

Enzymatic activities of Sir2 and chromatin silencing

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Heritable domains of generalized repression are a common feature of eukaryotic chromosomes and involve the assembly of DNA into a silenced chromatin structure. Sir2, a conserved protein required for silencing in yeast, has recently been shown to couple histone deacetylation to cleavage of a high-energy bond in nicotinamide adenine dinucleotide (NAD) and the synthesis of a novel product, O-acetyl-ADP-ribose. The deacetylase activity provides a direct link between Sir2 and the hypoacetylated state of silent chromatin. However, the unusual coupling of deacetylation to cleavage and synthesis of other bonds raises the possibility that deacetylation is not the only crucial function of Sir2.

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Abbreviations

NAD nicotinamide adenine dinucleotide
NaMN nicotinic acid mononucleotide
SIR silent information regulator

Introduction

Gene silencing is the inactivation of large domains of DNA by packaging them into a specialized inaccessible chromatin structure. This type of inactivation is involved in the regulation of gene expression and is also associated with the chromosome structures required for chromosome maintenance and inheritance. For example, in metazoan chromosomes, vast regions of DNA adjacent to centromeres and telomeres are packaged into an inactive type of chromatin called heterochromatin (reviewed in [1]). Mutations that disrupt the formation of heterochromatin compromise chromosome inheritance, very probably as a result of perturbed centromere function [2]. Silenced chromatin is also found in unicellular fungi. In fission yeast (*Schizosaccharomyces pombe*), similar to metazoans, large regions of DNA adjacent to centromeres are packaged into silenced chromatin, and the formation of these domains plays an important role in chromosome inheritance [3,4]. In both fission yeast and budding yeast (*Saccharomyces cerevisiae*), haploid cell identity is regulated by transcriptional silencing of DNA domains that contain mating-type information; also in both yeasts, telomeric DNA regions are packaged into silenced chromatin [5–8].

Genetic and biochemical studies have identified the main regulatory sites and proteins that collaborate to assemble silenced DNA in budding yeast (reviewed in [9]). A major recent development in this field has been the discovery

that the silencing protein Sir2 and other members of the Sir2 family are NAD-dependent enzymes [10••–14••]. The enzymatic activities of this protein family are unusual and are only now beginning to come into focus. Here, we discuss the enzymatic activities of Sir2 and the current speculation about the role of these activities in silencing.

The silencing machinery in yeast

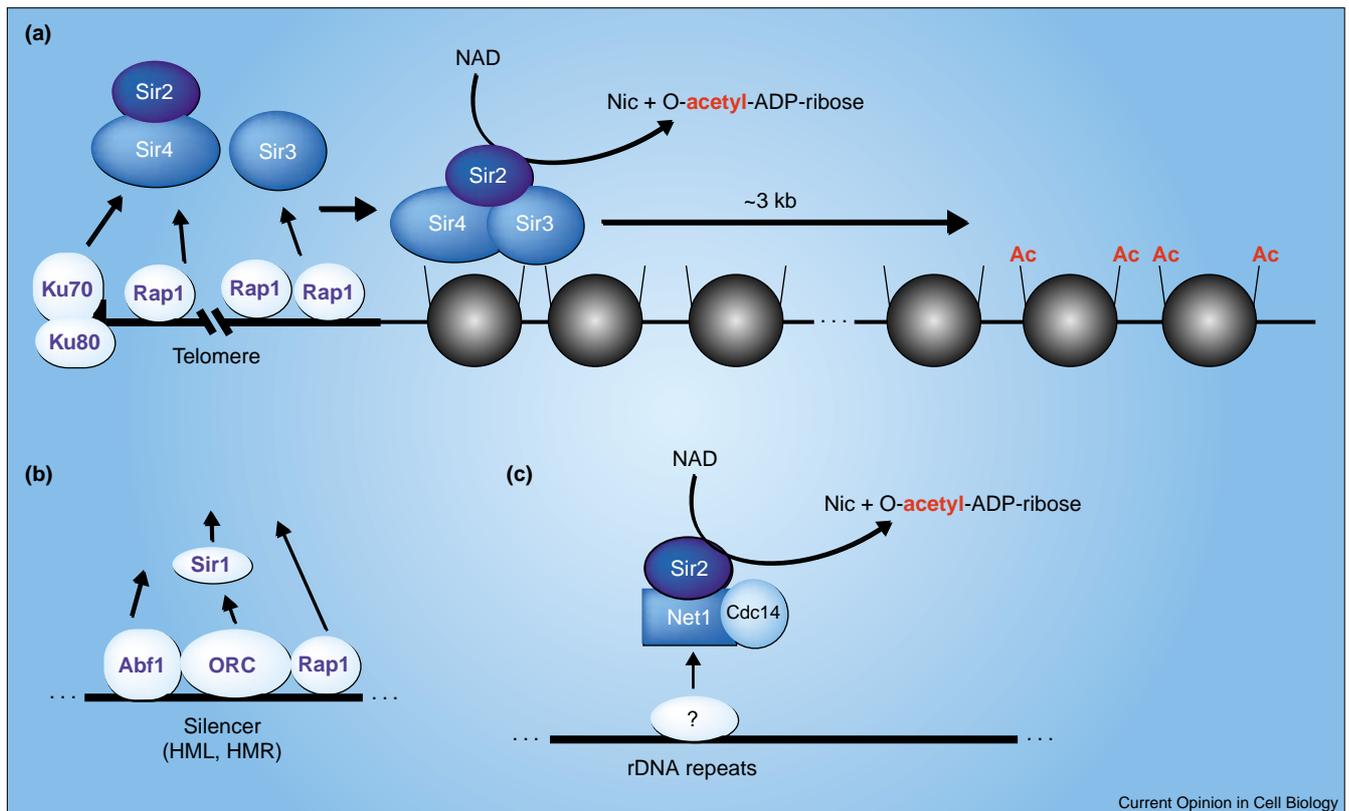
Sir2 is required for all known examples of silencing in yeast [15–19]. As a component of the SIR complex, it acts together with Sir3 and Sir4 to assemble silent chromatin at telomeric DNA regions and at the silent mating type loci (called HML and HMR, homothallic left and homothallic right, respectively; Figure 1; [20–22]). Together with Net1, Cdc14 and perhaps other proteins, in a complex called RENT (regulator of nucleolar silencing and telophase exit), Sir2 is involved in ribosomal DNA (rDNA) silencing (Figure 1; [23–25]). rDNA silencing is required for suppression of hyper-recombination within the highly repetitive rDNA array and repression of transcription of pol II reporter genes inserted in rDNA [18,19]. Suppression of hyper-recombination by Sir2 plays a primary role in extension of replicative life span in yeast [26,27].

The SIR and RENT complexes are recruited to their chromosomal targets via interactions with DNA-binding proteins, which bind to specific regulatory sites and initiate silencing. The DNA-binding protein(s) that target the RENT complex to rDNA have not been identified yet, but the mechanism of recruitment of SIR to DNA is relatively well understood (reviewed in [9]; see Figure 1). In addition to the two silencing complexes and DNA-binding proteins, the conserved and highly basic amino termini of histones H3 and H4 are required for silencing [28]. Mutations in several conserved lysines in histones H3 and H4 abolish silencing at the telomeres and the silent mating-type loci. These lysine residues are reversibly acetylated, and mutational analysis suggests that their deacetylated state is important for silencing [29–31]. Moreover, two of the proteins in the SIR complex, Sir3 and Sir4, are histone tail binding proteins, and their interactions with H3 and H4 are likely to play a key role in the assembly of silent chromatin domains [32]. Despite its general requirement for silencing, Sir2 had not been identified with a biochemical function until recently.

Ribosyltransferase activity of Sir2 and Sir2-like proteins

Sir2 is a member of an ancient family of proteins in organisms ranging from bacteria to complex eukaryotes [33,34]. Members of this family contain a 250 amino acid core domain that shares about 25%–60% sequence identity. The eukaryotic Sir2-like proteins have been implicated in a wide range of chromosome-associated phenomena,

Figure 1



Sir2-containing silencing complexes in budding yeast. (a) A model for the recruitment of the SIR complex (Sir2/3/4) to a telomere. The enzymatic activity of Sir2 is required for silencing and efficient spreading of the SIR complex along the chromatin fiber. Ac denotes acetylated histones in nucleosomes, which are shown as filled circles. Within the silent chromatin domain (spanning ~3 kb from the telomere) histones are hypoacetylated. See text and [9,52] for details and

additional references. (b) At the silent mating type loci (HML, HMR), a different set of proteins, bound to regulatory sites called silencers, recruits the SIR complex to DNA (reviewed in [9,53]). (c) The RENT complex mediates rDNA silencing and suppression of rDNA hyper-recombination. Protein(s) involved in its recruitment to DNA have not been identified. See text for a discussion of the NAD-dependent protein deacetylase activity of Sir2. Nic, nicotinamide.

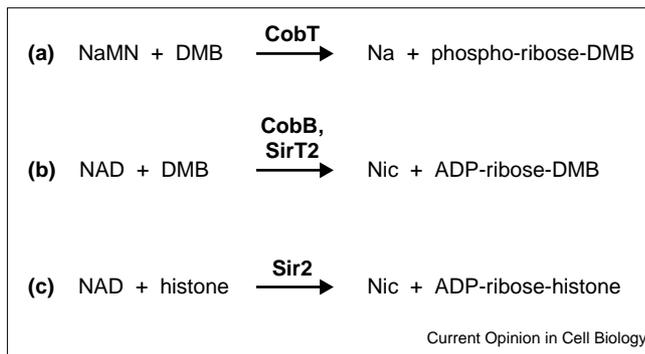
including gene silencing, cell cycle progression, chromosome segregation and DNA damage repair [33,35,36]. The first clues regarding the biochemical function of a Sir2-like protein came from studies of cobalamin (vitamin B12) biosynthesis in the bacterium *Salmonella typhimurium* by Escalante-Semerena and colleagues [32]. A precursor in the cobalamin pathway, DMB-5'-ribosyl-phosphate, is synthesized by transfer of phosphoribose from nicotinic acid mononucleotide (NaMN) to another small molecule, dimethyl benzimidazole (DMB; Figure 2a). The *Salmonella* CobT protein catalyzes this phosphoribosyl transfer reaction. However, Tsang *et al.* [32] discovered that CobB, the *Salmonella* Sir2-like protein, could partially compensate for the loss of CobT in the cobalamin pathway, suggesting that CobB could perform a similar enzymatic reaction.

The idea that Sir2-like proteins can perform phosphoribosyl transfer reactions, of the type described above, was directly tested by Frye [10••]. In examining various pyridine nucleotide derivatives as donors for the transferase

activity of Sir2, Frye made the key discovery that only NAD, but not NaMN or other NAD derivatives, could act as cofactors for *E. coli* and human Sir2-like proteins *in vitro* (CobB and SirT2, respectively; Figure 2b; [10••]). Sir2-like proteins were therefore suggested to be ADP-ribosyltransferases as they could transfer ADP-ribose from NAD to substrate molecules.

The above studies prompted several groups to examine whether the budding yeast Sir2 protein had a similar enzymatic activity and whether this activity was required for gene silencing. Using NAD as a donor, Tanny *et al.* ([11••]; J Tanny, D Moazed, unpublished data) tested the ribosyltransferase activity of Sir2 on a wide range of substrates, including histones and other silencing proteins. It was found that Sir2 could perform a weak ribosylation reaction in which it transferred the ADP-ribose moiety from NAD to itself and histones (Figure 2c). The weak ribosyltransferase activity was accompanied by an efficient histone-dependent NAD breakdown activity. Furthermore, a point mutation in Sir2 that abolished these activities *in vitro* resulted in a complete

Figure 2



Phosphoribosyltransferase activity of Sir2-like proteins.

(a) Phosphoribose transfer from NaMN to dimethyl benzimidazole (DMB) is catalyzed by the *Salmonella* CobT. CobB, the *Salmonella* Sir2-like protein, can compensate for loss of CobT function and has been proposed to perform the same reaction *in vivo* [37**].

(b) *E. coli* CobB and human SirT2 proteins perform an ADP-ribosyltransferase reaction, using NAD rather than NaMN as a donor molecule, to transfer ADP-ribose to DMB or to an artificial protein substrate *in vitro* [10**]. (c) The yeast Sir2 protein performs an NAD cleavage and weak ribosylation reaction using acetylated histones as a substrate [11**]. See text for further details.

Na, nicotinic acid; Nic, nicotinamide.

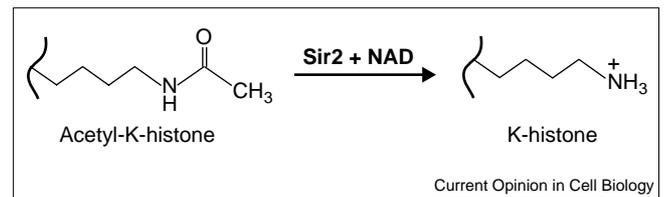
loss of silencing *in vivo*, suggesting that the ribosyltransferase (and/or NADase) activities of Sir2 were essential for silencing. However, as discussed below, the activities observed in this study were partial reactions that stemmed from the ability of Sir2 to catalyze some novel chemistry with NAD and acetylated substrates.

NAD-dependent protein deacetylase activity of Sir2

The reports of ribosyltransferase activity in Sir2 and Sir2-like proteins were followed by studies showing that Sir2-like proteins have an NAD-dependent histone deacetylase activity ([12**–14**]; Figure 3). In contrast to the ribosylation activity mentioned above, the deacetylase activity of Sir2 is very efficient, suggesting that it is the primary activity of the protein *in vitro*. This discovery has been greeted with enthusiasm, in part, because earlier evidence had suggested a role for histone deacetylation in assembly of silent chromatin domains [28,30]. In fact, Sir2 had earlier been suggested to be a histone deacetylase because its overexpression results in general histone hypoacetylation in yeast [38]. Deacetylation by Sir2 therefore provides a direct link between the silencing machinery and the observed hypoacetylated state of histones within silent chromatin domains.

Nonetheless, from a mechanistic point of view the requirement for NAD in a deacetylation reaction is difficult to explain. Deacetylation is an energetically favorable amide hydrolysis reaction. Similar reactions are catalyzed by numerous proteases without the need for a cofactor [39]. This reasoning, and a mutation in Sir2 that appeared to separate its two activities, led to the suggestion that the

Figure 3



The NAD-dependent deacetylase activity of Sir2. Sir2 and Sir2-like proteins from *E. coli*, yeast and mouse can deacetylate histones and other proteins [12**–14**]. Acetyl-K-histone, histone containing acetyl-lysine; K-histone, deacetylated histone.

ribosyltransferase/NAD breakdown and deacetylation activities of Sir2 are separable and fundamentally different [12**]. At this point, it was clear that Sir2 is a very unusual enzyme, but in the absence of a coherent reaction mechanism, its biologically significant activity remained in question. However, more recent studies, discussed below, provide evidence that the different enzymatic activities of this remarkable family of proteins are mechanistically related.

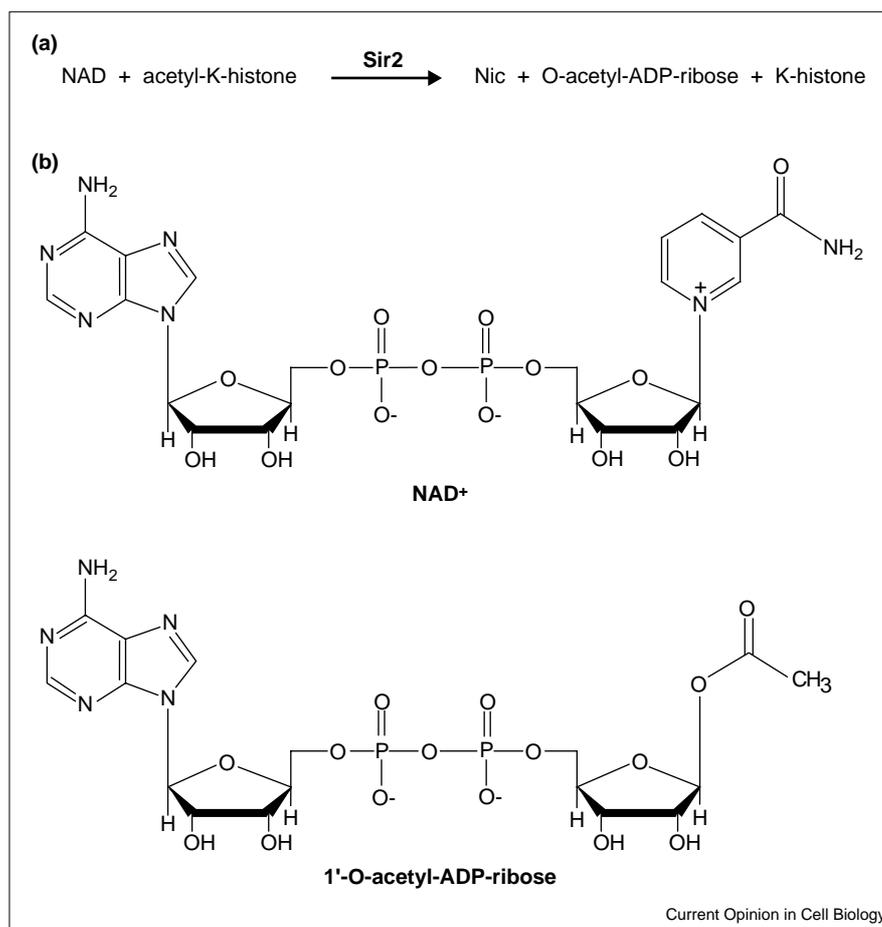
Acetyl transfer from substrate to ADP-ribose, the generation of O-acetyl-ADP-ribose

Although early experiments by Imai and colleagues [12**] had suggested that the ADP-ribosyltransferase and NAD cleavage activities of Sir2 are fundamentally distinct from its deacetylase activity, a number of observations suggested that these very different activities may be related. First, the ribosyltransferase and NAD breakdown activities of Sir2 required the presence of an acetylated substrate in the reaction ([11**,12**]; J Tanny, D Moazed, unpublished data), suggesting a relationship between these activities and acetyl-lysine (the substrate for the deacetylation activity of Sir2). Second, Landry *et al.* [13**] showed that, in addition to deacetylation, Sir2 could perform an exchange reaction in which it generated labeled NAD from ¹⁴C-labeled nicotinamide and unlabeled NAD. Although the efficiency of this exchange reaction is unknown, it required the presence of an acetylated histone substrate. Thus, acetyl-lysine was required for a second set of Sir2 activities, ribosylation, NAD breakdown and an exchange reaction involving breakage of the same C-N bond in NAD that is cleaved during ribosylation.

An important clue to the mechanism of Sir2 came from examination of the relationship between its deacetylation and NAD breakdown activities. The first crucial observation was that Sir2 couples deacetylation to cleavage of the high-energy glycosidic bond that links the ADP-ribose moiety of NAD to nicotinamide [40**–42**]. A precise stoichiometric relationship between deacetylation and NAD breakdown was uncovered, indicating that for every acetyl-lysine that is deacetylated one NAD molecule is cleaved. A second crucial observation in these experiments was the unexpected nature of the NAD cleavage

Figure 4

Coupling of deacetylation to NAD cleavage and acetyl transfer from substrate to ADP-ribose by Sir2 and Sir2-like proteins [41••,42••]. (a) The overall reaction scheme. (b) Structures of NAD and O-acetyl-ADP-ribose, the novel compound produced by Sir2 and Sir2-like proteins, are shown. This figure shows the most likely position of the O-acetyl group on the 1' ribose carbon; an alternative position for the O-acetyl group on the 2' ribose carbon has not been ruled out. See text and references [40••–42••] for details.



products. Straightforward cleavage of NAD at the glycosidic bond that links its ADP-ribose moiety to nicotinamide should result in the generation of ADP-ribose and nicotinamide. However, in addition to nicotinamide and small amounts of ADP-ribose, a third major reaction product, related to but distinct from ADP-ribose, was detected [41••,42••]. Label transfer experiments showed that this novel reaction product resulted from the transfer of the acetyl group from the substrate to the ADP-ribose moiety of NAD [41••]. Furthermore, at high pH, this product was shown to decay to ADP-ribose and acetate and was therefore concluded to be O-acetyl-ADP-ribose [41••]. In an independent study, Tanner *et al.* [42••] analyzed the reaction products produced by Sir2 and Hst2, a yeast Sir2-like protein, and found that O-acetyl-ADP-ribose was a primary product of deacetylation by these enzymes. This study also confirmed the identity of O-acetyl-ADP-ribose by mass spectroscopy analysis [42••]. These observations provide proof of the obligate coupling of deacetylation to NAD cleavage and demonstrate a direct role for NAD in histone deacetylation by Sir2. Consistent with these results, Landry *et al.* [40••] find that a non-hydrolyzable NAD analog, carba-NAD, is a competitive inhibitor of the

NAD-dependent deacetylase activity of Sir2. It is therefore abundantly clear that Sir2 has two coupled enzymatic activities, deacetylation and NAD breakdown, and produces a novel compound, O-acetyl-ADP-ribose (Figure 4).

As mentioned earlier, Sir2-like proteins also have a weak ribosyltransferase activity. Interestingly, this activity of Sir2 (as well as its NADase activity) requires the presence of an acetyl-lysine-containing substrate in the reaction, suggesting that it is linked to deacetylation. The above observations now provide an explanation for the weak ribosyltransferase activity of Sir2-like proteins. Tanner *et al.* [42••] suggest that, by analogy with NAD glycohydrolases, Sir2 first generates an oxo-carbenium ADP-ribose intermediate, which then acts as an acceptor for the acetyl group. This mechanism would involve the formation of an enzyme-ADP-ribose intermediate, providing an explanation for the detection of a ribosylated form of Sir2 [11••]. A similar reaction mechanism involves the cleavage of NAD by nucleophilic attack of the isoamide form of acetyl-lysine on the 1' ribose carbon position to release nicotinamide [42••]. Both the above mechanisms would also involve the generation of a transient substrate ADP-ribose intermediate, providing a possible explanation for the weak histone

ribosylation activity of Sir2. In either case, it is very likely that, at least *in vitro*, the ribosylation activity of Sir2-like proteins results from either the trapping of reaction intermediates or side reactions during deacetylation and NAD cleavage [41**,42**]

Significance for the mechanism of silencing

The coupling of deacetylation, itself an energetically favorable reaction, to cleavage of a high-energy bond is rather unusual. The free energy of hydrolysis of the glycosidic bond that links ribose and nicotinamide in NAD is ~8.2 kcal/mol, in the same range as the energy of hydrolysis of ATP to ADP [43]. One is forced to ask why does Sir2 do it this way. Is it just an unusual deacetylase or is there a role for the NADase activity and/or O-acetyl-ADP-ribose production in silencing? There is a long-standing correlation between gene inactivation and histone hypoacetylation [44,45]. In budding yeast, silent chromatin domains are hypoacetylated and mutational analysis of histones H3 and H4 suggests that the deacetylated state of one or more lysine residues in the amino termini of these histones is critical for silencing [29,30,38]. It is therefore easy to suppose that deacetylation is the important function of Sir2 in silencing. However, there is currently no direct evidence supporting this attractive hypothesis, and the possibility that one of the other two significant activities of Sir2, which are associated with deacetylation, NAD cleavage and O-acetyl-ADP-ribose synthesis, play a critical role in gene silencing must be considered.

Although histones associated with silent chromatin are generally in a hypoacetylated state, there is also evidence favoring a role for histone acetylation (rather than deacetylation) in transcriptional silencing in yeast and *Drosophila*. For example, deletion of the yeast *RPD3* gene, which encodes a histone deacetylase, results in a dramatic increase in silencing at telomeric, mating-type and rDNA regions [46-48]. Furthermore, deletion of the yeast type B histone acetyltransferase gene, *HAT1*, in combination with histone H3 amino-terminal tail mutations, results in a significant defect in telomeric silencing, and mutations in the MYST family of acetyl transferases have both positive and negative effects on silencing [49-51]. Contrary to general expectations, these results suggest that histone acetylation, as well as deacetylation, plays an important role in silencing.

Rpd3, Hat1 and MYST family acetyltransferases may be influencing the efficiency of silencing indirectly, for example through global effects on gene expression or chromatin assembly. However, an alternative possibility is that acetylation of a specific target protein(s) is required for Sir2 function and efficient silencing. In this model, acetyl-lysine (in histones or other proteins) would be required to trigger the NADase and O-acetyl-ADP-ribose synthesis activity of Sir2. The ultimate function of Sir2 in silencing would then be to use acetyl-lysine as a cofactor to breakdown NAD and/or to produce O-acetyl-ADP-ribose. In this regard, several intriguing possibilities have been proposed [41**,42**].

One possibility is that the energy of NAD breakdown might promote a step in the assembly of silent chromatin, such as the spreading of silencing complexes along the chromatin fiber [41**]. Another possibility is that O-acetyl-ADP-ribose could act as a local allosteric effector for other silencing proteins or as an acetyl donor for a novel class of protein acetyltransferases or as an ADP-ribose group donor for a novel ribosyltransferase [41**,42**]. Distinguishing between the above models requires examining the role of Sir2 in silencing under conditions that can separate its various activities.

Conclusions

Over the past year or so, Sir2 has travelled from being a protein of unknown biochemical function to being an enzyme with remarkable activities that produces a novel cellular metabolite. As is often the case, these new discoveries raise more questions than they answer. In the simplest case, the deacetylase activity of Sir2 provides a direct explanation for the hypoacetylated state of histones within yeast silent chromatin domains (see Figure 1). But whether this function of Sir2 is crucial for silencing or not is far from clear. The possible role of the NADase activity of Sir2 and the role of O-acetyl-ADP-ribose in silencing and cellular physiology remain to be determined. Finally, how any of the activities of Sir2 contribute to those unique properties of gene silencing, such as the spreading of repressors along the chromatin fiber or the stable inheritance of the silent state during chromosome duplication, remains the subject of future studies.

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