxygen and the deacylation rate by sirtuins. Indeed, only a 7-fold difference in rate was observed for an acetyl-lysine peptide between the sirtuin, Hst2, and the class I HDAC, HDAC8, but a 300,000-fold faster rate for HDAC8 compared to Hst2 for the corresponding trifluoroacetyl-lysine peptide. These differences were further manifested in

the divergent slopes of  $-1.57$  for Hst2 and  $+0.79$  for HDAC8 in the Taft plots of the substituent electron-withdrawing nature ( $\sigma^*$ ) (Hansch and Leo, 1979) versus the log deacylation rate.

In the future, differences in chemical mechanism between deacetylase classes might be exploited to develop selective deacetylase substrates. For example, substitution of an acetyl-lysine analog within existing fluorescent deacetylase substrates may provide fluorescent activity readout for a particular subset of HDACs. This approach has begun to be explored for class I and II HDACs by the Jung and Schwienerhorst labs utilizing propionyl-, butyryl-, trifluoroacetyl-, and other acetyl-lysine analogs (Heltweg et al., 2004; Hildmann et al., 2006; Riester et al., 2004). Combination of acetyl analog selectivity along with peptide sequence selectivity could afford highly selective HDAC substrates. These selective substrates could be useful to detect individual HDAC activity within live cells. Toward this end, a homogeneous assay suitable for high-throughput screening of class I/III/IV HDAC activity in mammalian cells was reported in which an acetyl-lysine fluorescent substrate was utilized (Ciossek et al., 2008). In addition, direct measurement of class I/III/IV HDAC activity in live cells via  $^{19}\text{F}$ -NMR has been reported with trifluoroacetyl-lysine (Sankaranarayananpillai et al., 2006).

The majority of existing HDAC fluorescent substrates utilize 7-amino-4-methylcoumarin (AMC), whose fluorescence is partially quenched through covalent attachment to the carboxyl of the acetyl-lysine residue and the amino group of AMC. Deacetylation of this substrate liberates a trypsin protease site that cleaves preferentially after deacetylated lysine to release fully fluorescent AMC. Other fluorophores could be utilized in this assay. For example, we have utilized a rhodamine 110-based fluorescent substrate to measure sirtuin activity (Borra et al., 2005). Oxazine dyes could also be used, as they have previously been employed in similar protease substrates (Boonacker et al., 2003). Selective substrates attached to diverse fluorescent dyes could allow measurement of multiple HDAC activities simultaneously.

#### Acetyl-Lysine Binding

Utilizing a series of ten acetyl-lysine analog peptides, the requirements for efficient acetyl-lysine binding were recently examined (Smith and Denu, 2007a). The various acetyl analogs varied in their nucleophile, electronic-withdrawing potential, size, charge, and hydrophobicity. Among the acetyl-lysine analog peptides, the binding constants ( $K_{\text{d}}$ ) to Hst2 varied 75-fold, from  $3.3$  to  $260 \mu\text{M}$ . The  $K_{\text{d}}$  values yielded the strongest correlation with the relative hydrophobicity of each acetyl analog, where the most hydrophobic analogs (i.e., thioacetyl and trifluoroacetyl) displayed the tightest binding. Modeling of the thioacetyl and trifluoroacetyl analogs into the Hst2 active site provided rationalization for the observed hydrophobicity correlation. In particular, the increased size and hydrophobicity of the trifluoromethyl group of trifluoroacetyl-lysine filled a hydrophobic pocket defined by Phe-67, Ile-117, and Ile-181, and the greater size and hydrophobicity of the sulfur in thioacetyl-lysine filled a hydrophobic pocket defined by Phe-184 and Val-228. Notably, these residues are conserved, suggesting that the trends with Hst2 will be applicable to other sirtuins.

The observed correlation of acetyl analog hydrophobicity and binding has application in the development of fluorescence polarization and other probes that bind within the acetyl-lysine binding pocket. Tight-binding thioacetyl-, trifluoroacetyl-, and



propionyl-lysine peptides are particularly well suited for this application. These probes would be useful in screening for compounds that specifically bind to the acetyl-lysine binding pocket of sirtuins. Such a screen would be particularly interesting for homologs that do not possess known deacetylase activity (e.g., Sirt4 and Sirt7) but may bind acetyl-lysine peptides. Recently, a similar fluorescent polarization assay was developed through attachment of a fluorescein label to SAHA, a general class I/II/IV HDAC inhibitor FDA approved for the treatment of cutaneous T cell lymphoma (Mazitschek et al., 2008).

#### Depropionylation and Debutyrylation

In addition to acetylation, there is growing evidence that the  $\epsilon$ -amine of lysine residues can be propionylated or butyrylated. Direct support for these modifications comes from two recent proteomics studies by Zhao and coworkers that revealed that histone lysine residues as well as the non-histone proteins p53, p300, and CREB-binding protein (CBP) are propionylated and butyrylated (Chen et al., 2007; Cheng et al., 2008). In addition, the authors found that p300 and CBP, which are known acetyltransferases, were capable of catalyzing propionylation and butyrylation. Garrity et al. (2007) have observed propionylation and regulation of propionyl-CoA synthetase by several acetyltransferases. Berndsen et al. demonstrated that the acetyltransferase Esa1 can catalyze efficient propionylation (Berndsen et al., 2007). Other evidence comes from the observation that rat liver extracts, as well as HDAC1, HDAC3, and HDAC6, can efficiently hydrolyze propionyl-lysine but not butyryl-lysine fluorescent substrates (Heltweg et al., 2004; Riestler et al., 2004).

We recently showed that Hst2, Sirt1, Sirt2, and Sirt3 catalyze efficient depropionylation and debutyrylation (Smith and Denu, 2007a). Among these sirtuins, deacetylase activity was the greatest, followed by depropionylase activity (29%–77% compared to deacetylation) and then debutyrylase activity (2%–26% compared to deacetylation). Hst2 exhibited the greatest depropionylase activity but the slowest debutyrylase activity, suggesting a more sterically restricted active site compared to Sirt1, Sirt2, and Sirt3. Comparison of the  $\text{NAD}^+$   $K_m$  values with Sirt2 using acetyl-, propionyl-, and butyryl-lysine peptides revealed a higher  $K_m$  value with propionyl- compared to acetyl-lysine peptide. This higher  $K_m$  value suggests that with propionyl-lysine peptide,  $\text{NAD}^+$  binding is adversely affected whereas nicotinamide-ribosyl bond cleavage is not significantly affected. Conversely, butyryl-lysine peptide displayed a lower  $K_m$  value, suggesting that nicotinamide-ribosyl bond cleavage is significantly perturbed compared to acetyl- and propionyl-lysine substrates (Smith and Denu, 2007a).

The extent and functions of propionylation and butyrylation throughout the cell requires further investigation. Given the different acyl-group substrate selectivity among sirtuins, propionylation and butyrylation could provide another level of regulation at sites of known acetylation, either in antagonistic or agonistic fashion. Additionally, it will be important to evaluate whether sirtuins that display little to no deacetylase activity (e.g., Sirt4–Sirt7) may more effectively catalyze depropionylation/debutyrylation. If these modifications prove to be a robust mode of regulation, then it will be essential to explore the links between short-chain fatty acyl-CoA metabolism and the enzymes that catalyze reversible lysine acylation.

#### Sir2 ADP-Ribosylation Mechanism

Although protein deacetylation is thought to be the primary activity displayed by sirtuins, several reports have suggested that some sirtuins (e.g., Sirt4 [Ahuja et al., 2007; Haigis et al., 2006] and Sirt6 [Liszt et al., 2005]) possess ADP-ribosyltransferase activity. Sirt6 was recently shown to also catalyze selective deacetylation of lysine 9 of histone H3 (Michishita et al., 2008), so it is reasonable to predict that yet unidentified deacetylase substrates may exist for Sirt4. The ability of sirtuins to catalyze multiple rounds of  $\text{NAD}^+$ -dependent protein ADP-ribosylation has not been demonstrated. Furthermore, the nature of the linkage and site of modification remains unclear. Among deacetylases reported to harbor both activities, deacetylase activity is three to five orders of magnitude greater than ADP-ribosylation (Kowieski et al., 2008; Tanner et al., 2000). Kowieski et al. recently determined the mechanism of ADP-ribosylation by a Sir2 homolog from *Trypanosoma brucei* first characterized by García-Salcedo and coworkers to possess both deacetylase and ADP-ribosyltransferase activity (García-Salcedo et al., 2003). Kowieski et al. suggested two distinct ADP-ribosylation mechanisms, both of which require an acetyl-lysine substrate (Kowieski et al., 2008). In one mechanism, acetyl-lysine reacts with  $\text{NAD}^+$  to form the *O*-alkylamidate intermediate. The ability of the *O*-alkylamidate intermediate to react with other exogenous alcohols has been demonstrated (Smith and Denu, 2006), indicating that amino acid side chains might intercept and react with the *O*-alkylamidate resulting in ADP-ribosylation instead of deacetylation. In a second ADP-ribosylation mechanism, the acetyl-lysine substrate and  $\text{NAD}^+$  proceed through deacetylation to form OAADPr, which then reacts nonenzymatically with a protein substrate resulting in ADP-ribosylation (Kowieski et al., 2008).

*Plasmodium falciparum* Sir2 (PfSir2), which has reported deacetylase and ADP-ribosyltransferase activity (Chakrabarty et al., 2008; Merrick and Duraisingh, 2007), was recently shown to hydrolyze  $\text{NAD}^+$  by two distinct mechanisms, one of which did not require an acetyl-lysine substrate and was insensitive to nicotinamide inhibition (French et al., 2008). Although it is unclear whether the acetyl-lysine-independent  $\text{NAD}^+$ -cleavage pathway is directly linked to protein ADP-ribosylation, this mechanism might reflect how some sirtuins catalyze direct ADP-ribosyltransfer to protein nucleophiles in the absence of an acetyl-lysine substrate and in a nicotinamide-insensitive manner. Interestingly, Sirt4 is also suggested to display ADP-ribosyltransferase activity in the absence of acetyl-lysine substrates (Ahuja et al., 2007; Haigis et al., 2006). In this case, it is possible that a protein side chain such as Asn or Gln could replace acetyl-lysine to form a similar *O*-alkylamidate intermediate or that a direct-transfer ADP-ribosylation mechanism is operative.

Acetyl-lysine analog peptides might serve as probes to distinguish between the above ADP-ribosylation mechanisms. In particular, a homocitrulline peptide could be valuable due to its ability to stall at the corresponding *O*-alkylamidate (or *O*-alkylisourea) intermediate, not proceed to deacylation, and accept alternative nucleophiles (Khan and Lewis, 2006; Smith and Denu, 2007a). For instance, substituting homocitrulline for acetyl-lysine might increase ADP-ribosylation in a mechanism that reacts with the *O*-alkylamidate (or *O*-alkylisourea) due to the increased

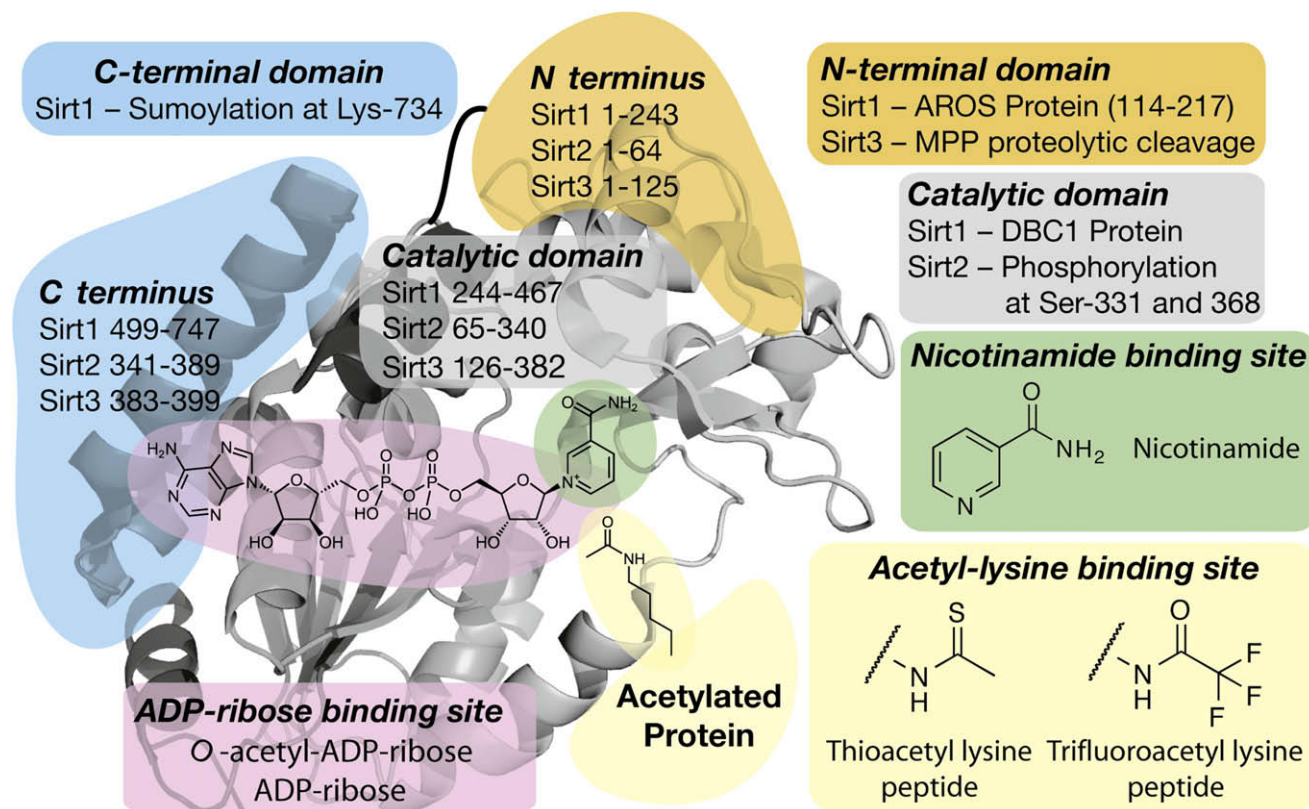


Figure 2. Known Posttranslational Modifications, Interacting Proteins, and Binding Sites of the Human Sir2 Homologs Sirt1, Sirt2, and Sirt3

lifetime of this intermediate. On the other hand, homocitrulline might decrease ADP-ribosylation in a mechanism that requires OAADPr.

#### In Vivo Modulation of Sirtuin Activity

**Endogenous Protein Modulators of Sirtuin Activity.** The variety of important functions involving Sir2 deacetylases underscores the need to understand the mechanisms that regulate their physiological activity. To date, both a specific protein inhibitor and activator of Sirt1 have been identified. Sirt1 was shown to interact with and be activated by active regulator of Sirt1 (AROS) (Figure 2) (Kim et al., 2007). AROS is a 142 amino acid nuclear localized protein that possessed no known function prior to its identification as a Sirt1 binding partner. AROS interacted with Sirt1 both in vitro and in cell extract, and the expression profiles of AROS and Sirt1 were similar. Interestingly, activation of Sirt1 by AROS appeared greater in cellular studies compared to in vitro experiments. This finding could indicate that AROS works with another protein factor in vivo or acts as a derepressor by displacing other inhibitory factors such as DBC1 (see below). Further work is needed to determine the mechanism of AROS-mediated Sirt1 activation. In particular, does AROS induce a conformational change in Sirt1 or does AROS binding affect other interacting proteins that regulate Sirt1 activity? Identification of tissues and protein targets that are affected by the AROS/Sirt1 interaction will be necessary to understand its physiological relevance. Discovery of compounds that disrupt AROS binding to Sirt1 would be valuable. Utilization of these compounds will allow researchers to determine the effect of AROS

activation on Sirt1-dependent processes, as well as what potential therapeutic benefits the AROS/Sirt1 interaction may hold.

Recent studies revealed that human deleted in breast cancer 1 (DBC1) is a specific Sirt1 inhibitor (Kim et al., 2008; Zhao et al., 2008) (Figure 2). DBC1 was previously identified from a region that is homozygously deleted in breast cancer (Hamaguchi et al., 2002). DBC1 was shown to bind and inhibit Sirt1-catalyzed deacetylation of p53 in vivo. Through siRNA knockdown of DBC1, Sirt1 was activated and the inhibition of apoptosis by p53 was decreased. In vitro assays revealed that inhibition occurred through binding of a leucine-zipper-like motif within DBC1 to the catalytic domain of Sirt1. Curiously, DBC1 also contains a catalytically inactive version of a Nudix hydrolase (MutT) domain (Anantharaman and Aravind, 2008). Homologs of the Nudix domain were shown to bind NAD<sup>+</sup>-like molecules and the product of sirtuin deacetylation, OAADPr (Grubisha et al., 2006). Anantharaman and Aravind proposed that OAADPr binding to DBC1 may facilitate Sirt1 inhibition and/or binding of DBC1 to Sirt1 (Anantharaman and Aravind, 2008). Understanding how NAD<sup>+</sup> and OAADPr levels regulate DBC1 binding to Sirt1 injects a fascinating possibility for allosteric regulation of Sirt1 activity. Another interesting aspect of regulation of Sirt1 may be the interplay between AROS and DBC1. In particular, are the binding of AROS and DBC1 to Sirt1 mutually exclusive, and/or dependent upon metabolites? In addition, the potential relationship between DBC1, Sirt1, and resveratrol is intriguing. The observed in vivo Sirt1 activation by resveratrol has proved difficult to reproduce in vitro (Borra et al., 2005; Kaeberlein et al., 2005). This is

potentially because resveratrol activates Sirt1 in vivo by displacing an inhibitory factor such as DBC1. Screening for novel Sirt1 activators through disruption of DBC1 binding to Sirt1 could afford compounds that treat age-related diseases.

#### **Posttranslational Modification of Sirtuins**

**Phosphorylation of Sirt2.** Sirt2 is phosphorylated at both Ser-368 and Ser-331 (Figure 2) (North and Verdin, 2007; Pandithage et al., 2008). Dryden et al. showed that Sirt2 protein abundance and phosphorylation decreased when the phosphatase CDC14B was overexpressed in cell culture, and that phosphorylation might inhibit Sirt2 degradation through the 26S proteasome (Dryden et al., 2003). However, the site of phosphorylation was not identified. In addition, Sirt2 phosphorylation at Ser-368 is involved in mitotic regulation from G2 to M phase. The kinase CDK1 and the phosphatases CDC14A and CDC14B reversibly regulated Sirt2 and therefore control of the cell cycle from G2 to M phase (North and Verdin, 2007). Interestingly, phosphorylation at Ser-331 inhibited the Sirt2 deacetylase activity, but the mechanism of inhibition is unknown (Pandithage et al., 2008). Ser-331 phosphorylation and inactivation was shown to interfere with cell adhesion and cell migration.

Phosphorylation is a newly discovered and interesting layer of sirtuin regulation, but determination of the role and extent of sirtuin phosphorylation requires further investigation. Using similar unbiased techniques as that used by Pandithage et al. (2008), one could elucidate other sirtuin phosphorylation sites. Furthermore, the use of specific kinase and phosphatase inhibitors in cellular and tissue studies will help elucidate the function of each sirtuin phosphorylation site.

**Sumoylation of Sirt1.** Sirt1 is modified at Lys-734 by the addition of a sumo group, which activates Sirt1 deacetylase activity (Yang et al., 2007) (Figure 2). An increase in Sirt1 sumoylation by reduction of the sumoylase SENP1 increased Sirt1 deacetylation activity. It was hypothesized that stress-inducing agents such as UV light and peroxide treatment counteract the anti-apoptotic activity of Sirt1 by recruiting SENP1 to Sirt1. SENP1 recruitment resulted in desumoylation and repression of Sirt1 deacetylase activity and consequent acetylation and activation of apoptotic proteins. Further studies are needed to determine how sumoylation affects other Sirt1 posttranslational modifications and interacting partners. Do sumoylation and AROS work together to activate Sirt1, are they tissue specific, or are they mutually exclusive? Can Sirt1 be sumoylated but inhibited by DBC1 or does sumoylation prevent DBC1 inhibition? What are the cellular cues that inhibit Sirt1 by DBC1 or activate Sirt1 by sumoylation and/or AROS? Critical toward answering these questions is the ability to obtain stoichiometrically sumoylated Sirt1. Chemical sumoylation could be accomplished through expressed protein ligation similar to the synthesis of stoichiometrically ubiquitinated histone H2B reported by the Muir lab (McGinty et al., 2008).

**Proteolytic Cleavage of Sirtuins.** Sir2 $\alpha$ , the mouse Sirt1 homolog, is cleaved by caspases during apoptosis (Ohsawa and Miura, 2006). Caspase activation during apoptosis controls cleavage of proteins during cellular degradation. In addition, Sirt1 has been linked to increased longevity through p53 (Lain et al., 2008). Therefore, Sir2 $\alpha$  proteolytic cleavage during apoptosis was suggested to inhibit the deacetylase activity and thus the protective effects of Sir2 $\alpha$ . Further work is needed to determine

whether Sir2 $\alpha$  proteolytic cleavage is consistent with human Sirt1 regulation, and whether caspase-dependent Sirt1 cleavage is required for apoptosis.

Sirt3 was shown to undergo proteolytic cleavage upon translocation into the mitochondria. In contrast to caspase-mediated cleavage of Sir2 $\alpha$ , cleavage of the Sirt3 mitochondrial localization sequence activates deacetylase activity. Full-length Sirt3 is proposed to be inactive until it is translocated to and proteolytically processed within the mitochondria to the active 28 kDa protein (Figure 2) (Schwer et al., 2002). The role of full-length inactivity is presumably to protect the cell from deacetylation until Sirt3 enters the mitochondria, because spurious deacetylation could cause misregulation of the multitude of cytoplasmic and nuclear processes potentially controlled by reversible acetylation. Similar to Sirt3, Sirt4 is proteolytically processed to remove the N-terminal 28 amino acids upon translocation to the mitochondria, although it is unclear what role this cleavage plays beyond mitochondrial localization (Ahuja et al., 2007).

#### **NAD<sup>+</sup> Salvage Pathway**

The role of the NAD<sup>+</sup> salvage pathway in physiological regulation of sirtuin activity was recently reviewed (Denu, 2007; Grubisha et al., 2005). Yet, in the last year, several studies reiterated the importance of NAD<sup>+</sup> metabolism in sirtuin regulation. Yang et al. suggested that increased NAD<sup>+</sup> levels in combination with genotoxic stress activate sirtuins resulting in cellular protective effects (Yang et al., 2007). Increased nicotinamide phosphoribosyltransferase (NAMPT) levels in fasted rats were effective for protection from apoptosis in the presence of Sirt3 and Sirt4. NAMPT is transcriptionally regulated by AMPK, which in turn can regulate NAD<sup>+</sup> levels and thus deacetylase activity (Fulco et al., 2008). Upon AMPK activation by reduced glucose, NAMPT is transcriptionally upregulated, which increases NAD<sup>+</sup> levels and subsequently Sirt1 deacetylase activity. This glucose-mediated signaling cascade was inhibited in mice that were heterozygous for the *SIRT1* gene, demonstrating that Sirt1 is integral to the cells' ability to sense reduced glucose and NAD<sup>+</sup> availability. Further knowledge of NAD<sup>+</sup> metabolism and the role of sirtuins could have great impact on understanding metabolic syndrome and other metabolic dysfunctions.

#### **Small-Molecule Modulation of Sirtuin Activity**

**Small-Molecule Sirtuin Activators and Inhibitors.** The mechanism of action and therapeutic potential of small-molecule sirtuin inhibitors and activators has been recently reviewed (Milne and Denu, 2008). As reviewed, the first putative sirtuin activators, specific to Sirt1, were polyphenols from plant metabolites (e.g., resveratrol). However, the in vivo Sirt1 activation by resveratrol has been difficult to reproduce in vitro (Borra et al., 2005; Kaeberlein et al., 2005), suggesting that resveratrol may activate sirtuins indirectly. Regardless, the identification of these sirtuin-specific activators has helped further understanding of sirtuin biology. More recently, Milne et al. have shown that Sirt1-specific activators, structurally unrelated to resveratrol, could provide novel treatments of type II diabetes (Milne et al., 2007). The identification and characterizing of specific sirtuin activators will be valuable to uncover the basic regulatory role of sirtuins in the cell and may be used as potential therapeutics to treat various diseases in which sirtuins are implicated.

As sirtuin inhibitors have been recently reviewed (Milne and Denu, 2008; Neugebauer et al., 2008), only a few recent studies



will be highlighted here. Mai et al. identified simplified analogs of eosin, a known inhibitor of both protein arginine and histone lysine methyltransferases, that also inhibited histone acetyltransferases and Sirt1/Sirt2 with similar potency in vitro (Mai et al., 2008). When tested on a human leukemia cell line, these inhibitors induced dose-dependent cytodifferentiation and high apoptosis levels, whereas single-target inhibitors (e.g., sirtinol and curcumin) were ineffective. This study suggested that promiscuous inhibitors of enzymes that control epigenetic modification might be more effective in some instances than their selective counterparts. However, as only arginine methylation was monitored in cells, further work is needed to determine whether the observed effect is due to promiscuous enzyme inhibition. Lain et al. carried out a cell-based screen to discover small-molecule p53 activators that decrease tumor growth (Lain et al., 2008). Their hit compounds, tenovin-1 and a more water-soluble analog, tenovin-6, were shown to inhibit Sirt1 and Sirt2 via yeast genetic screening, biochemical assays, and target validation studies. These tenovins were active on mammalian cells at low micromolar concentrations and decreased tumor growth in vivo as single agents. Catoire et al. reported evidence that sirtuin inhibition might provide a basis for the treatment of oculopharyngeal muscular dystrophy, which is caused by polyalanine expansion in the nuclear protein PABPN1 (Catoire et al., 2008). In particular, the authors showed that survival of mammalian cells expressing mutant PABPN1 was promoted by the sirtuin inhibitor, sirtinol, and decreased by the putative Sirt1 activator, resveratrol. Kiviranta et al. described new Sirt2 inhibitors based on the *N*-(3-phenylpropenoyl)-glycine tryptamide backbone and analyzed their binding modes through various computational methods (Kiviranta et al., 2008). Huhtiniemi et al. reported oxadiazole-carbonylaminothioureas that inhibited Sirt1 and Sirt2 with potency in the low micromolar range (Huhtiniemi et al., 2008). Chakrabarty et al. identified surfactin, a cyclic lipopeptide, as a novel inhibitor of *Plasmodium falciparum* Sir2 (PfSir2) (Chakrabarty et al., 2008). Kinetic analysis revealed that surfactin competes for NAD<sup>+</sup> binding but not acetyl-lysine substrate binding, with  $K_i$  values of 8  $\mu$ M and a  $K_d$  value of 20  $\mu$ M. Interestingly, surfactin was also shown to inhibit growth of malarial parasites in culture with an  $IC_{50}$  value of 9  $\mu$ M, but whether this effect is due to PfSir2 inhibition remains to be seen.

**Mechanism-Based Sirtuin Inhibitors.** Existing small-molecule sirtuin inhibitors rely on competitive binding with either acetyl-lysine or NAD<sup>+</sup> substrates. Inhibitors that take advantage of the unique sirtuin catalytic mechanism have been less exploited. Inhibition of Sirt1 by a thioacetyl-lysine peptide was first described by Fatkins et al. (2006). Subsequently, it was demonstrated that a similar thioacetyl-lysine peptide exhibited potent mechanism-based inhibition of Sirt1, Sirt2, Sirt3, and Hst2 (Smith and Denu, 2007b). For Hst2, this thioacetyl-lysine peptide yielded a  $K_{is}$  value of 17 nM, 280-fold lower than its  $K_d$  value of 4.7  $\mu$ M. This potent inhibition resulted from fast nicotinamide cleavage from NAD<sup>+</sup> ( $4.5 \text{ s}^{-1}$ ) but slow turnover ( $0.0024 \text{ s}^{-1}$ ) to products 1'-SH-OAADPr and deacetylated peptide, indicating an inhibitory mechanism in which thioacetyl-lysine peptide stalls at a catalytic intermediate after nicotinamide cleavage (Figure 3A). Solvent isotope effects, mass spectral analysis, density functional calculations, and general base mutational analysis

suggested a stalled  $\alpha$ -1'-S-alkylamidate intermediate. More recently, Hawse et al. reported crystallographic evidence in which this S-alkylamidate was directly observed bound to Sir2Tm (Hawse et al., 2008). The discovery of thioacetyl-lysine mechanism-based inhibitors allows for development of several unique probes to study sirtuin biology. Examples of these probes include novel sirtuin mechanism-based inhibitors, selective inhibitors, selective spectrophotometric substrates, and activity-based probes.

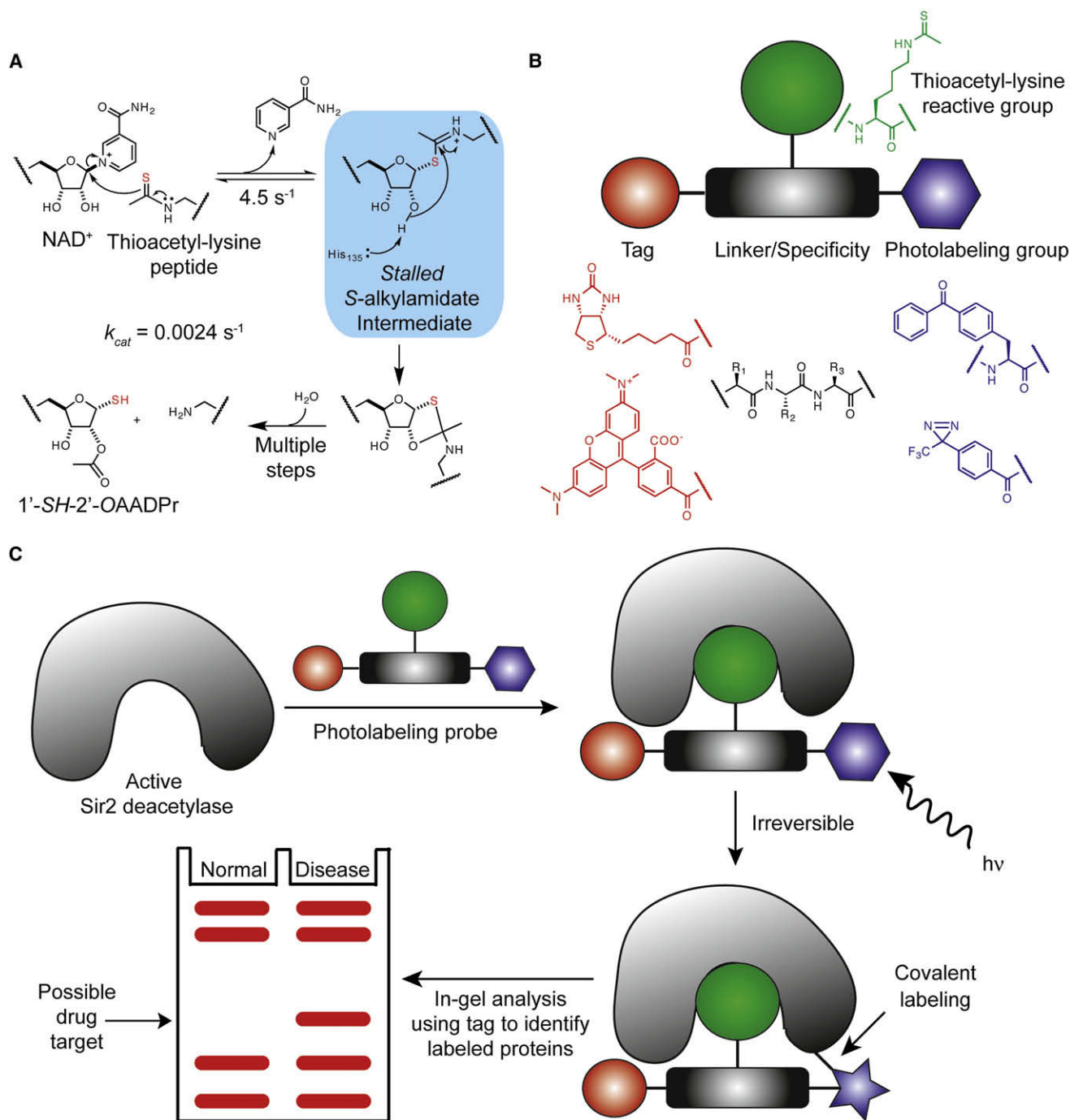
For further development of mechanism-based inhibitors, alternate sirtuin substrates that display more rapid nicotinamide formation or slower turnover (or formation of a dead-end complex) would exhibit greater sirtuin inhibition compared to thioacetyl-lysine. Selective sirtuin inhibitors might be developed through substitution of thioacetyl-lysine for acetyl-lysine into sirtuin-specific substrates. Fatkins and Zheng utilized the peptide sequence of  $\alpha$ -tubulin, a selective Sirt2 substrate (North et al., 2003), to discover a thioacetyl-lysine-based Sirt2 inhibitor that was 10- and 39-fold selective over Sirt1 and Sirt3, respectively (Fatkins and Zheng, 2008b). To move from peptide-based to small-molecule-based thioacetyl-lysine inhibitors, a substrate activity screening approach pioneered by the Ellman lab could be utilized (Patterson et al., 2007). In such an approach, a library of acetyl-lysine-based fluorescent substrates would be screened, and then the best substrates would be converted to inhibitors by replacement of acetyl-lysine with thioacetyl-lysine. A similar approach has been utilized to discover HDAC6-selective inhibitors through preparation of thiolate analogs of selected small-molecule fluorescent substrates (Suzuki et al., 2006).

Selective sirtuin substrates for a spectrophotometric assay could be developed if a sirtuin does not display a diminished rate of dethioacetylation compared to deacetylation. The 1'-thiol of the 1'-SH-OAADPr product could be detected with thiol-reactive reagents (e.g., Ellman's reagent) similar to an existing HDAC8 assay that detects thioacetate formed from dethioacetylation (Fatkins and Zheng, 2008a). To develop activity-based probes, addition of a tag (e.g., fluorophore or biotin) and a photolabeling group (e.g., benzophenone) to a thioacetyl-lysine peptide might allow simultaneous detection of deacetylase activity displayed by multiple sirtuins within cell extracts (Figures 3B and 3C). Alternatively, the photolabeling group could covalently label sirtuin-associated proteins, providing facile identification of sirtuin complexes. A similar approach has been reported by the Cravatt lab for class I and II HDACs through modification of the general HDAC inhibitor, SAHA, to incorporate a benzophenone photolabeling group and an alkyne tag (Salisbury and Cravatt, 2007, 2008). The authors found that both class I and II HDACs as well as other HDAC-associated proteins were labeled using this method. The modularity of the thioacetyl-lysine peptide inhibitor is well suited for these above applications because of the facile synthesis, control of sequence, and ease of peptide derivatization in solid-phase peptide synthesis.

### Conclusions and Future Directions

Recent research has furthered our understanding of the chemical and regulatory mechanisms catalyzed by sirtuins, but creative application of chemical biology will be necessary to develop molecular probes and therapeutic agents that target sirtuin





**Figure 3. An Example Application of the Mechanism-Based Inhibition Displayed by Thioacetyl-Lysine Peptides**

(A) Proposed inhibition mechanism of thioacetyl-lysine peptides.

(B) Modular design of a sirtuin activity-based photolabeling probe.

(C) Example experiment utilizing a sirtuin activity-based photolabeling probe to identify disease-specific sirtuin activities.

activity. Differentiating between individual sirtuins and across HDAC classes will be particularly difficult. However, understanding mechanistic differences between HDAC classes, especially in regard to acetyl binding/reactivity and the unique use of  $\text{NAD}^+$  by sirtuins, will prove fruitful. In this respect, mechanism-based sirtuin inhibitors such as thioacetyl-lysine and other

acetyl-lysine analogs are particularly promising. In addition, targeting *in vivo* modulators of sirtuin activity rather than sirtuins themselves may provide higher selectivity. Future development of specific sirtuin inhibitors, activators, and molecular probes will further understanding of sirtuin biology and treatments of age-associated diseases.

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