Characterization of the Histone Deacetylation Activity of the Yeast Silencing Gene SIR2

by
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ABSTRACT

A gene's location in the genome can have profound effects on its expression. In *Saccharomyces cerevisiae*, the gene SIR2 is crucial for silencing at the HM mating type loci, the telomeres, and the rDNA array. It is also necessary for maintaining a hypoacetylated state of the histones at these locations.

To silence genes, the Sir2 protein can deacetylate histones in the presence of NAD+. This is shown by the ability of Sir2p to remove the acetyl groups from peptides corresponding to the amino-terminal tail of histone H3 or histone H4. It selectively removes the acetyl group from lysine 16 on histone H4. This lysine plays a particularly crucial role in SIR2 dependent silencing. Sir2p can also ADP-ribosylate histones, but this activity is significantly weaker than its histone deacetylase activity.

Mutations in Sir2p that affect its ability to deacetylate histones can affect SIR2 function in vivo. Mutations that completely destroy

the ability of Sir2p to deacetylate histones also render SIR2 unable to silence. Two mutations, however, do not show a direct correlation between in vitro and in vivo activity. One mutant has nearly full levels of activity in vitro, but is unable to silence in vivo while the other has severely compromised in vitro activity, but show no sign of defect in vivo. Localization studies of the mutants suggest that the enzymatic activity of Sir2p is necessary for the SIR complex to localize to the telomeres although it is not necessary for Sir2p to localize to the rDNA. The results suggest that while the function of SIR2 appears to be to deacetylate histones at silent loci, that we do not yet fully understand how SIR2 does this.

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Table of contents

Chapter 1

Introduction

If you take the DNA out of a single cell from the human body and stretch it out into one long string it would measure 2 meters in length. Needless to say it is very important for the cell to pack this massive amount of DNA in as organized a fashion as possible. Failure to do so would result in a tangled mass of molecular fettuccini that the cell would not be able to replicate or transcribe from in timely fashion. The cell has solved this problem by wrapping the DNA around histones to form ordered structures called a nucleosome.

By forming nucleosomes, the cell has found a way to pack all of its DNA into the nucleus. In packing the DNA into tightly wound structures, however, the cell has created a new set of issues. In much the same way that it is difficult to access a particular length of string inside a ball of yarn, it is no easy task for the cell to access specific promoters located in the tightly wound chromosome. So while the nucleosomes do their job in efficiently packing a huge amount of DNA into a tiny cell, they get in the way of the transcriptional machinery in the process. This crisis that the cell faces has turned into an opportunity as the cell uses the nucleosome as a form of transcriptional regulation at many different levels.

Chromatin and Transcription

The nucleosome consists of about 200 bp of DNA wrapped around a histone octamer made up of the four core histones (H2A, H2B, H3, and H4) (reviewed in (Kornberg and Lorch 1999). Such a structure on the promoter of an active gene can impede the RNA

polymerase. In vitro transcription systems illustrate this when RNA is transcribed much less efficiently when nucleosomes are bound to the promoter (Knezetic and Luse 1986; Lorch et al. 1987). In vivo evidence that nucleosomes inhibit transcription comes from the observation that depletion of histones in yeast leads to increases in transcription of many genes (Han and Grunstein 1988).

The obstructive nature of histones necessitates that the cell loosen the chromatin to allow appropriate access to the promoter. The yeast SWI/SNF complex, one of the better studied transcriptional coactivators, loosens chromatin through remodeling nucleosomes at the promoter. Genetic screens have identified the SWI and SNF genes as transcriptional regulators (Neigeborn and Carlson 1984; Stern et al. 1984). Mutations in non-histone chromatin factors (Kruger and Herskowitz 1991) and in histones H2A and H2B (Hirschhorn et al. 1992) suppress deletions in SWI and SNF genes linking their activities to chromatin. Also, the chromatin structure at the promoter of the SWI/SNF regulated gene SUC2 is altered in SWI/SNF mutants (Hirschhorn et al. 1992).

Protein purification studies show that many of the SWI/SNF genes intereact in an 11 subunit complex that has a DNA dependent ATPase activity (Cairns et al. 1994; Cote et al. 1994). This complex enhances the binding of transcription factors to a nucleosomal DNA template in vitro (Cote et al. 1994). The SWI/SNF complex is one of the better-studied complexes that helps activate transcription through chromatin remodeling. Additional complexes that remodel chromatin include the RSC complex in yeast (Cairns et al. 1996), the

NURF complex in Drosophila (Tsukiyama et al. 1994), and the human SWI/SNF complex (Imbalzano et al. 1994).

In addition to the remodeling of nucleosomes at promoters, the acetylation of histones is an important modification that controls the level of gene expression. Histone acetylation occurs when the acetyl group from acetyl-CoA is transferred to a lysine residue on the amino-terminal tail of histone H3 or H4. This modification creates an acetyl lysine which has a neutral charge compared to the positively charged lysine. Histone acetylation and gene activation have long been connected (Allfrey 1977) and studies show that nucleosomes in transcriptional active regions are hyperacetylated (Hebbes et al. 1988). The first nuclear histone acetyl transferase (HAT) was identified in tetrahymena and discovered to be homologous to the yeast gene GCN5 (Brownell et al. 1996). GCN5 is a transcriptional coactivator that interacts with a set of coactivators call the ADAs (Georgakopoulos and Thireos 1992; Marcus et al. 1994; Georgakopoulos et al. 1995). This was the first evidence that histone acetylation is used by transcriptional coactivators to regulate gene expression. GCN5 also forms a complex with other important coactivators that play a role in varied tanscriptional processes (Grant et al. 1997).

In trying to understand how acetylation might actually affect gene expression, it is frequently speculated that the acetyl groups neutralize the lysine's positive charges and in so doing it weakens the affinity that histones have for DNA's phospho-diester backbone. Studies show that the affinity of the histone H4 tail for DNA is reduced when the lysines are acetylated (Hong et al. 1993). A high-

resolution crystal structure of the nucleosome gives a fuller picture though. Most of the contacts between DNA and the histones do not occur on the N-terminal tail but in the core of the histones (Luger et al. 1997). Reducing the charge on the tail should not strongly affect the histone's affinity to DNA in that case. In addition, while the N-terminal tails are the most poorly resolved part of the crystal, the structure does show that residues 16-25 of histone H4 extends into the adjacent nucleosome and interacts with a negatively charged part of histone H2A and H2B. Instead of reducing the histone's affinity for DNA, this argues that acetylation could reduce the affinity of nucleosomes for neighboring nucleosomes and perhaps in so doing it reduces the complexity of higher order chromatin structures.

While hyperacetylation of histones is associated with actively expressed genes, the hypoacetylation of histones is associated with unexpressed genes. As such there are enzymes that deacetylate histones to repress gene expression. By doing this, the cell is actively using the repressive nature of chromatin to decrease transcription. The first histone deacetylase was found by looking for proteins that bind to the deacetylatase inhibitor trapoxin (Taunton et al. 1996). The trapoxin binding protein deacetylates histones and, interestingly, is homologous to the yeast gene RPD3. RPD3 had previously been identified as a gene that is required for the full repression as well as full activation of some genes (Vidal and Gaber 1991). Molecular studies in yeast showed that recruitment of Rpd3p to promoters represses transcription. Rpd3p appears to act in a corepressor complex with Sin3p that is recruited to promoters

by DNA binding proteins like Ume6p (Kadosh and Struhl 1997). Mammalian RPD3 shows a similar corepressor role (Alland et al. 1997; Hassig et al. 1997; Heinzel et al. 1997; Laherty et al. 1997; Nagy et al. 1997; Zhang et al. 1997). These studies suggest that repression can occur through the recruitment of histone deacetylases to the promoter which is the first molecular mechanism of transcriptional repression to be elucidated.

The relationship between RPD3 and transcription is more complicated than that of simply a corepressor as RPD3 is required not only for the repression of genes but also the activation of genes (Vidal and Gaber 1991). Micro-array expression analysis has shown that genome-wide, there are as many genes that are downregulated in rpd3 deletion strains as there are genes that are upregulated (Bernstein et al. 2000). This is very curious, as, in general, hyperacetylation of histones is associated with active genes while hypoacetylation is associated with inactive genes. Some have theorized that supposed gene activation by Rpd3p could be through indirect effects. Time course studies using inhibitors of the deacetylase activity of Rpd3p have suggested that for some genes this may be the case, but for others the inhibitors act far too quickly for the effects to be indirect (Bernstein et al. 2000). This suggests that the deacetylation activity of Rpd3p is directly responsible for the activation of genes. Why this happens is unclear. Some think it has to do with the precise acetylation pattern of histones (De Rubertis et al. 1996). This will be discussed in more detail later.

Position Effects on Gene Expression

At specific genes, the cell has to overcome chromatin's repressive nature to increase transcription and at the same time it has co-opted chromatin to repress transcription. Chromatin also plays a crucial role in regional effects where a gene's expression is affected not just by its promoter, but also by its location in the genome. Perhaps the oldest know example of this phenomenon is position effect variegation (PEV) in Drosophila. PEV was first observed in a mutant fly that shows patches of red and white in its eye (reviewed in (Reuter and Spierer 1992; Wallrath 1998). In this mutant the gene normally responsible for eye pigmentation was translocated to a pericentric region of the genome. Observations of chromosomes under the microscope show that pericentric regions tend to be look more condensed. Condensed regions such as these are called heterochromatin. The patchy eye color is explained by the pericentric heterochromatin creeping into the eye pigmentation gene. In some groups of cells, the heterochromatin is able to silence the gene while in other groups it cannot.

There are several characteristics about PEV that highlight the nature of heterochromatin. One, the phenomenon is moderately heritable, that is level of expression in cells of common origin is the same, although there is occasional switching. This is why the eyes are patchy; the patches of red and white are the result of daughter cells having the same repressed state as their progenitor cells. If it were random from cell division to cell division, you would expect a dilution effect instead of patching. Two, the effect of

heterochromatin tends to be repressive. Three, PEV does not depend on the promoter of the gene being repressed.

In light of the generally repressive effect that histones have and the repressive effects of heterochromatin, it should not come as a big surprise that the two are highly related. Genetically this is seen in the observation that mutations in histones (Moore et al. 1979) or heterochromatin proteins (James et al. 1989; Eissenberg et al. 1990) lead to a weakening of heterochromatin's repressive effects and a decrease in PEV. Also, histone deacetylase inhibitors, like butyrate, lead to a decrease in PEV suggesting that histone deacetylation is important for the repression (Mottus et al. 1980). At a more structural level, genes in heterochromatic regions are nuclease resistant and are less accessible to nucleases, a hallmark of repressive nucleosomal arrangements (Wallrath and Elgin 1995).

Another well-known example of heterochromatin and locus specific gene regulation is X-inactivation in mammals. To maintain the proper dosage of genes in XX mammalian females, one of the X-chromosomes is silenced (reviewed in (Marin et al. 2000). Chromatin plays a role in this process at several levels. For one, promoters on the inactive X are hypoacetylated compared to the active X (Gilbert and Sharp 1999). Also, the nucleosomes of the inactive X are enriched for a histone H2 variant called macroH2A further suggesting that the cell is altering the chromatin to inactivate the chromosome (Costanzi and Pehrson 1998). Similar to PEV, the inactive X shows clonal patterns suggesting that it is moderately heritable most dramatically illustrated in mosaicism of some X-linked traits in women.

Unlike higher eukaryotes, the small size of chromosomes in Saccharomyces means that heterochromatic regions of the chromosome cannot be observed under the microscope. Despite this, we have probably learned more about heterochromatin and position effects in yeast than any other organism. The first recognized position effect in yeast is silencing at the mating type loci (reviewed in (Laurenson and Rine 1992). In yeast, there are three loci that contain mating type information. The MAT locus can either have the a1 and a2 genes or the α1 and α2 genes (see Figure 1). To the right of MAT is the HMR locus that normally has the a1 and a2 genes and to the left is the HML locus that normally has the α1 and α2 genes. There is no sequence difference between the a or α genes at the MAT locus or HM loci; however, the genes at the MAT locus are expressed, while the genes at the HM loci are kept silent. In fact any, marker gene placed in the HM regions is silenced regardless of the nature of the promoter is driving its expression (Brand et al. 1985).

The mating type loci are just two of several loci that are silenced in yeast. Gottschling and colleagues showed that when they integrated marker genes into the telomeres the marker's expression levels are dramatically reduced (Gottschling et al. 1990). While the expression level of genes placed at the telomeres is much lower than that seen at their native loci, the silencing is not a strong as that at HM where most genes are totally repressed. Investigators also saw that when RNA polymerase II genes are transposed or integrated into the rDNA array their expression is silenced (Bryk et al. 1997; Smith and Boeke 1997).

Similar to PEV in Drosophila and X-inactivation, the silencing of genes in yeast is moderately heritable. At the HM loci this is seen most clearly in Δsir1 mutant strains. In these strains only 20% of the cells successfully silence the HM loci while 80% do not (Pillus and Rine 1989). These two states are, however, fairly stablely inherited as daughter cells usually have the same level of silencing as their mother cells. Similar results at the telomeres are seen in the sectoring phenotype of an ADE2 marker placed there (Gottschling et al. 1990). The sectoring indicates that the silenced state is fairly stable from generation to generation with only rare switching events occurring.

The SIR genes

Studies of silencing in yeast have benefited from the ease of doing both genetics and biochemistry in the organism. The first genes to be linked to silencing were the SIR genes (SIR1, SIR2, SIR3, and SIR4). These genes were identified in genetic screens for mutations that derepress the HM loci (Rine and Herskowitz 1987). SIR1 appears to be important for establishment, but not maintenance, as its deletion, as describe earlier, leads to a partial loss of silencing yet the strain does appear to be able to maintain it once it has been established (Pillus and Rine 1989). SIR2, SIR3, and SIR4, on the other hand, appear to be crucial for the maintenance of HM silencing as deletions of these genes lead to a complete desilencing of the loci (Rine and Herskowitz 1987). Deletions of SIR2, SIR3, or SIR4 also lead to a complete loss of silencing at the

telomeres suggesting a general role in silencing while SIR1 deletions have no effect suggesting a more specialized role for it at HM (Aparicio et al. 1991).

Another gene that plays an important role in silencing is RAP1. RAP1 is an interesting gene because in addition to its role in silencing it also is involved in the activation of genes by binding to the UAS of many promoters. Rap1p also binds to the telomeres (Berman et al. 1986) and HM loci (Shore and Nasmyth 1987; Buchman et al. 1988). Removal of the RAP1 sites from the HM loci leads to a partial derepression of the silencer (Brand et al. 1987; Kimmerly et al. 1988) and rap1 mutants partially derepress HM silencing (Kurtz and Shore 1991) and fully abrogate telomere silencing (Kyrion et al. 1993). All this suggests that Rap1p binding to silent loci may lead to some of the specificity of silencing.

Other genes that affect silencing include the ORC genes and the N-terminal acetylases NAT1 and ARD1. The ORC proteins were originally identified by their ability to bind to the yeast origins of replication (Bell and Stillman 1992) but were later shown to bind to sites at the HM silencer (Bell et al. 1993; Foss et al. 1993; Micklem et al. 1993). Mutations in the ORC2 gene also lead to partial defects in HM silencing (Foss et al. 1993; Micklem et al. 1993). Together, these results suggest that ORC binding, like RAP1 binding, may play a role in the specificity of HM silencing. NAT1 and ARD1 are N-terminal acetylases and deletions of either results in desilencing at HM (Whiteway et al. 1987; Mullen et al. 1989) and the telomeres (Aparicio et al. 1991). Not much more is known however, how these acetylases affect silencing.

The interaction of these various genes plays a crucial role in silencing. Among the SIR genes themselves, two-hybrid experiments (Moretti et al. 1994) and protein affinity experiments show that Sir2p, Sir3p, and Sir4p associate (Moazed and Johnson 1996). Co-immuno-precipitation experiments show in vivo that Sir2p, Sir3p, and Sir4p interact, but that the Sir2-Sir3 interaction is Sir4p dependent (Strahl-Bolsinger et al. 1997) while the Sir2-Sir4 and Sir3-Sir4 interactions are probably direct (Moazed et al. 1997; Strahl-Bolsinger et al. 1997). This suggests that the Sir2-Sir3 interaction is largely mediated through Sir4p. This SIR complex appears to localize to the telomeres as witnessed by immunofluoresence experiments (Gotta et al. 1996; Gotta et al. 1997) and chromatin IP experiments (Hecht et al. 1996; Strahl-Bolsinger et al. 1997).

Although the Sir proteins do not directly bind to DNA themselves, there is evidence that they receive their locus specificity in part through their ability to interact with proteins that do bind DNA like Rap1p. Two-hybrid and protein binding experiments show that Rap1p and Sir3p interact directly (Moretti et al. 1994). To further illustrate the importance of this interaction, mutations in RAP1 that affect its ability to silence at the telomeres and HM also decrease its affinity for Sir3p (Moretti et al. 1994). Rap1p also co-localizes with Sir3p and Sir4p to the telomeres in immunofluorescence (Gotta et al. 1996) and chromatin IP experiments (Hecht et al. 1996). The interactions suggests a model where the Sir proteins form a complex that is brought to the telomeres and HM loci through ability of Rap1p to specifically bind to these loci (see Figure 2a,b). As RAP1

deletions only partially repress silencing at HM it is likely other proteins including the ORC complex and Sir1p (Triolo and Sternglanz 1996) are involved in recruiting the SIR2/3/4 complex to HM.

Once the SIR genes are brought to the telomeres or HM, chromatin plays a vital role in their ability to silence. The first indications that silencing and chromatin are strongly connected comes from genetic studies that have shown that deletion of or mutations in the N-terminal tail of histone H4 leads to derepression of the HM loci (Kayne et al. 1988). The observation that mutations in SIR3 can suppress silencing defective histone H4 mutants show a genetic interaction between the SIR genes and chromatin (Johnson et al. 1990). Studies of silencing at the telomeres have shown that mutations in histone H4 also derepress silencing there (Aparicio et al. 1991). Initially, it was thought that only histone H4 played a direct role in silencing but later studies have shown that mutations in histone H3 can also disrupt silencing (Thompson et al. 1994). The observation of the importance of histones in silencing has led to investigations into the physical nature of nucleosomes in silent chromatin. Nucleosome accessibility studies suggest that the nucleosomes at HMR are significantly less accessible than those at MAT (Chen et al. 1991). Site specific mutational analysis shows that lysines that are normally acetylated are important for silencing (Johnson et al. 1990; Megee et al. 1990; Park and Szostak 1990). This of course suggests the acetylation state of histones could play an important role in silencing. To test this, Braunstein and colleagues have used antibodies that specifically recognize

acetylated histones to show that the histones at the HM loci and telomeres are hypoaceyted compared to histones at non-silenced loci (Braunstein et al. 1993; Braunstein et al. 1996). Importantly, this hypoacetylation of histones is dependent on SIR2, SIR3, and SIR4 as deletion of any of these genes leads to levels of histone acetylation at the telomeres and HM seen in more actively transcribed regions (Braunstein et al. 1993; Braunstein et al. 1996). Interaction studies trying to understand how the histones and SIR complex work together to silence show that Sir3p and Sir4p can interact with the N-terminal tails of histone H3 and H4 although Sir2p and Rap1p do not (Hecht et al. 1995). When lysines 5, 8, 12, and 16 on histone H4 are mutated to glutamine, Sir3p no longer interacts with the histone tail. These lysines are known to be acetylated and mutating them to glutamine loosely mimics their aceylation. These results suggest a model where the SIR complex interacts with hypoacetylated histones at silent loci and thereby leads to a tightly packed heterochromatic structure that can silence genes.

Although in general there is a direct relationship between silencing and low levels of acetylation, studies with the yeast histone deacetylase RPD3 show the story is not so simple. As mentioned, RPD3 is involved not only in the repression of genes, but also in their activation (Vidal and Gaber 1991). RPD3 appears to play a role in derepression of telomere silencing as its deletion actually increases silencing there (De Rubertis et al. 1996). This is counter-intuitive as logically losing a histone deacetylase that represses promoters should decrease silencing, not increase it. One

explanation for this paradox suggests that a precise acetylation pattern is important for silencing. Studies of the exact residues that show decreases in acetylation at silent regions suggests that residues 5, 8, and especially 16 are unacetylated there while residue 12 is acetylated (Braunstein et al. 1996). Interestingly, Δrpd3 mutants show increased acetylation of residue 12 and 5 suggesting it has a preferences for those residues (Rundlett et al. 1996). Perhaps deacetylation of residue 12 by Rpd3p is partially countering silencing which would explain the increase in silencing that occurs when it is disrupted. On the other hand, because RPD3 does control transcription of a variety of genes, its desilencing activity could merely be an indirect effect. Micro-array analysis suggests that Δrpd3 strains have higher levels of histone expression and this could lead to increased levels of silencing (Bernstein et al. 2000). Time course experiments in the same study, however, suggest that the response time is too fast for indirect effects to be completely responsible.

Compared to what is known about HM and telomere silencing, rDNA silencing is a fairly new area, but an understanding of the factors are starting to take shape. The first link between the SIR genes and the rDNA came when it was observed that mutations in SIR2 lead to an increase in recombination at the rDNA array (Gottlieb and Esposito 1989). Later studies have shown that RNA polymerase II transcribed genes are partially silenced when located within the rDNA array (Bryk et al. 1997; Smith and Boeke 1997). In some ways it is counter-intuitive that silencing occurs at the rDNA as the rDNA genes are some of the most heavily transcribed in the genome.

Investigations into the SIR gene's role in rDNA silencing suggest that SIR2 is a central player there as it is the only SIR gene required for silencing at that locus (Bryk et al. 1997; Smith and Boeke 1997). Immuno-fluoresence and chromatin IP studies also show that SIR2 localizes to the rDNA (Gotta et al. 1997). Among the other SIR genes, deletion of SIR4 leads to an increase in rDNA silencing (Smith and Boeke 1997). This is probably a result of SIR2 being freed up from the telomeres and moving to the rDNA as the Δsir4 effect is SIR2 dependent and overexpressing SIR2 leads to a strengthening of rDNA silencing (Smith et al. 1998).

As SIR2 is the only SIR gene necessary for silencing at the rDNA it would be expected that a SIR2 complex separate from the SIR2/3/4 complex plays a role at the rDNA (see Figure 2c). The NET1 gene is able to interact with SIR2 but not SIR4 in GST pull downs (Straight et al. 1999). Disruption of NET1 leads to a loss of rDNA silencing and NET1 localizes with SIR2 to the rDNA (Shou et al. 1999; Straight et al. 1999). Highlighting the importance of this interaction, some mutations in SIR2 that affect rDNA silencing but not telomere silencing cannot bind NET1 and fail to localize to the rDNA (Cuperus et al. 2000). This second Sir2 containing complex is called the RENT complex and also contains Cdc14 and appears to play a role in exit from mitosis as well as silencing (Shou et al. 1999; Straight et al. 1999).

Other effects of SIR2

While the SIR genes seem to be primarily involved in silencing and chromatin, there are other processes they are involved in that may or may not be related to their roles in chromatin. One process is the DNA non-homologous end joining (NHEJ) reaction. SIR4 interacts with the Ku complex, a complex important in NHEJ (Tsukamoto et al. 1997). The Sir proteins also relocalize from the telomeres to sites of DNA breakage (Martin et al. 1999; Mills et al. 1999). In vivo, the deletions of the SIR genes can lead to less efficient ligation of broken DNA ends (Tsukamoto et al. 1997; Martin et al. 1999; Mills et al. 1999), but it is possible that this effect could be mediated through a/α expression that is a result of desilencing of the HM loci (Astrom et al. 1999; Lee et al. 1999). It is possible that the SIR genes are merely holding the Ku factors at the telomeres or it is possible that they have a more direct link to NHEJ. The observation that they relocalize to broken sites suggests that their role may be more complex than that of mere holding factors.

SIR2 levels also affect Saccharomyces life span. The life span of yeast that have no SIR2 are several fold shorter than yeast with wildtype SIR2 while expression of a single extra copy of SIR2 leads to a 40% increase in the yeast life span (Kaeberlein et al. 1999). A clue to how SIR2 is linked to life span came with the observation that it and NAD are necessary for life span extension by caloric restriction (Lin et al. 2000). It is also known that the retention of rDNA circles by mother cells can lead to aging (Sinclair and Guarente 1997) and, in light of SIR2's role in rDNA silencing and recombination, old Δsir2 cells show a faster rate of rDNA circle accumulation (Kaeberlein et al. 1999). Life span in other organisms is also

affected by SIR2 as seen in *Caenorhabditis elegans* where overexpression of the homologue SIR-2.1 increases life span by as much as 50% (Tissenbaum and Guarente 2001). This leads to the tantalizing prospect of SIR2 having universal effects on life span.

SIR2 and its homologues

Of the factors involved in silencing, SIR2 has received special interest in recent years for several reasons. As seen above, SIR2 is the only SIR gene critical for silencing at the telomeres, the HM loci, and the rDNA, making its role in silencing more universal. In addition, studies of acetylation and silencing have shown that SIR2 has stronger affects on the acetylation state of silent chromatin than the other SIR genes and its overexpression leads to global decreases in histone acetylation levels (Braunstein et al. 1993). Also, SIR2 is the most highly conserved of the SIR genes with homologues in organisms ranging from bacteria to humans (Brachmann et al. 1995).

Studies of SIR2 homologues have shown that in Saccharomyces itself there are four homologous genes called HST1, HST2, HST3, and HST4 (Brachmann et al. 1995). Disruptions of any one of these genes do not by themselves affect silencing but the genes do have subtle effects on silencing. HST1 is the most homologous to SIR2 and its overexpression can actually suppress a SIR2 disruption's defect in HM silencing. Also, the double deletion of HST3 and HST4 is defective for telomere silencing. The hst3/hst4 double mutant has several other phenotypes including an increase in UV sensitivity

and an increase in chromosome loss. This could suggest that HST3 and HST4 play a role in DNA repair or chromosome stability.

The in vivo function of the HST genes are not well understood but studies have shown that HST1 plays a role in transcriptional repression. HST1, along with the gene SUM1, is necessary for the repression of mid-sporulation specific genes (Xie et al. 1999). Unlike the regional affects of SIR2 on transcription, the effects of HST1 appear to be gene specific. HST1 and SUM1 appear to act through the mid-sporulation promoter element (MSE) that is known to be required for repression of mid-sporulation specific genes. Hst1p does not appear to bind to DNA but instead is probably recruited to the MSE by Sum1p. Interestingly, SUM1 was originally identified as a dominant mutation (SUM1-1) in screens for mutations can suppress Δsir defects in HM silencing (Klar et al. 1985; Lin et al. 1990). This ability to suppress deletions of the SIR genes is dependent upon HST1 suggesting that the SUM1-1 silencing allele acts by bringing the SIR2-like gene HST1 gene to the silent loci (Rusche and Rine 2001; Sutton et al. 2001).

As mentioned, SIR2 homologues are found in many species, however, relatively little is known about what their cellular functions are. Chromatin profiling techniques in Drosophila have tried to identify where a Drosophila SIR2 homologue localized (van Steensel et al. 2001). The results show that Sir2 does not strongly localize anywhere, although there are few loci that show a modest enrichment of Sir2. What does seem clear though is that Sir2 does not localize to heterochromatic regions making it much different from what is seen with yeast SIR2.

It is also unclear what the mammalian SIR2 homologues are doing, although a few experiments have tried to address this. Experiments that have replaced the yeast SIR2 gene with a human homologue called hSIR2A show the human version is unable to silence (Sherman et al. 1999). On the other hand, a chimeric protein with the human core domain in a yeast SIR2 gene is able to restore HM silencing in a Δsir2 strain suggesting some level of functional similarity between the genes. Ultimately though, the studies with the fly and human SIR2 homologue suggest we cannot assume that in organisms other than yeast that SIR2 plays the same role in silencing and heterochromatin.

Studies with CobB, a bacterial homologue of SIR2, have led to the first major breakthrough in discovering an enzymatic activity for SIR2. Mutations in CobB disrupt the biosynthetic pathway involved in cobalamin biosynthesis suggesting that it may play an enzymatic role in the pathway (Tsang and Escalante-Semerena 1998). This possibility was investigated by showing that extracts from cells overexpressing CobB are able to catalyze a phosphoribose transferase reaction that is in the cobalamin pathway.

The observation that a SIR2 homologue has an enzymatic activity suggests SIR2 homologues in eukaryotes might have a similar activity. CobB and a human homologue to SIR2 called SIRT2 can transfer an ADP-ribose moiety from NAD to BSA and thereby ADP-ribosylate the protein (Frye 1999). This raises the attractive possibility that SIR2 could be acting by modifying important chromatin factors through an ADP-ribosylation activity. When investigators examined this possibility they found that yeast Sir2p is

indeed capable of ADP-ribosylating histones (Tanny et al. 1999). Mutations that disrupt this enzymatic activity also lead to a defect in silencing. This suggests that Sir2p acts by ADP-ribosylating histones and this modification plays an important role in the chromatin structure. On the other hand, the ADP-ribosylation activity is relatively weak and ADP-ribosylation of histones has never been associated with silent chromatin in yeast. On top of that, no evidence has linked histone ribosylation to transcriptional repression. So many questions remain about the exact correlation between this enzymatic activity of SIR2 and how SIR2 works in the cell.

The focus of this project has been to understand the enzymatic activity of Sir2 and how it relates to the function of SIR2 in vivo. In the process of trying to understand the histone ADP-ribosylation activity of Sir2, we have discovered that SIR2 is capable of deacetylating histones in the presence of NAD and that this activity is more robust than the ADP-ribosylation activity. Given that histones at HM and the telomeres are hypoacetylated in a SIR2 dependent manner, people had long suspected that Sir2p may be a histone deacetylase, but no one had ever detected such an activity. The unexpected link had been that Sir2p would require NAD to deacetylate histones.

In light of the fact that histone acetylation levels are affected by mutations in SIR2 and that Sir2p can deacetylate histones in vitro, seems likely that the activity is relevant to the function of SIR2. The third chapter of this thesis describes mutations in the

core domain. Among the things we have discovered are that one of the mutations affects the in vitro activity of Sir2p, but does not affect the ability of SIR2 to silence. Also we found a mutant with the opposite effect, it does not affect the in vitro activity, but in vivo it is incapable of silencing. These two mutations tell us that we do not know everything there is to know about SIR2 and that its role in the cell may be more complicated than simply that of a histone deacetylase.

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FIGURES

Figure 1

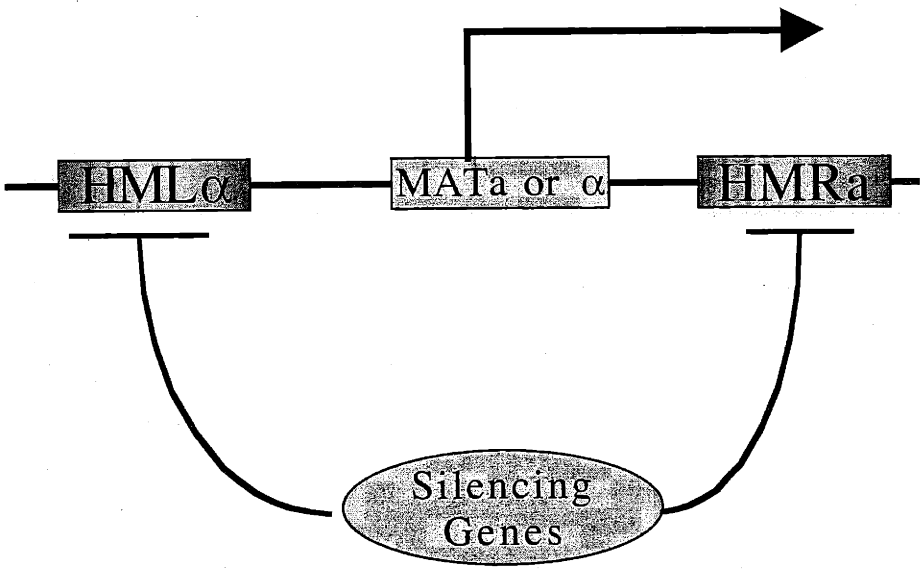
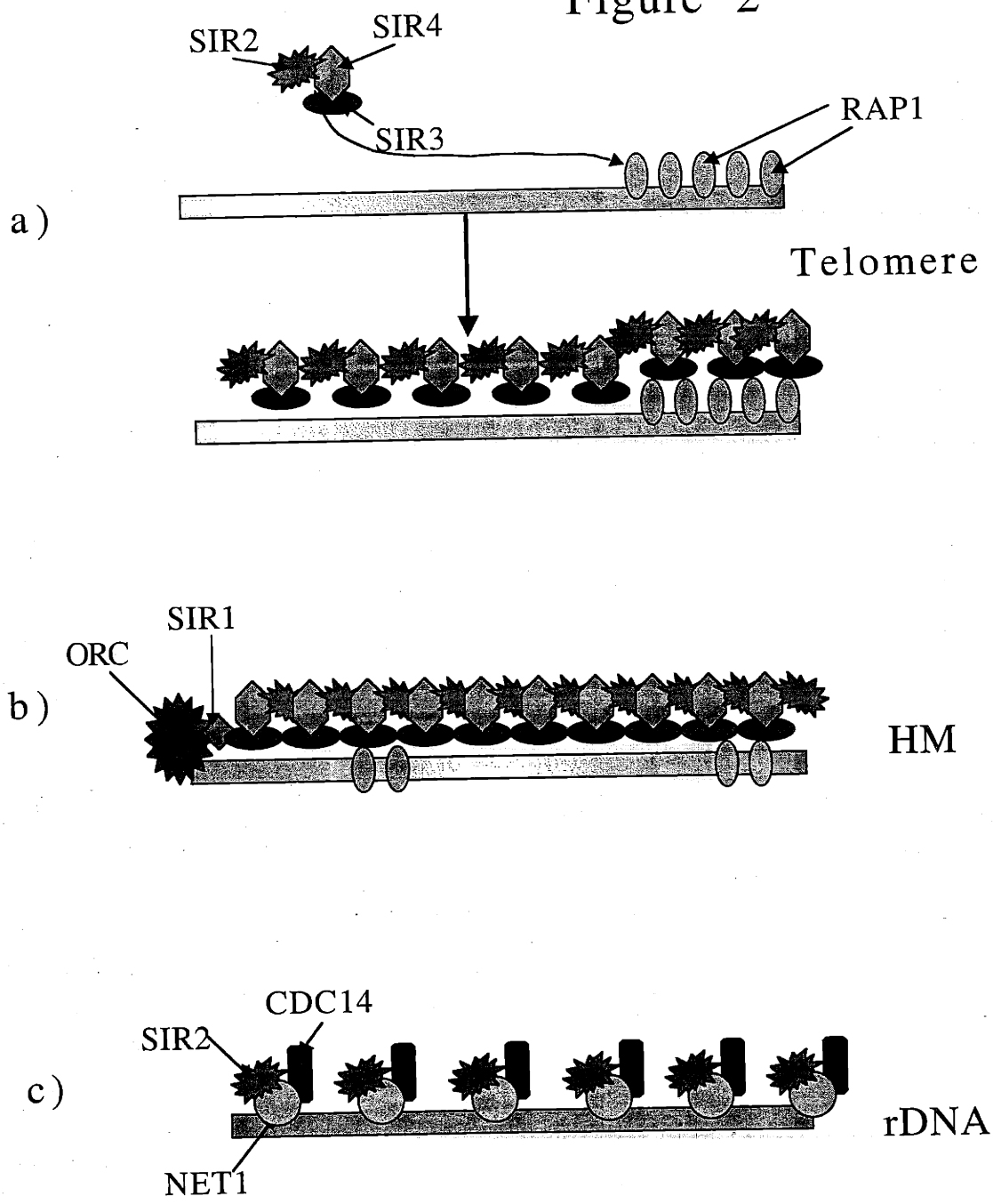


Figure 1. The HM and MAT loci in yeast. The MAT locus may contain either the a or α mating type genes and the yeast mating type is determined by which of the two is located there. HML contains the α mating type genes while HMR contains the a mating type genes, but their expression is suppressed by silencing genes. This way the cell can have both a and α mating type information but only expressed what is located at the MAT locus.

Figure 2. Interaction of Silencing Factors. a) Silencing at the telomeres. Rap1p recognizes RAP1 binding sites and binds to the telomeres. Sir3p binds to Rap1 and brings the SIR complex to the telomeres. The SIR complexes continue to come to the telomere and create a silent state. b) Silencing at the HM loci. Rap1p and the ORC complex bind to sequences at HM. Sir1p binds to ORC and the SIR complex is recruited to HM by Rap1p and ORC/Sir1p. The SIR complex then silences the HM loci. c) Silencing at the rDNA. Sir2 is recruited to the rDNA by the Net1p and silences there.

Figure 2


SIR2
SIR4
SIR3
RAP1
a)
Telomere
SIR1
ORC
b)
HM
CDC14
SIR2
c)
NET1
rDNA

Chapter 2

Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase

This chapter was previously published in *Nature*, Vol 403, pages 795-800, February 2000. The authors were Shin-ichiro Imai, Christopher M. Armstrong, Matt Kaeberlein, and Leonard Guarente. My contribution to this work included the making of the recombinant Sir2p used in the biochemical experiments, some of the biochemical assays, the making of the mutants and testing HM and telomere silencing.

Summary

Yeast Sir2p is a heterochromatin component that silences transcription at silent mating loci (Rine and Herskowitz 1987), telomeres (Gottschling et al. 1990), and the ribosomal DNA (rDNA) (Bryk et al. 1997; Smith and Boeke 1997), and also suppresses recombination in the rDNA (Gottlieb and Esposito 1989) and extends replicative life span (Kaeberlein et al. 1999). Mutational studies indicate that Lys16 in the amino-terminal tail of histone H4 and Lys9, 14, and 18 in H3 are critically important in silencing, while Lys5, 8, and 12 of H4 play more redundant functions (Thompson et al. 1994; Hecht et al. 1995; Braunstein et al. 1996). Lys9 and 14 of histone H3 and Lys 5, 8, and 16 of H4 are acetylated in active chromatin and hypoacetylated in silenced chromatin and overexpression of Sir2p promotes global deacetylation of histones (Braunstein et al. 1993; Braunstein et al. 1996), suggesting that Sir2p could be a histone deacetylase. Deacetylation of Lys16 of H4 is necessary for binding of the silencing protein, Sir3p (Hecht et al. 1995). Here we show that yeast and mouse Sir2 proteins are novel NAD-dependent histone deacetylases, which deacetylate Lys9 and 14 of H3 and specifically Lys16 of H4. Our analysis of two *SIR2* mutations supports the notion that this deacetylase activity accounts for silencing, recombination suppression, and extension of life span in vivo. These findings provide a molecular framework of NAD-dependent histone deacetylation that connects metabolism, genomic silencing and ageing in yeast and, perhaps, higher eukaryotes.

Results and Discussion

Sir2p is a limiting component promoting longevity in yeast mother cells. Cells lacking Sir2p have a reduced replicative life span and cells with an extra copy of *SIR2* display a much longer life span than wild type (Kaeberlein et al. 1999). This extension likely results from a hyper-silencing in the rDNA which reduces recombination and the production of extrachromosomal rDNA circles, a known cause of senescence in ageing mother cells (Sinclair and Guarente 1997). *SIR2* homologs have been identified in many organisms ranging from bacteria to humans (Brachmann et al. 1995). The Salmonella homolog, cobB, has been implicated in a pyrimidine transfer reaction (Tsang and Escalante-Semerena 1998) and both cobB and eukaryotic Sir2 proteins were shown to possess ADP-ribosyltransferase activity (Frye 1999; Tanny et al. 1999).

Because Sir2 proteins have been shown to use NAD as a substrate in a ADP-ribosylation reaction, we examined whether NAD could be a cofactor required for deacetylase activity. To test this possibility, we used purified recombinant Sir2p in a reaction with NAD and a peptide of the histone H3 amino-terminal tail (residues 1-20) di-acetylated at lysines 9 and 14. Thus, we reacted 5 μg of recombinant yeast Sir2p (79 pmoles) (Fig. 1a), 10μg of the H3 peptide (4.2 nmoles) and increasing concentrations of NAD and analyzed the products by high-pressure liquid chromatography (HPLC). As shown in Fig. 1b, the peptide reacted in the absence of NAD gave rise to two peaks (3 and 5) which were analyzed by electron-spray mass spectroscopy (see supplementary data) and correspond to monomer (MW2370) and dimer (MW4740) peptide, the

latter likely due to oxidation of the peptide at the carboxyl cysteine residue. The same species were observed in reactions with a control bacterial preparation ("pET" in Fig. 1a) in the presence of NAD (see Fig. 5a-D).

The addition of NAD to the reaction containing Sir2p gave rise to three additional peaks (1, 2, and 4), as well as an alteration in peak 3, which were also analyzed by electron-spray mass spectroscopy (Fig. 1c-f and supplementary data). Strikingly, these peaks did not correspond to ADP-ribosylated species, but, rather, to deacetylated species of peptide (Fig. 1g). Peak 4 corresponded to the singly-deacetylated dimer (MW4698), peak 3 now also contained the doubly-deacetylated dimer (MW4656), peak 2 corresponded to the triply deacetylated dimer (MW4614), and peak 1 to the singly-deacetylated monomer (MW2328). We estimate that at least 27% of the input peptide was deacetylated by Sir2p. The concentration of NAD at which the reaction proceeded to about 50% of the maximal level was about 100μM.

To analyze these reaction products further, peak 4, the singly deacetylated dimer, along with the input peak 5 were subjected to N-terminal protein sequencing by Edmann degradation (Fig. 2). The only differences between the input and reacted peaks occurred at lysines 9 and 14. 23-27% of the acetyl lysines at each position were deacetylated by Sir2p in the presence of NAD. The unacetylated lysine 18 of peaks 4 and 5 is also shown for comparison. NADH, NADP, and NADPH did not promote a significant level of deacetylation by Sir2p, and further, neither NADH nor NADP inhibited the activity of NAD in this reaction (see supplementary data). Thus, Sir2p is an NAD-dependent histone deacetylase which can deacetylate either lysine 9 or 14 of the H3 N-

terminal tail. The number of moles of peptide deacetylated greatly exceeds that of Sir2p, indicating that Sir2p catalyzes multiple reaction cycles.

We next examined the Sir2p deacetylase activity using a 20-residue peptide of the amino-terminus of histone H4 completely acetylated at Lys5, 8, 12, and 16 and containing a carboxyl cysteine. In the absence of NAD, this peptide gave rise to two prominent peaks (1 and 2) by HPLC corresponding to dimeric and monomeric peptides, respectively (Fig. 3a). Addition of NAD elicited a third prominent peak (3, Fig. 3b) corresponding to a singly deacetylated species, which was collected and analyzed by Edmann degradation. As shown in Fig. 3c-f, deacetylation selectively occurred at Lys16. About 32% of the Lys16 was deacetylated as opposed to less than 6% deacetylation at the other lysines. This specificity of Sir2p corresponds well with the primary importance of Lys16 of histone H4 in silencing.

We tested the effect of a potent inhibitor of histone deacetylases, trichostatin A (TSA) (Yoshida et al. 1990). As shown in Fig. 4a and b, TSA was totally incapable of inhibiting deacetylation by Sir2p at 400nM, a concentration known to inhibit histone deacetylases, including Rpd3 family members (Taunton et al. 1996). Consistent with its reported ADP-ribosyltransferase activity, we found that Sir2p transferred ^{32}P from NAD to intact histone H3 (Fig. 4c) or to the di-acetylated H3 peptide (Fig. 4d). Transfer of label to the peptide was assayed using thin layer chromatography (TLC), which revealed not only this transfer but also a substantial amount of NAD hydrolysis. We next tested a known inhibitor of mono-ADP-ribosyltransferases, coumermycin A1 (Banasik and Ueda 1994). Both the ADP-ribosylation (Fig. 4c and 4d) and NAD hydrolysis

(Fig. 4d) were inhibited by this drug at 200μM, a concentration known to inhibit other mono-ADP-ribosyltransferases. Strikingly, coumermycin A1 was *not* capable of inhibiting deacetylation of the H3 tail by Sir2p (Fig. 4e). Thus, the proposed activity of ADP-ribosyltransferase (as well as the NAD hydrolase) is fundamentally distinct from this NAD-dependent deacetylation activity.

We next characterized a mouse *SIR2* homolog termed *mSir2α* (GenBank accession number, AF214646). The conserved region of this protein resembles yeast Sir2p more closely than do other mouse Sir2 proteins (Fig. 1h and supplementary data). To determine whether the mouse Sir2p homolog mSir2α would catalyze this deacetylation reaction, we incubated purified recombinant mSir2α with the di-acetylated H3 peptide and analyzed the reaction products by HPLC (Fig. 1a and i). The murine protein gave rise to the same array of products with a similar yield as the yeast enzyme, suggesting that mammalian Sir2 proteins are also mediators of transcriptional silencing.

In order to determine whether the histone deacetylase activity of Sir2p is required for in vivo functions, two alanine mutations in highly conserved residues of the core domain (Gly-270 and Asn-345) were introduced by site-directed mutagenesis (Fig. 5a-A). 6XHis tagged proteins were purified from E. coli (Fig. 5a-B) and assayed for deacetylase activity with the di-acetylated H3 peptide (Fig. 5a-C-F). Mutant 345 was inactive, providing strong evidence that the deacetylase activity is an inherent property of Sir2p. Alternatively, mutant 270 showed a high level of activity, about 80% of wild type. These proteins were also analyzed for ADP-ribosyltransferase activity using the intact histone H3 as substrate (Fig.

5a-G). Mutant 345 was inactive and mutant 270 now displayed only a very weak activity, about 7% of wild type.

To examine the functions of these mutants in vivo, we used a yeast indicator strain in which the endogenous *SIR2* had been deleted and the wild type or mutant *SIR2* genes were integrated back into the genome. The mutant proteins were stably expressed (Fig. 5b-A). Silencing of the *HMLα* locus was assayed by mating with a haploid strain of opposite mating type and monitoring the appearance of diploids on selective media (Fig. 5b-B). Mutant 270 was mating proficient, indicating that silencing was intact, and mutant 345 was defective. Telomere silencing was determined by repression of the telomere-positioned *URA3* gene on media containing 5-fluoro-orotic acid (FOA) and this assay showed that mutant 270 had partial silencing and 345 was defective (Fig. 5b-C). Silencing in the rDNA was determined by repression of rDNA-positioned *ADE2* on adenine-lacking media, showing that mutant 270 silenced as well as the wild type and 345 was defective (Fig. 5b-D).

Recombination in the rDNA was assayed by loss of *ADE2* on media limiting in adenine, giving rise to a red pigment. Half-red/white sectored colonies indicate *ADE2* loss in the first generation after plating, and the frequency of these colonies compared to Ade+ colonies is a direct measure of the recombination rate in the rDNA. Wild type *SIR2* suppressed recombination about 12-fold compared to the *sir2* deletion strain, mutant 270 showed a high degree of suppression in the range of wild type, and mutant 345 was as defective as the *sir2* deletion (Fig. 5b-E). Replicative life spans were also determined as another measure of *SIR2* function in the rDNA (Fig. 5b-F). The *sir2* deletion shortened life span about 50% compared to wild type, as expected. Mutant 270

complemented the *sir2* deletion to give a wild type life span and mutant 345 was completely defective in complementing the life span defect.

Although NAD and NADH are frequent enzyme cofactors in oxidation/reduction reactions, this is the first example to our knowledge in which NAD drives a distinct enzymatic reaction, i.e. deacetylation by Sir2p of lysines in the amino-terminal tails of histones H3 and H4. Our findings are consistent with studies that show the importance of Lys9 and 14 in the tail of histone H3, and, most critically, Lys16 of H4, in silencing (Thompson et al. 1994; Hecht et al. 1995; Braunstein et al. 1996). We suggest that deacetylation by Sir2p of Lys16 of H4, and Lys9 and 14 of H3 is critical for silencing, while deacetylation of other lysines in these histone tails may be a passive consequence of residence in silent chromatin.

What is the relationship between histone deacetylation and ADP-ribosylation? It was recently reported that the ADP-ribosyltransferase activity of Sir2p was essential for silencing in vivo (Tanny et al. 1999). Backing this claim was the finding that Sir2 mutant H364Y loses ADP-ribosyltransferase activity in vitro and fails to support silencing in vivo. However, we find that mutation of this same histidine also greatly reduces the NAD-dependent deacetylase activity (not shown), much like our Asn-345 mutant. Moreover, the Gly-270 mutant, which is highly defective for ADP-ribosyltransferase but retains 80% of the wild type deacetylase activity, is largely proficient for silencing, recombination suppression, and life span extension in vivo. Therefore, we surmise that the NAD-dependent deacetylase plays a vital role in these Sir2 functions in vivo, while the ADP-ribosyltransferase does not. Sir proteins are known to move to sites of DNA breaks (Martin et al. 1999; Mills et al. 1999) to aid

their repair by non-homologous end joining (Tsukamoto et al. 1997; Boulton and Jackson 1998). We propose that the ADP-ribosyltransferase activity of Sir2p may function in DNA repair. Consistent with this hypothesis, the ADP-ribosylation of histones is known to occur when cells are treated with DNA damaging agents (Adamietz and Rudolph 1984; Kreimeyer et al. 1984; Pero et al. 1985) and antibodies against mono ADP-ribose react with mammalian nuclei only when cells are treated with DNA-damaging agents (Meyer and Hilz 1986).

The NAD requirement of Sir2p for deacetylation suggests that this protein may be a sensor of the energy or oxidation state of cells. It is interesting to note that the anti-aging regimen of caloric restriction is effective in extending life span in a variety of organisms, including yeast (Muller et al. 1980), C. elegans (Lakowski and Hekimi 1998), rodents (Weindruch et al. 1986), and probably primates (Roth 1999). We speculate that the slower metabolic rate in calorically restricted cells may exert part of its effect by increasing the availability of NAD, which, in turn, up-regulates the deacetylation activity of Sir2 proteins and chromatin silencing. This persistence of genomic silencing may slow aging-related processes, such as genome instability and inappropriate gene expression.

METHODS

Production of recombinant proteins

The yeast *SIR2* gene or the *mSir2α* full-length cDNA was engineered to be cloned into pET28a vector (Novagen, WI). Site-directed mutations were generated in the plasmid pRS305-SIR2 using the Gene Editor system

(Promega, Madison, WI) according to the procedure provided by the manufacturer. Sequences were verified by Sanger sequencing methods. The mutants were then subcloned into pET28a. BL21(DE3) and BL21(DE3)pLysS with an extra copy of arginine tRNA gene was transformed with the *SIR2* and *mSir2α* plasmids, respectively. Each transformed bacterial clone was induced in 1mM IPTG at 37^{0}C for 1hr. The induced 6XHis-tagged proteins were purified with Ni-NTA agarose under native conditions. The control eluate was prepared from a bacterial clone carrying pET28a vector only. The recombinant proteins were aliquoted and kept at –70^{0}C.

Deacetylation and ADP-ribosylation assays

The typical reaction of Sir2 deacetylase activity was performed in 50 µl of buffer containing 50mM Tris-HCl [pH 9.0], 4mM $MgCl_2$, 0.2mM DTT, variable concentration of cold nicotinamide adenine dinucleotide (NAD) or NAD derivatives (SIGMA, MO), 5-10µg of the purified recombinant Sir2 proteins, and 10µg of the N-terminal tail peptide of histone H3 (amino acid 1-20+Cys) ARTKQTAR(AcK)STGG(AcK)APRKQLC di-acetylated at positions 9 and 14 or of *Tetrahymena* histone H4 (amino acid 2-19+Cys) AGG(AcK)GG(AcK)GMG(AcK)VGA(AcK)RHSC tetra-acetylated at positions 5, 8, 12, and 16 (Upstate Biotechnology, NY). The starting peptide material of H3 contains a contaminant with 100 Da smaller molecular weight, which also showed exactly the same patterns of deacetylation (data not shown). To detect the ADP-ribosylation activity, 8µCi of NAD 5'-[α-^{32}P]triphosphate (~1000Ci/mmol, Amersham Pharmacia Biotech, NJ) was added to the same reaction containing 1µM cold NAD. 4µg of intact histone H3 protein (Roche Molecular Biochemicals, IN) was also used for

this assay. All reaction mixtures were incubated at room temperature for 1hr. Trichostatin A and coumermycin A1 (SIGMA) were prepared in dimethylsulfoxide (DMSO, SIGMA), and 5μl of solvent or inhibitor was added to the reactions prior to adding Sir2 proteins.

Analysis of deacetylated or ADP-ribosylated products

After the incubation, the products were precipitated at -20^{0}C overnight by adding 50μl of distilled water and 25μl of 100% trichloroacetic acid (TCA) solution. For high pressure liquid chromatography (HPLC), the precipitates were reconstituted in 5% CH_3CN and 0.1% trifluoroacetic acid (TFA) and run in the gradient concentration between 0.05% TFA and 0.043% TFA plus 80% CH_3CN on Hewlett Packard Model 1100 HPLC system with 214TP52 column (VYDAC, CA). The chromatograms at the absorbance of 210nm were digitally recorded and analyzed by Hewlett Packard ChemStation system (version A.06.03[509]). Fractions of samples were collected every 1min by Gilson Fraction Collector Model 203. Peptide sequencing was done by the Applied Biosystems Procise 494 HT protein sequencing system. PTH amino acid chromatograms were recorded and analyzed by the ABI Model 610A2.1 data integration/analysis system. Electron-spray mass spectroscopy was done on the PE Sciex Model API365 system. The mass spec data were analyzed by the BioMultiView program (version 1.3.1). To detect the ADP-ribosylated intact H3 protein, the pellets were dissolved in 15μl of Leammli's sample buffer, boiled for 1.5min and electrophoresed in a 10-20% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue to check equal loading of histones, dried, and exposed to Kodak X-OMAT film. To analyze the ADP-ribosylated H3 peptides, 10μl of each

reaction mixture was spotted on a cellulose TLC plate (EM Science, NJ). The chromatography was performed for 9-10hrs in a TLC chamber containing the 65:5:3:2:29 mixture of isobutyric acid, pyridine, acetic acid, butanol and water. The plate was dried and exposed to Kodak X-OMAT film. The peptide spots were checked by ninhydrin staining.

Molecular cloning of mSir2α

The mouse 15-day embryo 5'-STRETCH PLUS cDNA library (Clontech, CA) was screened with the EST cDNA fragment of AA199012 as a probe. Five positive clones were obtained from approximately one million independent plaques. One of the five clones contained a 3.9kb cDNA fragment. The nucleotide sequence of this fragment was determined with an Applied Biosystems 374 automated sequencer. We concluded that the cDNA clone we isolated encodes the full-length mSir2α protein, because the in vitro-translated protein from this cDNA clone showed an indistinguishable size (110-120kD) to the protein in the mouse NIH3T3 extract recognized by a specific polyclonal antibody against a N-terminal portion of mSir2α (data not shown). The amino acid sequences of mSir2α and other *SIR2* family members were aligned in the Clustal X program and a phylogenetic tree was generated by using the NJPLOT program (supplementary data).

Strains, plasmids, and antibodies

All strains used were derivatives of W303a *sir2Δ*: W303R *sir2Δ(MATa, ade2-1, leu2-3,112, trp1-1, ura3-52, his3-11, sir2::TRP1, rDNA-ADE2)*, W303RT *sir2Δ(MATa, ade2-1, leu2-3,112, trp1-1, ura3-52, his3-11, rad5-535, sir2::TRP1, rDNA-ADE2, TELVIIL-URA3)*, and W303R

sir2Δ/rpd3Δ(MATa, ade2-1, leu2-3,112, trp1-1, ura3-52, his3-11, rdp3::URA3, sir2::TRP1, rDNA-ADE2). pRS305-SIR2, an integrating plasmid that contains *SIR2* driven by its native promoter, was used. Mutant *SIR2* genes were also cloned into these vectors. *SIR2* and mutant *sir2* strains were generated by cutting the plasmid within the *LEU2* gene and integrated using standard yeast transformation protocols. Rabbit antibodies to Sir2p were generated by using full-length recombinant Sir2p purified under denaturing conditions.

Silencing, life span and rDNA recombination assays

To test silencing at the telomeres and rDNA, 10-fold dilutions of the derivatives of either W303RT or W303R *Δrpd3* were spotted on media containing 5-FOA or media lacking adenine, respectively. To assay for HM silencing, W303R derivatives were patched onto YPD with the tester strain CKy20 (*MATα, arg1, tsm11*) and after overnight growth were replica plated to minimal media with no supplemented amino acids. Life span and rDNA recombination rates were measured as in Kaeberlein, et. al. (Kaeberlein et al. 1999).

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FIGURES

Figure 1. In vitro deacetylation assays of the H3 peptide (residues 1-20) di-acetylated at lysines 9 and 14 by recombinant yeast Sir2p. **a**, Coomassie blue-stained gel after SDS-PAGE of purified recombinant yeast (rSir2p) and mouse Sir2 (mSir2α) proteins. Full length proteins are indicated by dots. Vector controls (pET) were prepared analogously to recombinant proteins. **b-f**, HPLC chromatograms showing absorbance at 220 nm of products of deacetylation assays with yeast Sir2p and the indicated concentrations of NAD. The efficiencies of the reactions are calculated as a fraction of the areas under peaks 1, 2, and 4 compared to the area under all of the peaks. **g**, Scheme of contents of peaks 1-5 shown in chromatograms. From the relative abundance of peaks, it is likely that the dimer peptide is a better substrate for Sir2p than the monomer peptide. **h**, mSir2α, yeast Sir2p (ySir2p), and CobB are schematically compared. Core domains are shown as shaded boxes with the percentages of amino acid identity to the ySir2p core domain. **i**, The HPLC chromatogram of the product of deacetylation assay with 10μg of recombinant mSir2α protein at 1mM NAD. The calculated efficiency of the reaction is indicated.

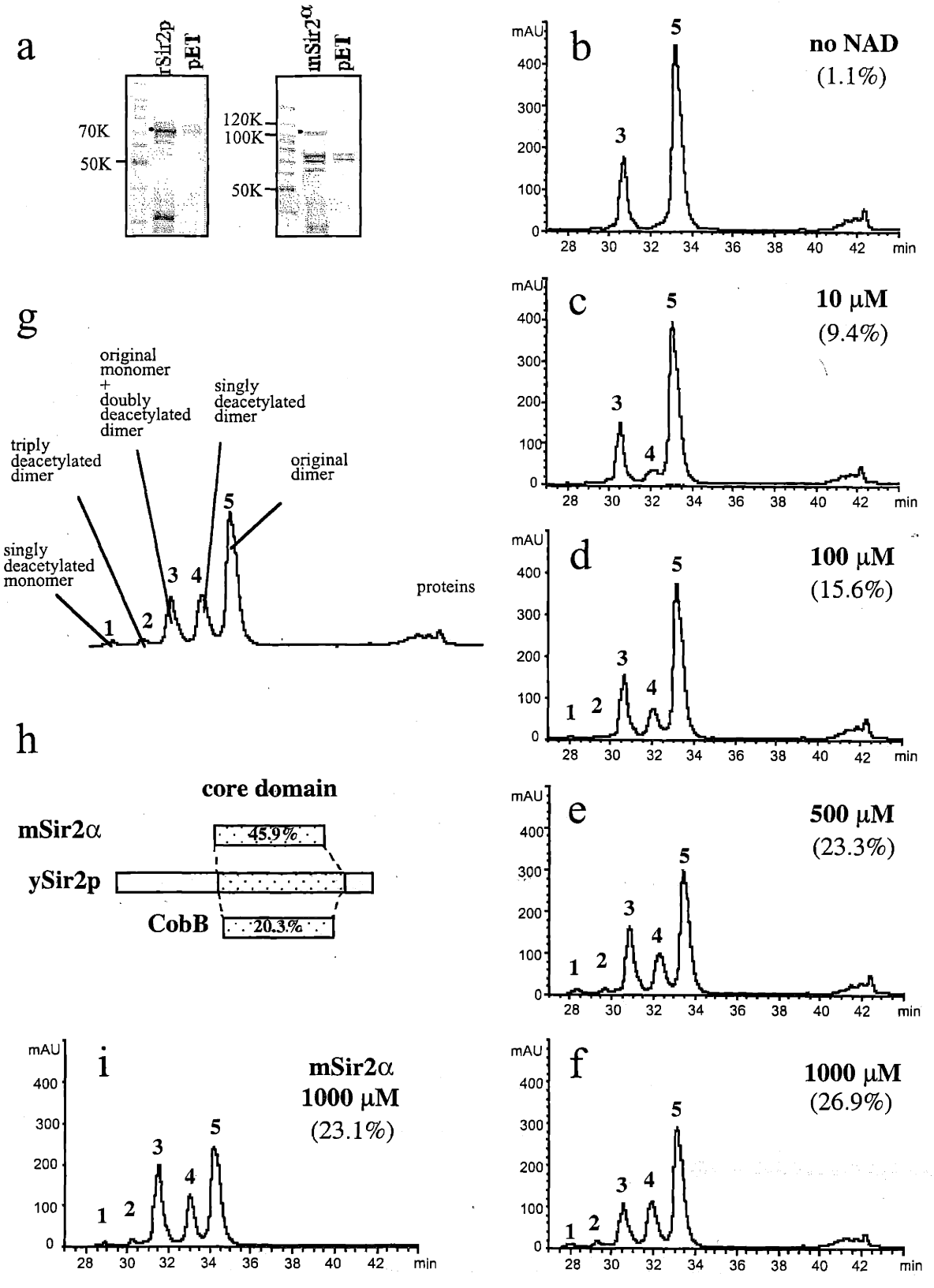

a
rSir2p
pET
mSir2α
pET
70K
50K
120K
100K
50K
b
no NAD
(1.1%)
c
10 μM
(9.4%)
d
100 μM
(15.6%)
e
500 μM
(23.3%)
f
1000 μM
(26.9%)
mAU
min
g
original monomer + doubly deacetylated dimer
singly deacetylated dimer
triply deacetylated dimer
original dimer
singly deacetylated monomer
proteins
h
core domain
mSir2α
45.9%
ySir2p
CobB
20.3%
i
mSir2α
1000 μM
(23.1%)

Figure 2. Amino-terminal sequencing of peaks 4 and 5 of the Sir2p deacetylase reaction at 1 mM NAD as determined by Edmann degradation. Chromatograms at positions 9, 14, and 18 are shown. In peak 4, about 23% of Lys9 (**a**) and 27% of Lys 14 (**b**) are deacetylated. In peak 5, both Lys9 and 14 are essentially all acetylated (**d** and **e**). The unacetylated Lys18 of both peaks 4 and 5 are shown for comparison (**c** and **f**). The peak to the right of the acetylated lysine at position 14 corresponds to the chromatographic position of alanine, and represents preview of Ala15 in cycle 14.

Figure 2

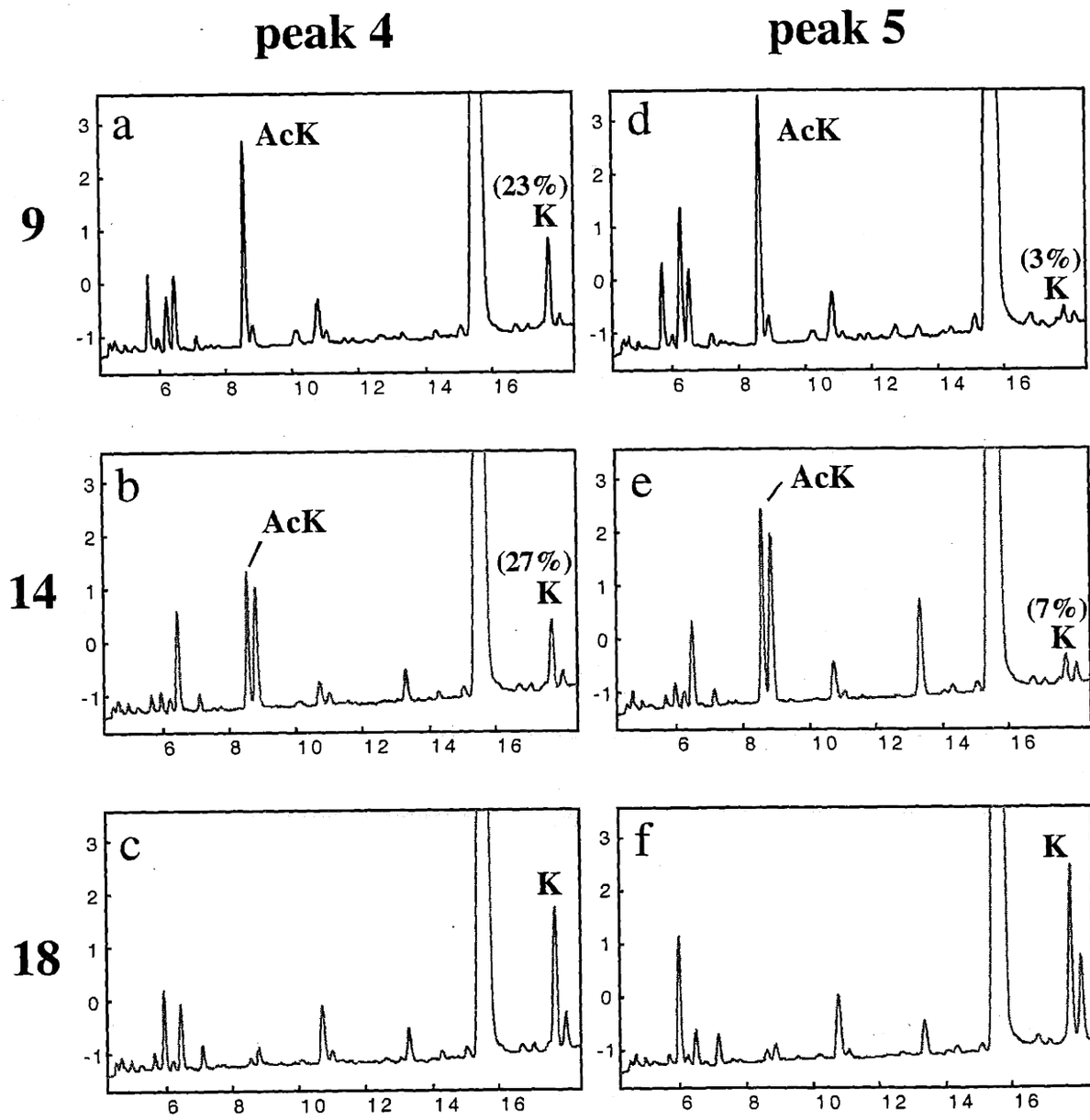

peak 4
peak 5
9
14
18
a
AcK
(23%)
K
d
AcK
(3%)
K
b
AcK
(27%)
K
e
AcK
(7%)
K
c
K
f
K
3
2
1
0
-1
6
8
10
12
14
16

Figure 3. The deacetylation activity of yeast Sir2p on the H4 peptide (residues 2-19) tetra-acetylated at lysines 5, 8, 12, and 16. **a-b**, HPLC chromatograms of products of deacetylation assays with Sir2p and the indicated concentrations of NAD. The efficiencies of the reactions are calculated from the areas under peaks. Peaks 1 and 2 correspond to the dimeric and monomeric forms of the input peptide. Peak 3 corresponds to the deacetylated product. **c-f**, Amino-terminal sequencing of peak 3. Chromatograms of positions 5, 8, 12, and 16 are shown. Substantial deacetylation (32%) is observed for only Lys16. The sum of deacetylation of all four residues, about 40%, is less than the theoretical 50% expected for the singly deacetylated dimeric peptide, most likely due to contamination of peak 3 by peak 2. As a control, peak 1, corresponding to the original dimer peptide, shows minimal deacetylation at positions 5, 8, 12, and 16 - 0.1%, 0.1%, 0.7%, and 0.5%, respectively.

Figure 3

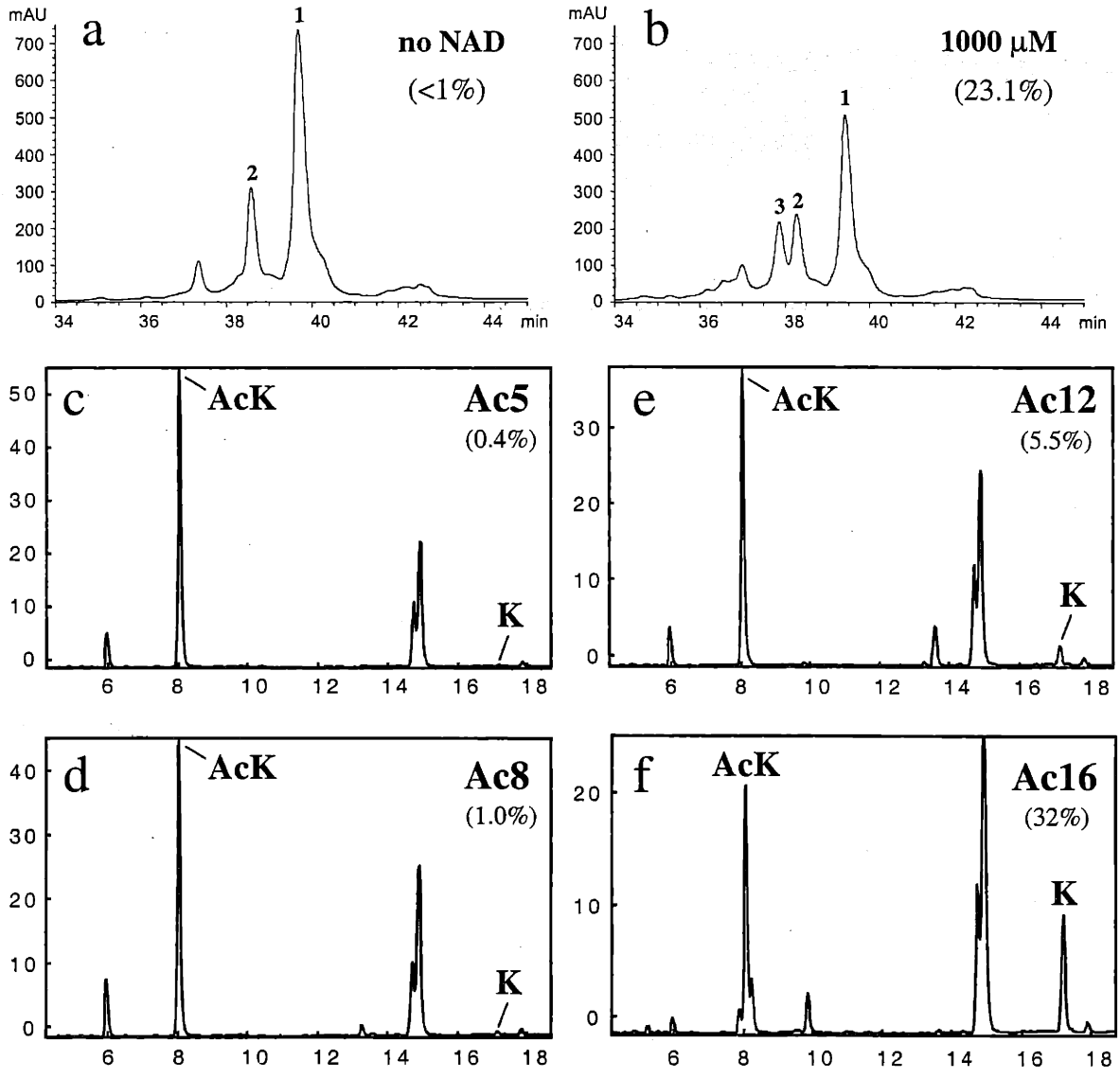

a
no NAD
(<1%)
b
1000 μM
(23.1%)
mAU
min
c
AcK
Ac5
(0.4%)
K
d
AcK
Ac8
(1.0%)
K
e
AcK
Ac12
(5.5%)
K
f
AcK
Ac16
(32%)
K

Figure 4. Effects of inhibitors on the deacetylase and ADP-ribosyltransferase activities of recombinant Sir2p (rSir2p). The HPLC chromatograms of deacetylation reactions at 1mM NAD in the presence of solvent only (**a**), 400nM trichostatin A (TSA) (**b**), and 200μM coumermycin A1 (Coumer) (**e**) are shown. The calculated efficiencies of the reactions are indicated. The effect of 200μM coumermycin A1 on ADP-ribosylation at 1μM NAD of the intact histone H3 (**c**) and the H3 peptide (**d**) are examined on SDS-PAGE and TLC, respectively. pET corresponds to the vector control. In **d**, the ADP-ribosylated peptide is indicated by a bracket on a longer exposure (left), and NAD and its hydrolysed product are indicated by an arrow and an arrowhead, respectively, on a shorter exposure (right).

Figure 4

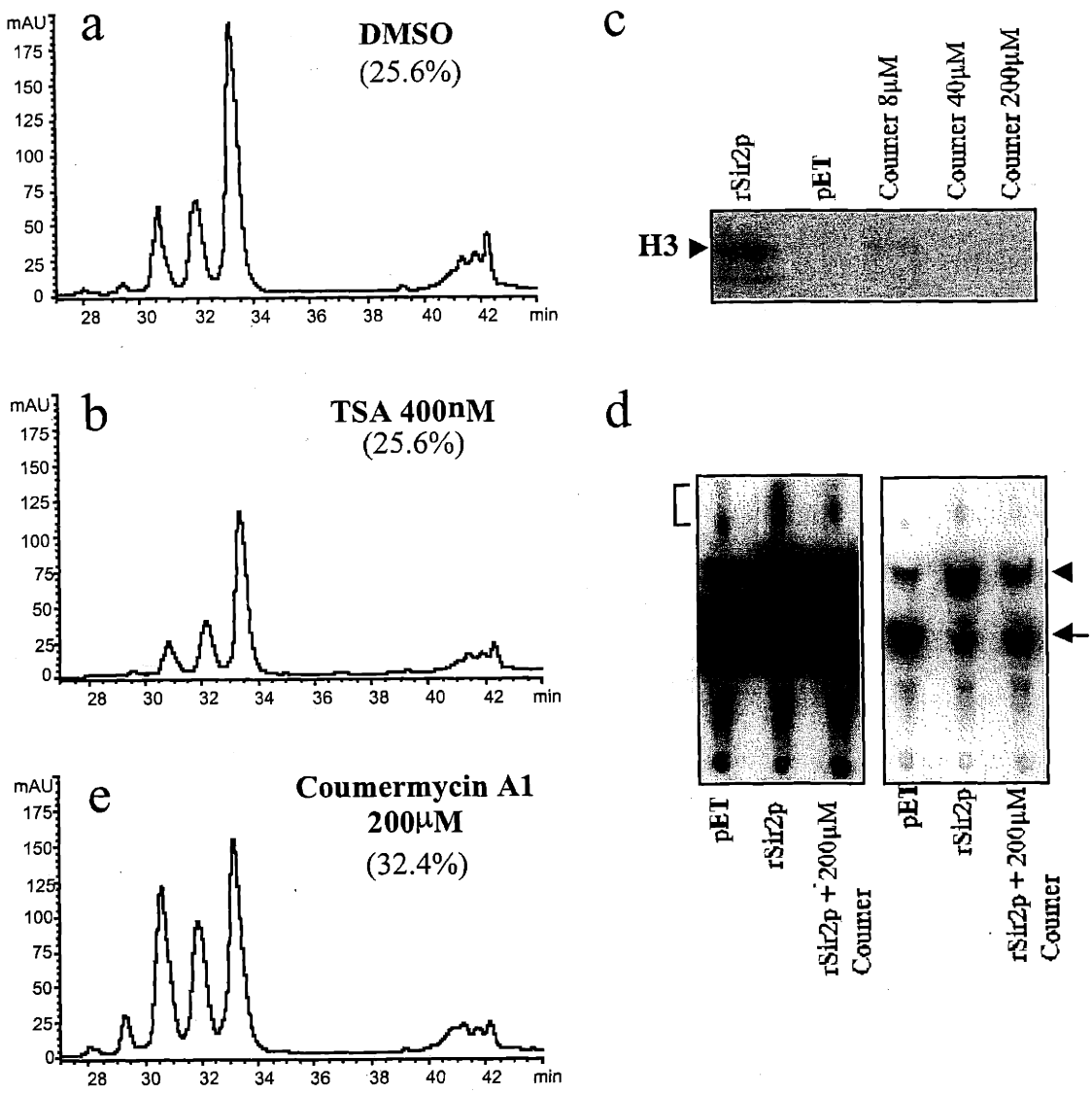
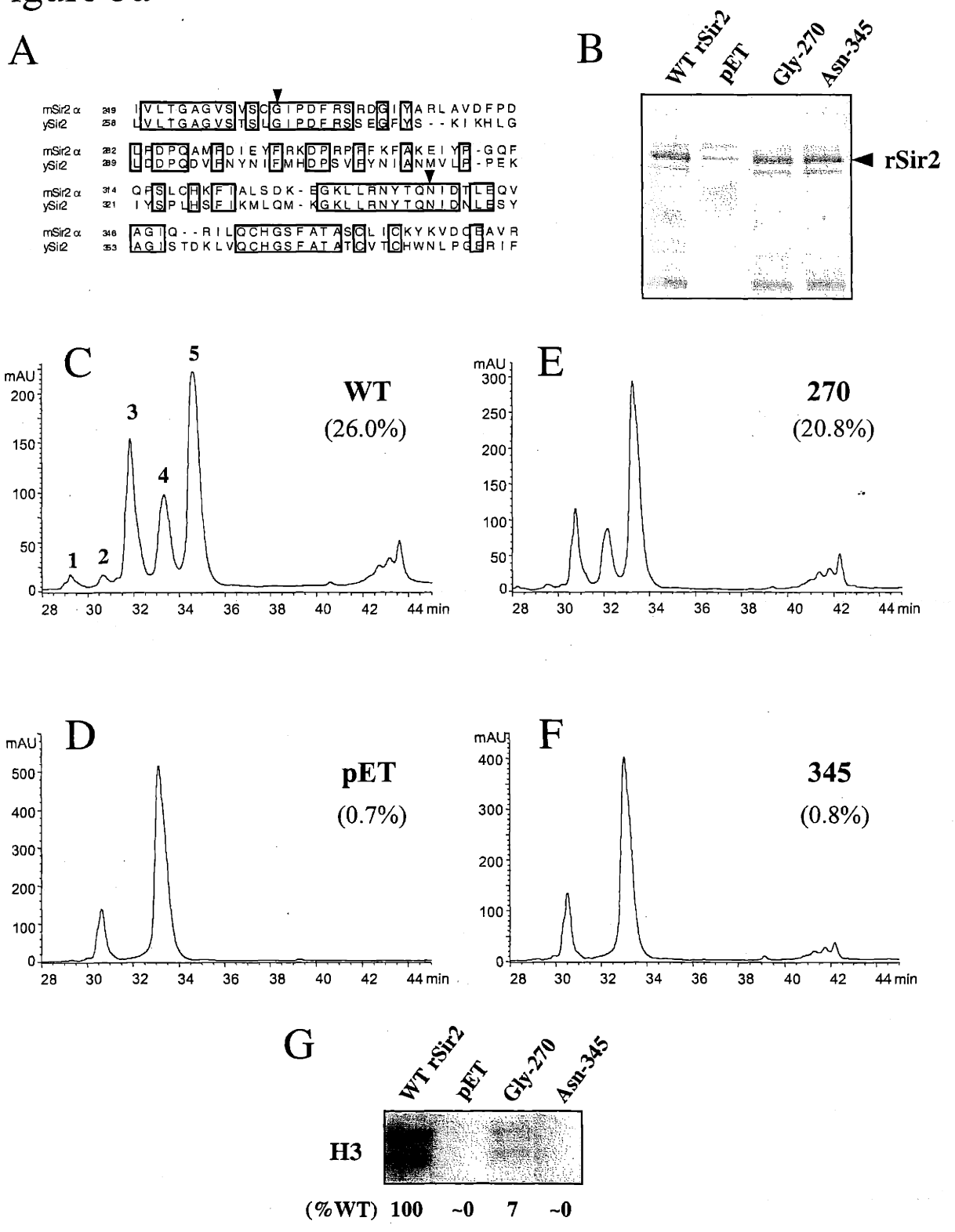

a
DMSO
(25.6%)
mAU
175
150
125
100
75
50
25
0
28
30
32
34
36
38
40
42
min
b
TSA 400nM
(25.6%)
e
Coumermycin A1
200μM
(32.4%)
c
rSir2p
pET
Coumer 8μM
Coumer 40μM
Coumer 200μM
H3
d
pET
rSir2p
rSir2p + 200μM Coumer
pET
rSir2p
rSir2p + 200μM Coumer

Figure 5 Deacetylation activity of Sir2p is essential for silencing, recombination suppression, and life span extension in vivo. **a**, In vitro assays of Sir2 enzymatic activities. **A**, Two highly conserved residues (Gly-270 and Asn-345) between yeast Sir2p and mSir2α (indicated by arrowheads) were mutated to alanine. **B**, 6XHis tagged versions of wild type and mutant Sir2 proteins along with a vector control (pET) were expressed in E. coli, purified over a Ni-NTA column, and run on a 7% polyacrylamide SDS gel stained with Coomassie blue. **C-F**, Effects of mutations in Sir2p on NAD-dependent histone H3 deacetylation activity. Indicated recombinant Sir2 proteins and a vector control were incubated with a peptide corresponding to the di-acetylated amino-terminal tail of histone H3 and 1mM NAD. HPLC chromatograms at 220 nm absorbance and efficiencies of the reactions are shown. **G**, Effects of mutations in Sir2p on ADP-ribosylation activity. 1μg of the purified proteins were tested for the ability to ADP-ribosylate intact histone H3 with ^{32}P labeled NAD. The lower band corresponds to a degraded histone product present in the preparation. Reaction efficiencies determined by quantitation in the Phosphorimager are shown below. **b**, In vivo assays for *SIR2* functions. **A**, Western blotting on 25 μg of yeast whole cell extracts from wild type, *sir2Δ* and the two mutants was performed using an anti-Sir2 antibody. The upper band corresponds to Sir2p and a lower background band is included as a loading control. **B**, Silencing at *HMLα* was tested by mating the strains with a tester strain of the opposite mating type and monitoring growth of diploids on selective media. **C**, Telomere silencing was assayed by the ability of strains with telomeric *URA3* to grow on media containing 5-FOA, which is toxic when *URA3* is expressed, but harmless when *URA3* is silenced. Strains were spotted in 10-fold dilutions

on media lacking FOA (complete) and containing FOA. **D**, rDNA silencing was monitored in a strain with the *ADE2* marker located within the rDNA array. *RPD3* was disrupted to enhance silencing differences between the wild type and *sir2Δ* strains. *sir2* mutant strains were spotted in 10-fold dilutions on complete and adenine minus media to monitor effects on silencing. **E**, W303R with *ADE2* at the rDNA was tested for rDNA recombination rates by counting the number of half-sectoring colonies lacking the marker in the first generation after plating. **F**, The mother cell life span of wildtype, *sir2Δ*, and mutants 270 and 345 were determined in *hmlΔ* strains in which **a**/α effects are eliminated.

Figure 5a


A
mSir2α
ySir2
B
WT rSir2
pET
Gly-270
Asn-345
rSir2
C
WT
(26.0%)
mAU
min
D
pET
(0.7%)
E
270
(20.8%)
F
345
(0.8%)
G
WT rSir2
pET
Gly-270
Asn-345
H3
(%WT) 100 ~0 7 ~0

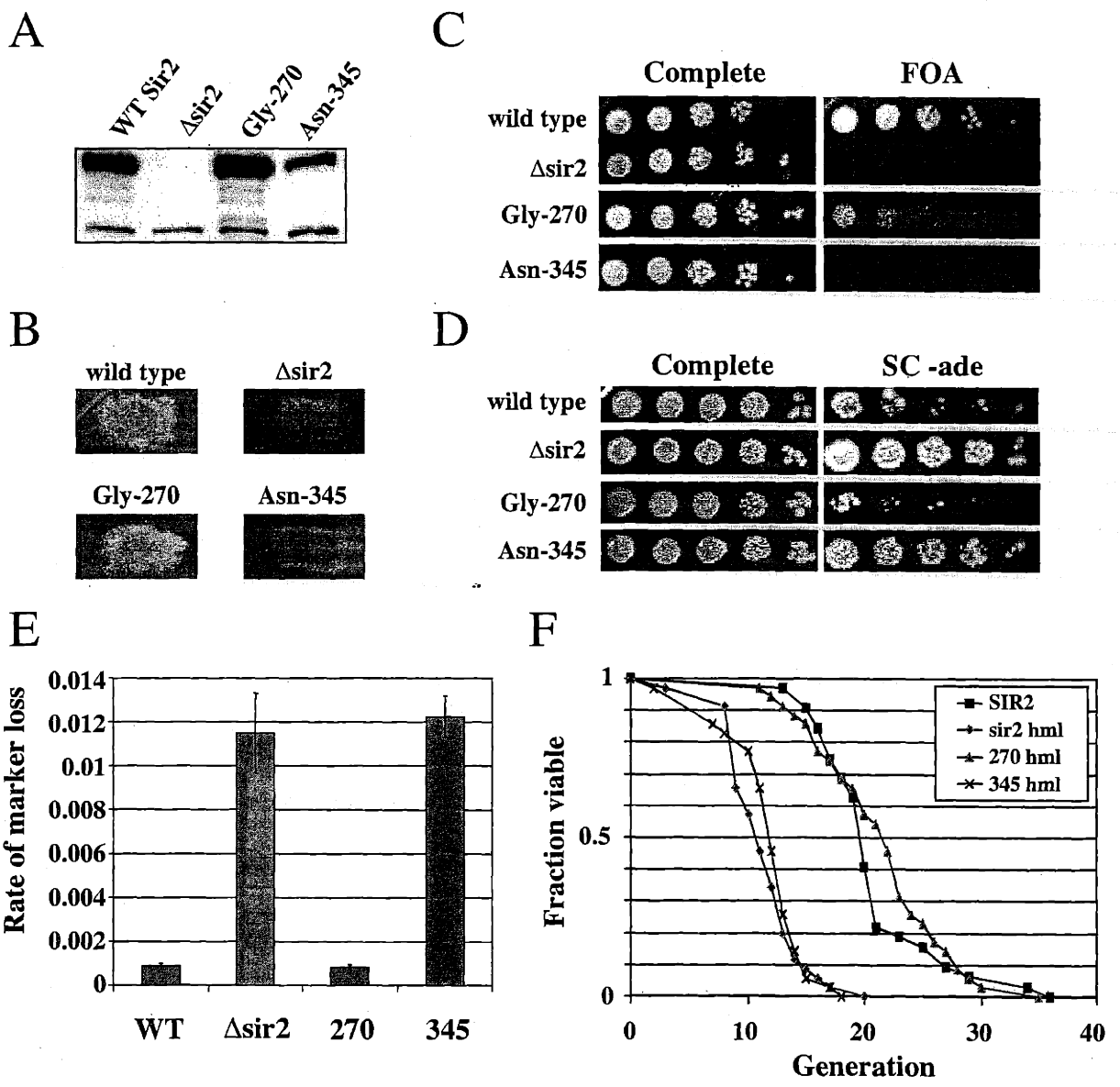

A
WT Sir2
Δsir2
Gly-270
Asn-345
B
wild type
Δsir2
Gly-270
Asn-345
C
Complete
FOA
wild type
Δsir2
Gly-270
Asn-345
D
Complete
SC -ade
wild type
Δsir2
Gly-270
Asn-345
E
Rate of marker loss
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
WT
Δsir2
270
345
F
Fraction viable
1
0.5
0
0
10
20
30
40
Generation
SIR2
sir2 hml
270 hml
345 hml

Figure 5b

Chapter 3

Effects of mutations in SIR2 on histone deacetylation and silencing

SUMMARY

The yeast SIR2 gene and many of its homologues have been identified as NAD+ dependent histone deacetylases (Imai et al. 2000; Landry et al. 2000b; Smith et al. 2000). To get a broader view of the relationship between the histone deacetylases activity of Sir2p and its in vivo functions we have mutated 8 highly conserved residues in the core domain of SIR2. These mutations have a range of effects on the ability of Sir2p to deacetylate histones in vitro and to silence genes at the telomeres and HM loci. Interestingly, there is not a direct correlation between the in vitro and in vivo effects in some of these mutations. We also show that the histone deacetylase activity of Sir2p is necessary for the proper localiztion of the SIR complex to the telomeres.

INTRODUCTION

SIR2 affects processes as wide spread as silencing (Rine and Herskowitz 1987), recombination (Gottlieb and Esposito 1989), DNA repair (Tsukamoto et al. 1997), and aging (Kaeberlein et al. 1999). SIR2 was first identified with the other SIR genes in a screen for mutations that derepress the silent mating type loci (HM) (Rine and Herskowitz 1987) and later shown to silence markers located in the telomeres (Aparicio et al. 1991). SIR2 is also the only SIR gene that is necessary for silencing at the rDNA (Bryk et al. 1997; Smith and Boeke 1997).

Clues to the functions of SIR2 and the other SIR genes first came from genetic studies that linked HM silencing to chromatin. Mutations in the N-terminal tail of histone H3 and H4 can derepress silencing at HM and the telomeres (Kayne et al. 1988; Park and Szostak 1990; Aparicio et al. 1991; Thompson et al. 1994). In addition, the histones in silenced regions of the genome are hypoacetylated in a SIR dependent manner (Braunstein et al. 1993; Braunstein et al. 1996). These observations emphasize an important connection between the SIR genes, the deacetylated state of silent chromatin, and the ability to repress. SIR2 appears to play an especially important role in this regard as its overexpression lead to decreases in global acetylation levels of histones (Braunstein et al. 1993).

The first in vitro clue for an activity came when CobB, a bacterial homologue to SIR2, was shown to be involved in

transferring a phospho-ribose moiety from Nicotinate mononucleotide (NaMN) to 5,6-dimethylbenzimidazole (Me_2Bza) (Tsang and Escalante-Semerena 1998). It was then discovered that the human homologue to SIR2 could use NAD+ to ADP-ribosylate protein (Frye 1999) and that yeast SIR2 could ADP-ribosylate histones, albeit inefficiently (Tanny et al. 1999). Sir2p was then shown also to deacetylate histone tails in an NAD dependent manner in a robust fashion (Imai et al. 2000). Related studies showed that HST2, a yeast genes that has sequence homology to SIR2, and CobB were also capable of deacetylating histones in an NAD+ dependent manner (Landry et al. 2000b; Smith et al. 2000).

Recent investigations into the mechanism of the histone deacetylation activity of Sir2 suggest that it is linked to the hydrolysis of NAD+ (Landry et al. 2000a; Tanner et al. 2000; Tanny and Moazed 2001). These two activities are coupled in a reaction that transfers the acetyl group from histones to the ADP-ribose from NAD+ and forms an 1-O-acetyl-ADP-ribose moiety. It is currently speculated that this deacetylation reaction requires high-energy yielded from the hydrolysis of NAD+ (Tanny and Moazed 2001) or that the 1-O-acetyl-ADP-ribose moiety itself could have a signaling function in the cell (Tanner et al. 2000).

NAD+ could also play a vital link between SIR2 and aging. Deletions of SIR2 decrease yeast lifespan while overexpressing SIR2 leads to an extension (Kaeberlein et al. 1999). SIR2 is also necessary for the increase in lifespan in caloric restricted yeast and that NAD+ levels are crucial for this increase (Lin et al. 2000). When the *Caenorhabditis elegans* homologue of SIR2 Sir-2.1 is overexpressed,

the worm's lifespan is increased by as much as 50% (Tissenbaum and Guarente 2001).

In order to understand this gene that affects so many processes, we have mutated SIR2 and investigated its effects in vitro and in vivo. Previous studies have shown that mutations in SIR2 can affect both the enzymatic activity and its silencing phenotypes (Tanny et al. 1999; Imai et al. 2000). Other studies have shown that mutations outside the most conserved part of SIR2 create locus specific alleles that silence either the telomeres or the rDNA but not both (Cuperus et al. 2000). In this study we mutated residues in the highly conserved core domain of SIR2 to try to better understand the relationship between the in vivo phenotypes and its in vitro enzymatic activity of SIR2.

MATERIAL AND METHODS

Yeast strains, Plasmids and Antibodies

Yeast strains are listed in Table 1. pRS305-SIR2, an integrating plasmid that contains SIR2 driven by its native promoter, was used. Mutant sir2 genes were also cloned into these vectors. SIR2 and mutant sir2 strains were generated by cutting pRS305-SIR2 within the LEU2 gene at an Afl II site and integrated using standard yeast transformation protocols. SIR2 or mutant sir2 cloned into the pET28a vector was used for the production of recombinant protein. The HA-tagging of SIR4 was done with the pSF323-SIR4-3XHA vector (a gift from Steve Bell) which integrates a tagged version of SIR4 into the native SIR4 locus. Rabbit antibody to Sir2p and Sir3p have been

previously described (Mills et al. 1999; Imai et al. 2000). The 12CA5 antibody to the HA epitope was obtained from Covance and the acetylated histone H3 and acetylated histone H4 were obtained from Upstate Biotechnology.

Site directed Mutagenesis of SIR2

Site directed mutants were generated in pRS305-SIR2 as per Imai et. al., 2000 and subcloned into pET-28a (Imai et al. 2000). The mutations were sequenced to insure that the mutagenesis was successful. Expresion of Sir2p in yeast was monitored by western blot analysis of whole cell extracts probe with anti-Sir2p antibody.

Purification of Recombinant Protein and Enzymatic Assays

6-his tagged recombinant Sir2p and mutant Sir2p were purified from BL21 bacteria that overexpressed the gene on a pET28a plasmid as described previously (Imai et al. 2000). ADP-ribosylation of histones was detected as described previously (Imai et al. 2000). Histone deacetylation activity was measured using a peptide corresponding to the N-terminal tail of Histone H4 (SGRGKGGKGLGKGGAKRHRC) labeled with tritiated acetate using the Histone Deacetylase Assay Kit from Upstate Biotechnology. The assay was performed by incubating 2 μg of recombinant protein with the labeled peptide in 1mM NAD overnight. Ethyl Acetate was then used to separate acetyl groups freed by the reaction from those still bound to the peptide. Deaceytlation activity was then measured by counting the free triated acetate in a scintilation counter.

Silencing and rDNA recombination Assays

To test silencing at the telomeres, 10-fold dilutions of the derivatives of W303RT were spotted on media containing 5-FOA. To assay for HM silencing, W303R derivatives were patched onto YPD with the tester strain CKy20 and after overnight growth were replica plated to minimal media with no supplemented amino acids. rDNA recombination rates were measured as previously described (Kaeberlein et al. 1999).

Immuno-precipitation of HA-SIR4 and SIR2

Whole cell extracts were prepared from cells grown in 100ml of YPD to an OD of 1.0 (Strahl-Bolsinger et al. 1997). 200 μl of extract was diluted to 500 μl with lysis buffer to which 3 μl of anti-HA antibody was added and incubated at 4° overnight. Protein A beads were then added and further incubated at 4° for 1 hour. The beads were washed 3 times with lysis buffer and then boiled in 60 μl of SDS running buffer. 10 μl were run on a 7.5% PAGE gel for western blotting analysis.

Chromatin Immuno-precipitation

Yeast were grown in 100 ml of YPD to and OD of 1.0. Immuno-precipitation of cross-linked extract was performed essentially as described (Strahl-Bolsinger et al. 1997), using 2.5 μl of anti-SIR3 polyclonal antibody or 5.0 μl of anti-SIR2 polyclonal, anti-acetylated histone H3 antibody, or anti-aceytlated histone H4 antibody. PCR analysis of immuno-precipitated DNA was performed in 50 μl

reaction volumes using 1:25, 1:75, and 1:225 of the total immuno-precipitated DNA. PCR reaction conditions were as described using the following primers: TEL-300.fwd—GGATATGTCAAAATTGGATACGCTTATG, TEL-300.rev—CTATAGTTGATTATAGATCCTCAATGATC, TEL-3000.fwd—TGATTCTGCTTTATCTACTTGCGTTTC, TEL-3000.rev—AGAGTAACCATAGCTATTTACAATAGG, XV-internal2.fwd—GTAGTTCGTTAGGTATGGACATTGATTTGGCC, XV-internal2.rev—AAATGAAATGTATTGGGGCCTAGGTTCGCA. Slot blot analysis was performed by blotting 10 μl of IP DNA or 5 μl of input DNA to a Zeta-Probe® membrane using a Bio-Rad slot blot apparatus. The blot was then probed with a 32P labeled DNA fragment corresponding to the 5S rDNA sequence.

RESULTS

Mutations in the core domain of SIR2 affect enzymatic activity

We chose 8 amino acid residues that are absolutely conserved between yeast SIR2, mouse SIR2α, and CobB for mutational analysis (figure 1a). We mutated the following residues in the yeast SIR2 gene to alanine: Thr-261, Gly-270, Ile-271, Phe-274, Arg-275, Asn-345, Asp-347, and His-364. Each mutant was expressed in bacteria and purified over a Ni-column (figure 1b).

To test the effect of these mutations on the NAD+ dependent histone deacetylation activity of Sir2p, we performed a histone

deacetylation assay in the presence of NAD+ by incubating the recombinant proteins with an histone H4 peptide with tritiated acetyl groups and counting the amount of tritium that was freed in the reaction (figure 2a). The mutations fell into 3 different categories based on their ability to deacetylate. The first category including the most C-terminal mutations, Asn-345, Asp-347, and His-364, destroyed the histone deacetylation activity of Sir2p. The second category, Arg-275, showed almost no loss of histone deacetylation activity, releasing about 1900 cpm of acetate compared to wildtype's 2400 cpm. The third category including the most N-terminal core domain mutants Thr-261, Gly-270, Ile-271, and Phe-274, drastically decreased the histone deacetylation activity, yet did not completely obliterate it, with activities ranging from 5% to 20% the level of wildtype.

We also investigated the ADP-ribosyl transferase activity of a subset of the mutants (261, 270, 271, 275, 345, and 347) (figure 2b). Once again the mutations appear to fall into 3 different categories. The most C-terminal mutations (345 and 347) had no noticeable level of activity. Mutation 275 was as robust as wildtype. The N-terminal mutations (261, 270 and 271) had levels of activity that were dramatically weakened, but higher than the non-activity of mutants 345 and 347 or the empty vector control. The effect of mutations on ADP-ribosylation correlated well with their effects on deacetylation.

Mutations affect in vivo functions of SIR2

We integrated wildtype SIR2, empty vector, and each of the mutants into a Δsir2 strain. We performed western blots on yeast whole cell extract and saw that each of the mutants expresses at levels comparable to the wild-type allele suggesting that none of the mutations alters the stability of the protien in vivo (figure 3a).

To test silencing at the HM loci, we crossed each mutant with a mating tester strain to see if they could successfully mate (figure 3b). Wildtype, mutants 261, 270, and 271 formed diploids while the other strains failed to mate suggesting that mutants 261, 270 and 271 silence the HM loci while mutant 274, 275, 345, 347, and 364 are unable to silence.

To investigate silencing at the telomeres, we used a strain in which the URA3 marker has been integrated into the telomere at the left arm of chromosome VII. When SIR2 is functional, the URA3 gene is repressed and the strain can grow on media containing 5-fluoro-orotic acid (5-FOA), a toxic substrate to cells that express the URA3 gene. We measured telomere silencing in the sir2 mutant strains by spotting the mutants onto 5-FOA media (figure 3c). The mutants fell into 3 categories based on their ability to silence telomeres. The first class, mutants 271, 274, 275, 345, 347, 364, were incapable of silencing the URA3 marker. The second class, mutant 261, grew as well as wildtype suggesting no defect in telomere silencing. The third class, mutant 270, showed very limited but noticeable growth on 5-FOA suggesting it could partially silence the marker.

SIR2 suppresses recombination at the rDNA and silences markers integrated there. To investigate how mutations affect the

role of SIR2 at the rDNA, we measured the recombination rate in a strain with the ADE2 marker in the rDNA array by counting marker loss in half-sectoring colonies (figure 3d). Mutants 274, 275, and 345 had a rate of marker loss comparable to the sir2 disrupted strain indicating they had lost the ability to suppress rDNA recombination. Mutants 261 and 270 had low rates of marker loss comparable to wildtype. Finally mutant 271 was intermediate with twice the rate of marker loss as wildtype, but 5 times lower than a sir2 deletion.

Overall the mutants fell into three phenotypic categories. The first class, which only contained mutant 261, showed the same phenotypes as the wild-type allele. The second class, mutants 270 and 271, showed some ability to silence as well as wildtype in some assays, yet they were either partially or completely defective in silencing at other loci. The final class, mutants 274, 275, 345, 347, and 364, was incapable of silencing at any locus tested.

As mentioned earlier, the mutations also affected Sir2p's enzymatic activities. Interestingly, there was not perfect correlation between the in vivo and in vitro effects for some of the mutants. There were, in particular, two puzzling mutants, mutant 261 and mutant 275. Mutant 261 had a weak enzymatic activity, yet appeared to be as strong as wild type in all of its in vivo phenotypes. Alternatively, mutant 275 had near wildtype levels of activity in vitro, but was totally defective in vivo.

Acetylation State of histones in vivo

Because mutant 261 showed low activity in vitro despite behaving like wild-type in vivo and mutant 275 had the opposite effect, we determined the acetylation state of silent chromatin by chromatin immuno-precipitation (ChIP). After cross-linking, we used both anti-acetylated H3 and anti-acetylated H4 to pull down the acetylated histones that had been cross-linked to DNA. After reversing the cross-linking we probed for telomere sequences by using primers that specifically recognize the telomere on the right arm of chromosome VI (figure 4a). In strains with a wild-type allele of SIR2, very little telomeric sequence was pulled down as compared to a sir2 disruption. This result was expected, as regions that are silenced tend to be hypo-acetylated in a SIR-dependent manner (Braunstein et al. 1993; Braunstein et al. 1996). Histones at the telomeres in mutant 261 were hypoacetylated much like wild-type while those in mutant 275 were hyper-acetylated much like the sir2 disruption.

We also checked histone acetylation levels at the rDNA by blotting the ChIP DNA to a filter using a slot blot apparatus and then probing the blot for rDNA. The blot showed the same pattern we saw at the telomeres, hypoacetylation in wild type and mutant 261, hyper-acetylation in Δsir2 and mutant 275 (figure 4b). Thus, for these unusual mutants, the histone acetylation state correlated with the observed degree of silencing.

Mutations are dominant negative and do not affect formation of a Sir2p/Sir4p complex

A mutation that affects the enzymatic activity of Sir2 has been shown be dominant negative in vivo (Tanny et al. 1999). This led us to test if our mutations were dominant negative. We integrated each mutant into a wild type strain with URA3 at TEL-VIIL so that each strain would have both a wild type and mutant copy of SIR2 present. We spotted the cells on FOA media to test their ability to silence the telomere (figure 5a). We saw that mutants 271, 274, 275, 345, 347, and 364 were unable to silence the telomere even though a wild type copy of SIR2 was present. This is interesting and suggests that even though these mutants fail to silence, they are probably forming a SIR complex, but the mutants are poisoning the complex in such a way as to inhibit its ability to silence.

Because of the dominant negative results and the possibility that mutant 275 may fail to deacetylate histones at the telomeres because it is unable to form the SIR complex in vivo we wanted to see directly if some of these mutants form a proper SIR complex. It is known that Sir2p and Sir4p interact directly and co-immuno-precipitate from whole cell extracts (Moazed et al. 1997; Strahl-Bolsinger et al. 1997). To test whether these mutants form the SIR complex, we tagged the native SIR4 gene with HA in SIR2 wild type, Δsir2, mutant 261, mutant 275, and mutant 347 strains. We then isolated whole cell extract from these strains and used anti-HA antibody to pull down Sir4p. HA-Sir4p was precipitated from all of the strains as seen by western blot (figure 5b). We then re-probed the western to see if Sir2p co-precipitated (figure 5c). Sir2p co-precipitated from wild-type extract but not from extracts with untagged SIR4. Moreover, Sir2p was co-precipitated with Sir4p in all

three mutant extracts. This suggests that all mutant Sir2p tested assemble with Sir4p.

Mutations affect Sir complex's ability to localize to the telomeres

While the mutants appear to form the SIR complex, it is possible they fail to localize to the telomeres or other silenced loci. To investigate this possibility we immuno-precipitated with anti-Sir3p antibody from extracts prepared after cross-linking and probed the co-precipitated DNA using PCR with primers that recognize sequence around 300 bp from the end of the right arm of chromosome VI (figure 6a). As expected, a wild type strain supported immuno-precipitation of the telomeric sequence while a Δsir2 strain did not. Extracts from the sir2 mutants 261 and 270 gave a comparable signal to wild type. However, in the remaining mutants: 271, 274, 275, 345, 347, and 364, the telomere signal was absent, indicating Sir3p does not localize to the telomeres. We observed the same pattern using anti-Sir2 antibody (data not shown). As a control for specificity, we re-probed the immuno-precipitated DNA with primers corresponding to internal chromosomal sequences and saw the same low level of pull down from all of the extracts (figure 6b). This localization data shows a strong correlation between the silencing activites at telomeres and the ability of the Sir complex to localize properly in the mutant strains. Because mutant 270 only partially silences the telomeres, we checked more distal regions of the telomere by probing

sequences that are 3000 bp from the end (figure 6c). The SIR complex in mutant 270 localized much more poorly than wild type at this distal site suggesting that this mutant SIR complex is weakened in its ability to further polymerize along the telomeres.

At the rDNA, Sir2 interacts with a novel set of proteins termed the RENT complex while the other SIR genes do not have a direct effect on the rDNA (Shou et al. 1999; Straight et al. 1999). We determined whether Sir2p was localizing to the rDNA in the mutant strains. The DNA from anti-Sir2 ChIPs was blotted to a filter and then probed for rDNA sequences (figure 6d). As expected DNA from a wildtype strain contained rDNA sequences while DNA from a sir2 disruption did not. Surprisingly, all of the mutants localized to the rDNA, even though their ability to elicit hypoacetylation of histones and repress recombination varied at that site. This finding contrasts to telomeres where there is a strong correlation between the silenced state and the localization of the Sir proteins.

DISCUSSION

In this paper we investigated the effect of core domain mutations on the in vitro enzymatic activity and in vivo phenotypes of SIR2. A simple model would have predicted that mutations that affect the histone deacetylase activity of Sir2p would also affect the ability of SIR2 to silence, and further, that the most severe mutations would show the strongest de-silencing phenotypes. While most mutations do indeed affect the in vivo and in vitro functions of SIR2 in similar ways, two of the mutations showed unexpected

differences between in vivo and in vitro activity. A summary of the mutations and their effects in vivo and in vitro is listed in Table 2.

Correlations between mutations effects on enzymatic activity and silencing phenotypes

When we examined the effects the mutations had on the enzymatic activity of Sir2p and the mutations fell into 3 categories: totally inactive, partially active, and fully active. The first class of mutants had three members, mutants 345, 347, and 364. When we looked at the effect these mutations had in vivo, we saw that none of them were capable of silencing in any of the assays tested, even though they properly formed complexes and properly localized to some chromosomal loci.

The only mutant showing full enzymatic activity is mutant 275. Surprisingly, this mutant was incapable of silencing in any of the in vivo assays. This finding defies simple explanation, as you would expect a mutation that does not affect the ability of Sir2p to deacetylate histones in vitro to be able to deacetylate chromatin and silence the appropriate regions. The mutant may, however, have a subtler defect that only manifests itself in vivo.

The other class of mutants: 261, 270, 271, and 274, partially disrupted the enzymatic activity of Sir2p showing anywhere from 5% to 20% of wildtype levels of histone deacetylase activity. Of these only mutant 274 was completely inactive in all of the in vivo assays tested. Mutants 270 and 271 may not have silenced at wild type levels because their enzymatic activity was reduced by 5 to 10

fold. Mutant 261, however, was exceptional because it had even lower activity than mutants 270 and 271, yet it had no noticeable silencing defects.

The Paradoxes of Mutants 261 and 275

The lack of a direct correlation between the histone deacetylation activity and the silencing phenotypes of mutants 261 and 275 suggest that the role of Sir2p is more complex than that solely of a histone deacetylase. One explanation would be the in vitro histone deacetylation activity of SIR2 is not crucial for silencing. Instead, perhaps in vivo the relevant substrate for the deacetylation activity of Sir2p is something other than histones. By this scenario mutant 261 would weaken activity for histones but not for the other substrate while mutant 275 would have the opposite effect. Lending support to this possibility is the observation that Hst2p appears to provide most of the NAD+ dependent histone deacetylase activity in cell extracts (Smith et al. 2000). New evidence has also suggested that SIR2 homologues can deacetylate proteins other than histones (Imai, Park, and Guarente, unpublished data and (Sutton et al. 2001). On the other hand to say this histone deacetylation reaction is just an in vitro artifact ignores what has been observed about SIR2 and histone acetylation. For one hypoacetylation of histones at silent loci is dependent upon SIR2 which would make sense if SIR2 was a histone deacetylase. Also Sir2p preferentially deacetylates residue 16, the residue that plays the most important role in silencing (Imai et al. 2000; Tanny and

Moazed 2001). These observations strongly support the idea that Sir2p has histone deacetylase activity in vivo.

If Sir2p is deacetylating histones in vivo then how can we explain the effects of mutants 261 and 275? Mutant 275 may create a functional defect unrelated to its enzymatic activity such as an inability to interact with other proteins. When we did co-IP experiments with Sir4p we saw that Sir2p and Sir4p interacted in mutant 275 strains suggesting that the mutation did not disrupt that crucial interaction. It does not, however, rule out the possibility that another protein interaction is disrupted by mutant 275. In mutant 261, it is possible that the mutation causes a folding defect in E. coli that does not exist in yeast. Purification of the mutant from yeast extract, however, showed that it was still a poor histone deacetylase making that explanation unlikely (Armstrong unpublished data). It is possible that the limited activity that mutant 261 possesses is adequate to silence in vivo but this would seem unlikely in light of the results with mutants 270, 271, and 275. Another reason that could explain both mutants might be a difference in how they interact with chromatin as opposed to histones. Mutant 275 is a very effective deacetylase with histone peptides as the substrate, but perhaps it is unable to deacetylate nucleosomes in the full chromatin context that is seen in the cell. On the other side perhaps mutant 261 is far more efficient at using full chromatin as the substrate. While difficult to test, such a possibility could explain the unusual nature of these mutations.

SIR2 Mutations and their effects on localization

We were also curious about what effect the mutations would have on where the SIR complex localized. Mutants that failed to silence at the telomeres also failed to localize there. This included mutant 275 along with the mutations that render the enzyme inactive. Is the mutants' failure to silence a result of their failure to localize? This could explain why mutant 275 does not silence. However, all of the mutants localize to the rDNA regardless of their ability to affect rDNA recombination. If mutant 275 were not silencing merely because it failed to localize then one would expect mutant 275 to be able to successfully suppress rDNA recombination yet it cannot.

Conversely it seems plausible that the failure of these mutants to deacetylate could lead to their inability to localize to the telomeres. This implies a model in which Sir2p first deacetylates the histones and then the SIR complex tightly binds to the telomeres forming a fully functional silencing complex. This fits observations that showed that Sir3p and Sir4p were capable of binding to the tails of histone H4 (Hecht et al. 1995). Mutants 345, 347 and 364 lend the strongest support to the model because they are completely incapable of deacetylating histones in vitro and they fail to localize to the telomeres. If telomere localization were independent of the acetylation state, then these mutants should localize properly. The failure of the SIR complex in mutant 275 to localize to the telomeres could also be a byproduct of the mutant's failure to deacetylate in vivo. In light of the observation that Sir3p and Sir4p bind to histone tails, others have suggested that a silenced state can be

achieved in part by the binding of the Sir proteins to the unacetylated histone tail to create heterochromatin (Wu and Grunstein 2000). If this is true then the telomere localization data suggests that the deacetylation of histones by Sir2p is necessary for the SIR complex to polymerize along the telomere and form proper heterochromatin. Even if histone deacetylation is a crucial step for the proper localization of the SIR complex to the telomeres, it is not necessary for Sir2p to localize to the rDNA. This suggests another difference between silencing at the telomeres and the rDNA on top of the different complexes that Sir2p interacts with at the two loci.

The Structure of SIR2

The crystal structure of the *Archaeglobus fulfidus* homologue to SIR2 bound to NAD+ has recently been solved allowing us to compare our mutants to the crystal structure (Min et al. 2001). There are four major parts to the structure: a Rossman fold made up of 6 parallel strands and 6 helices, a loop that appears to be the most structurally flexible part of the protein, a helical domain, and a Zn binding domain. NAD+ binds in a pocket between the helical domain and the Rossman fold and flanked by the loop.

Mutant 261 is on the border between the first part of the Rossman fold and the loop. Mutants 345 and 347 fall in the border region between the second part of the Rossman fold and the Zinc binding domain. Mutant 364 falls in the middle of the Zinc binding domain. Mutants 270, 271, 274, and 275 all fall within the loop. Mutant 275's location is interesting as it corresponds to residue

Arg33 in *Archaeglobus fulfidus* which the authors see form a hydrogen bond with NAD+ in one of their crystals suggesting an important role in NAD+ binding. If mutant 275 where crucial for NAD+ binding, then one would expect this mutation to destroy the enzymatic activity, but it does not. On the other hand, in the monoclinic crystal the authors do not see the residue binding to NAD+ challenging the importance of that residue in NAD+ binding. It is interesting to note that mutants 261 and 275 both lie in or near the loop suggesting that learning more about this loop region could reveal more about the function of SIR2.

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Figures and Tables

Table 1. Yeast Strains

Strain	Genotype
W303R5-Δsir2	MATa ade2-1 leu2-3,112 can1-100 trp 1-1 ura3-52 his3-11,15 RAD5 rDNA-ADE2 sir2::TRP1
W303RT	MATa ade2-1 leu2-3,112 can1-100 trp 1-1 ura3-52 his3-11,15 rad5-535 TELVIIL-URA3 rDNA-ADE2
W303RT-Δsir2	W303RT sir2::TRP1
W303R5-SIR2-wt	W303R5-Δsir2 leu2::LEU2-SIR2
W303R5-Δsir2-pRS305	W303R5-Δsir2 leu2::LEU2
W303R5-sir2T261A	W303R5-Δsir2 leu2::LEU2-sir2T261A
W303R5-sir2G270A	W303R5-Δsir2 leu2::LEU2-sir2G270A
W303R5-sir2I271A	W303R5-Δsir2 leu2::LEU2-sir2I271A
W303R5-sir2F274A	W303R5-Δsir2 leu2::LEU2-sir2F274A
W303R5-sir2R275A	W303R5-Δsir2 leu2::LEU2-sir2R275A
W303R5-sir2N345A	W303R5-Δsir2 leu2::LEU2-sir2N345A
W303R5-sir2D347A	W303R5-Δsir2 leu2::LEU2-sir2D347A
W303R5-sir2H364A	W303R5-Δsir2 leu2::LEU2-sir2H364A
W303RT-SIR2-wt	W303RT-Δsir2 leu2::LEU2-SIR2
W303RT-Δsir2-pRS305	W303RT-Δsir2 leu2::LEU2
W303RT-sir2T261A	W303RT-Δsir2 leu2::LEU2-sir2T261A
W303RT-sir2G270A	W303RT-Δsir2 leu2::LEU2-sir2G270A
W303RT-sir2I271A	W303RT-Δsir2 leu2::LEU2-sir2I271A
W303RT-sir2F274A	W303RT-Δsir2 leu2::LEU2-sir2F274A
W303RT-sir2R275A	W303RT-Δsir2 leu2::LEU2-sir2R275A
W303RT-sir2N345A	W303RT-Δsir2 leu2::LEU2-sir2N345A
W303RT-sir2D347A	W303RT-Δsir2 leu2::LEU2-sir2D347A
W303RT-sir2H364A	W303RT-Δsir2 leu2::LEU2-sir2H364A
W303R5-SIR2-wt-HASIR4	W303R5-SIR2-wt sir4::URA3-SIR4HA
W303R5-Δsir2-HASIR4	W303R5-Δsir2-pRS305 sir4::URA3-SIR4HA
W303R5-sir2T261A-HASIR4	W303R5-sir2T261A sir4::URA3-SIR4HA
W303R5-sir2R275A-HASIR4	W303R5-sir2R275A sir4::URA3-SIR4HA
W303R5-sir2D347A-HASIR4	W303R5-sir2D347A sir4::URA3-SIR4HA
W303RT-SIR2/SIR2	W303RT-leu2::LEU2-SIR2
W303RT-SIR2/sir2T261A	W303RT-leu2::LEU2-sir2T261A
W303RT-SIR2/sir2G270A	W303RT-leu2::LEU2-sir2G270A
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W303RT-SIR2/sir2N345A	W303RT-leu2::LEU2-sir2N345A
W303RT-SIR2/sir2D347A	W303RT-leu2::LEU2-sir2D347A
W303RT-SIR2/sir2H364A	W303RT-leu2::LEU2-sir2H364A
Cky20	MATα arg1 tsm11

Table 2. Summary of Mutant phenotypes

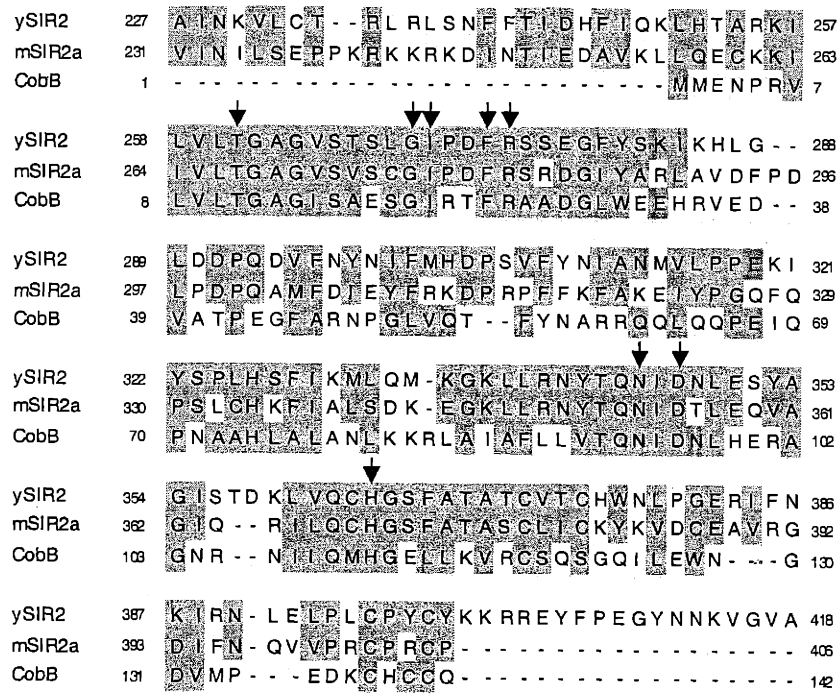
mutant	ADP-Rib. Activity (% of wt)	Deacetylase Activity*	HM Silencing	Telomere Silencing	rDNA recom. rate	ChIP at Telomeres
wt	100%	100%	+	+	0.1%	+
Δsir2	N/A	N/A	-	-	1.2%	-
Thr-261	4%	5%	+	+	0.1%	+
Gly-270	7%	21%	+	+/-	0.1%	+/-
Ile-271	8%	9%	+	-	0.2%	-
Phe-274	ND	9%	-	-	0.9%	-
Arg-275	100%	78%	-	-	1.0%	-
Asn-345	0%	0.3%	-	-	1.2%	-
Asp-347	0%	0.4%	-	-	ND	-
His-364	ND	0.4%	-	-	ND	-

*as a % of wild type minus the pET negative control

Figure 1. Mutagenesis of the core domain of SIR2. (A) Eight amino acid residues that are conserved between bacterial CobB, Sacharomyces SIR2 and mouse SIR2α were mutated to alanine in ySIR2. They correspond to the following residues: Thr-261, Gly-270, Ile-271, Phe-274, Arg-275, Asn-345, Asp-347, and His-364. (B) rSir2p from E. coli was purified over a Ni+ column. The purified protein was run on a polyacrylamide gel to test for purity and stability.

Figure 1

a)


ySIR2 227 AINKVLCT--RLRLSNFFTIDHFIQKLHTARKI 257
mSIR2a 231 VINILSEPPKRKKRKDINTIEDAVKLLQECKKI 263
CobB 1 ----------MMENPRV 7
ySIR2 258 LVLTGAGVSTSLGIPDFRSSEGFYSKIKHLG-- 288
mSIR2a 264 IVLTGAGVSVSCGIPDFRSRDGIYARLAVDFPD 296
CobB 8 LVLTGAGISAESGIRTFRAADGLWEEHRVED-- 38
ySIR2 289 LDDPQDVFNYNIFMHDPSVFYNIANMVLPPEKI 321
mSIR2a 297 LPDPQAMFDIEYFRKDPRPFFKFAKEIYPGQFQ 329
CobB 39 VATPEGFARNPGLVQT--FYNARRQQLQQPEIQ 69
ySIR2 322 YSPLHSFIKMLQM-KGKLLRNYTQNIDNLESYA 353
mSIR2a 330 PSLCHKFIALSDK-EGKLLRNYTQNIDTLEQVA 361
CobB 70 PNAAHLALANLKKRLAIAFLLVTQNIDNLHERA 102
ySIR2 354 GISTDKLVQCHGSFATATCVTCHWNLPGERIFN 386
mSIR2a 362 GIQ--RILQCHGSFATASCLICKYKVDCEAVRG 392
CobB 103 GNR--NIIQMHGELLKVRCSQSGQILEWN----G 130
ySIR2 387 KIRN-LELPLCPYCYKKRREYFPEGYNNKVGVA 418
mSIR2a 393 DIFN-QVVPRCPRCP---------- 406
CobB 131 DVMP---EDKCHCCQ---------- 142

b)

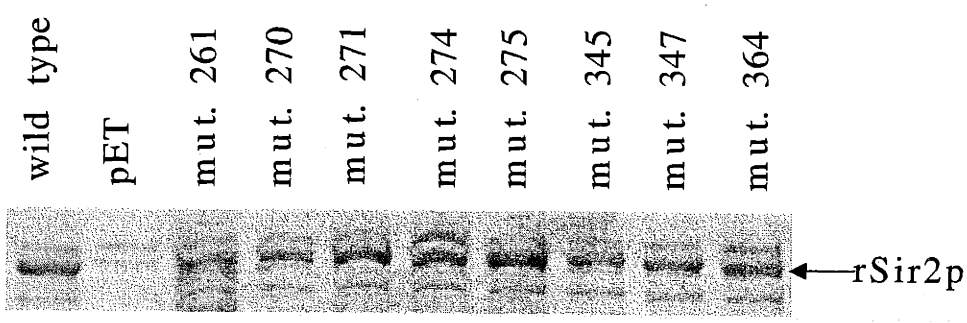
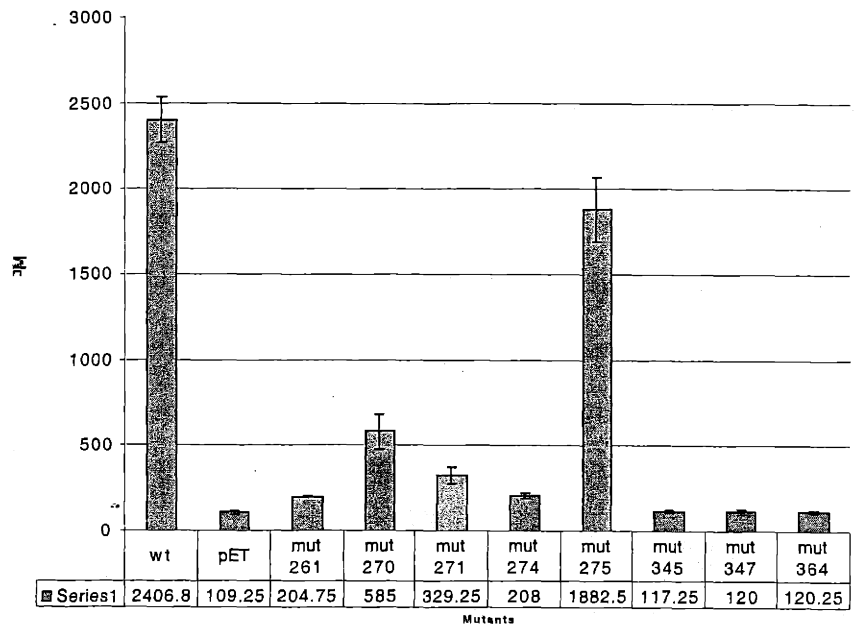

wild type
pET
mut. 261
mut. 270
mut. 271
mut. 274
mut. 275
mut. 345
mut. 347
mut. 364
rSir2p

Figure 2. Analysis of Sir2p's enzymatic activities. (A) Tritiated H4 peptide was used to measure the efficiency of the mutants in a NAD+ dependent deacetylation reaction. The peptide was incubated with 1mM NAD^+ and 2μg of recombinant protein. The graph measures deacetylation activity by counting the amount of tritiated acetate released from the peptide. (B) The capability of the mutant rSir2 proteins to ribosylate Histone H3 was measured by incubating recombinant enzyme with ^{32}P-NAD and Calf Histone H3. The products were run out on a polyacrylamide gel and the gel was exposed to film to see if the histones incorporated label from the ^{32}P-NAD^+.

Figure 2

a)


3000
2500
2000
1500
1000
500
0
wt
pET
mut 261
mut 270
mut 271
mut 274
mut 275
mut 345
mut 347
mut 364
Series1
2406.8
109.25
204.75
585
329.25
208
1882.5
117.25
120
120.25
Mutants

b)

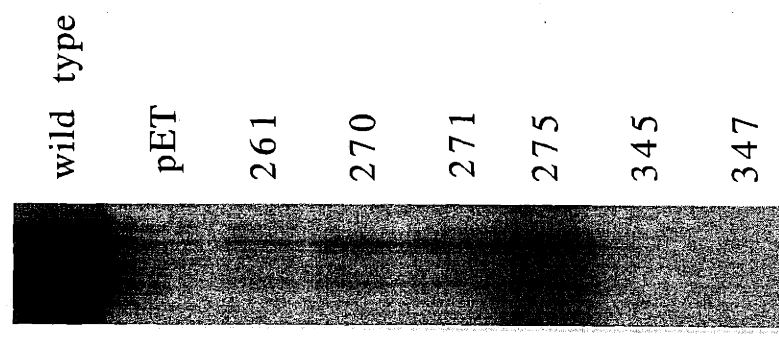

wild type
pET
261
270
271
275
345
347

Figure 3. Testing sir2 mutant phenotypes in vivo. (A) Whole cell protein extracts were made from each of the indicated strains. 15 μg of WCE was run on a polyacrylamide gel and blotted to a PVDF membrane for Western blot analysis. The blot was probed with anti-Sir2p to measure the level of Sir2p in each of the mutant strains. The upper band is the Sir2p band. (B) To investigate silencing at the HMLα locus, the mutant strains were mated with a mating tester strain and grown on selective media to select for diploids. (C) To test for telomere silencing, SIR2 was mutated in a strain background with the URA3 marker at the end of Telomere VII. Each mutant strain was tested for its ability to silence the marker by growing on media containing 5-FOA (a substrate that is toxic to yeast expressing URA3). (D) rDNA recombination rates were measured in a strain background with the ADE2 marker located in the rDNA array. The rate of marker loss was measured for each mutant by counting the number of colonies that lost the marker in the first generation after plating.

Figure 3

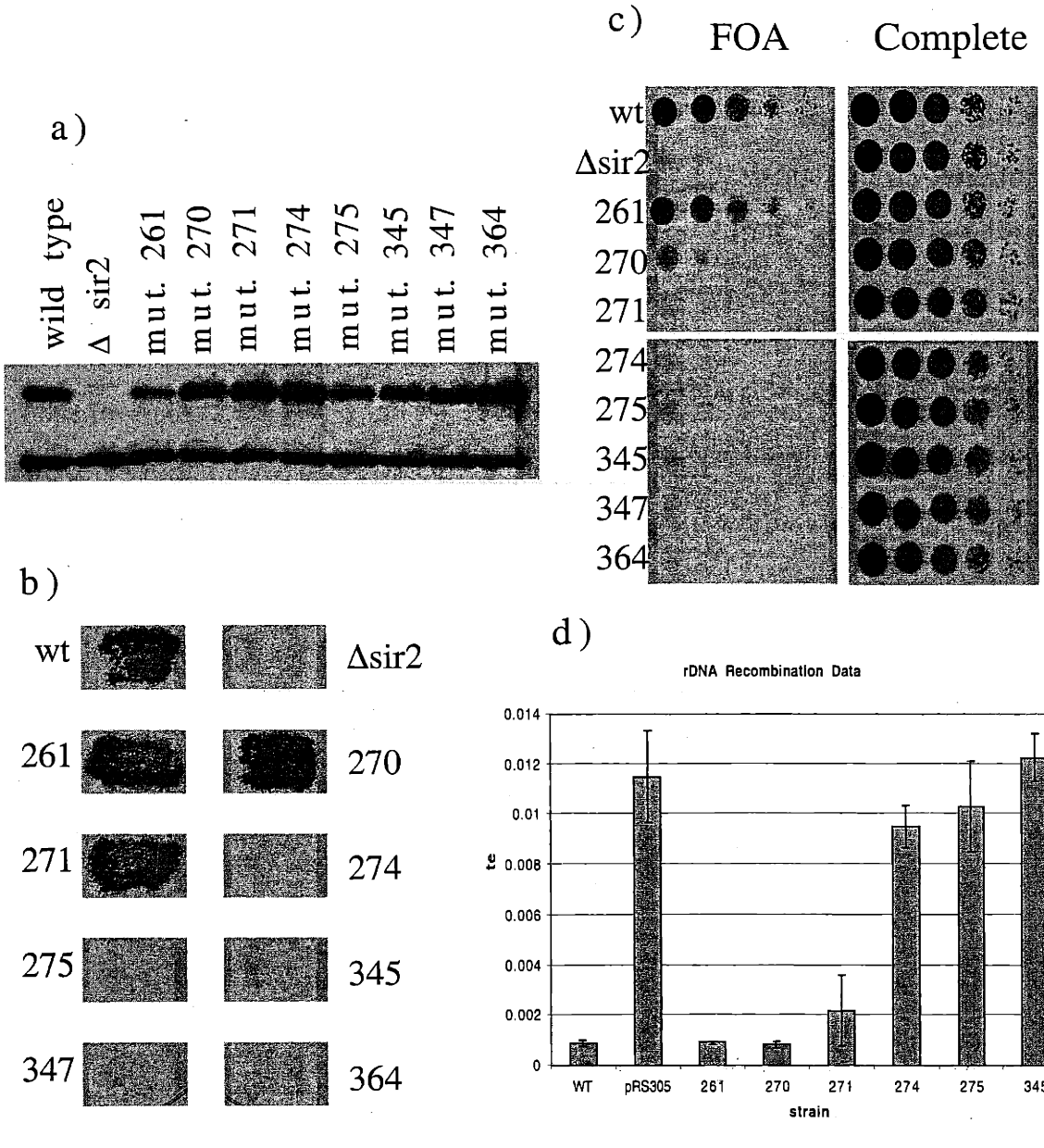
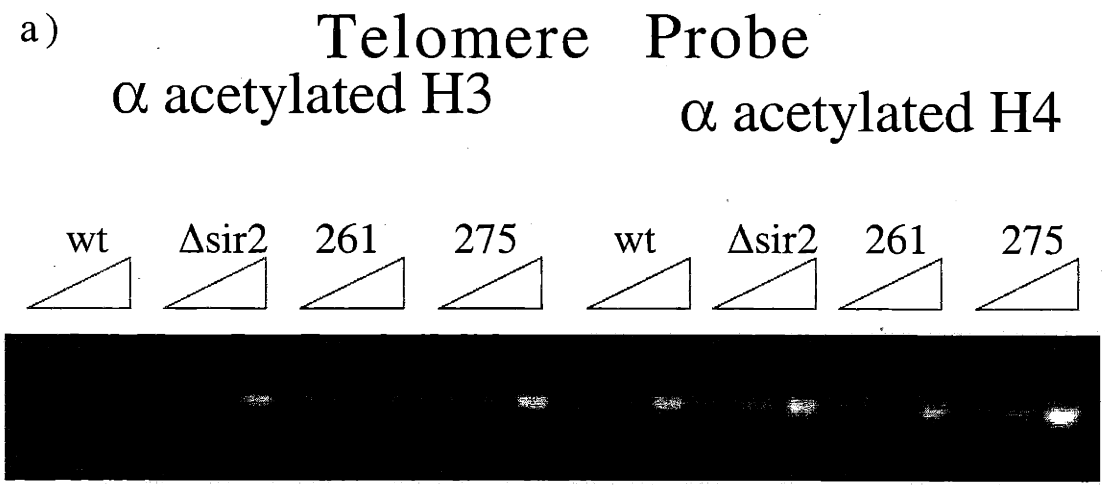

a)
wild type
Δ sir2
mut. 261
mut. 270
mut. 271
mut. 274
mut. 275
mut. 345
mut. 347
mut. 364
b)
wt
Δsir2
261
270
271
274
275
345
347
364
c)
FOA
Complete
wt
Δsir2
261
270
271
274
275
345
347
364
d)
rDNA Recombination Data
te
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
WT
pRS305
261
270
271
274
275
345
strain

Figure 4. Chromatin IP using anti-acetylated histone antibody to measure histone acetylation at silent loci. (A) Chromatin IP was used to investigate the acetylation state of the histones at the telomeres in mutants 261 and 275. Three fold diluations of anti-acetylated H3 and anti-acetylated H4 IP DNA were probed for telomeres using PCR primers corresponding to the telomere on the right arm of chromosome VI. (B) IP DNA from above was blotted to a membrane using a slot blot apparatus. The DNA was then probed for rDNA sequences to test the level of acetylation of histone H3 and H4 at the rDNA in the mutant strains

Figure 4


a)
Telomere Probe
α acetylated H3
α acetylated H4
wt
Δsir2
261
275
wt
Δsir2
261
275

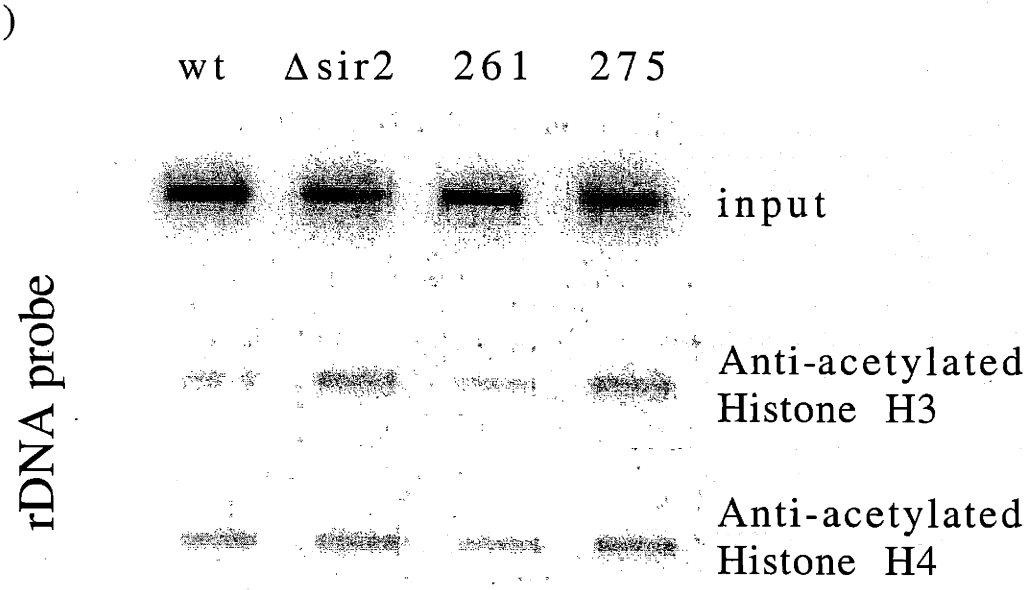
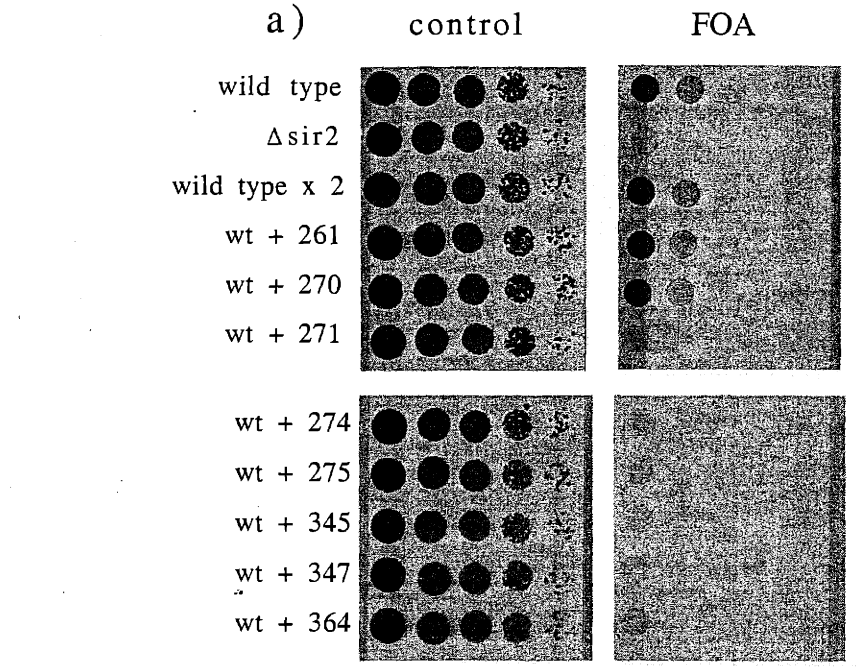
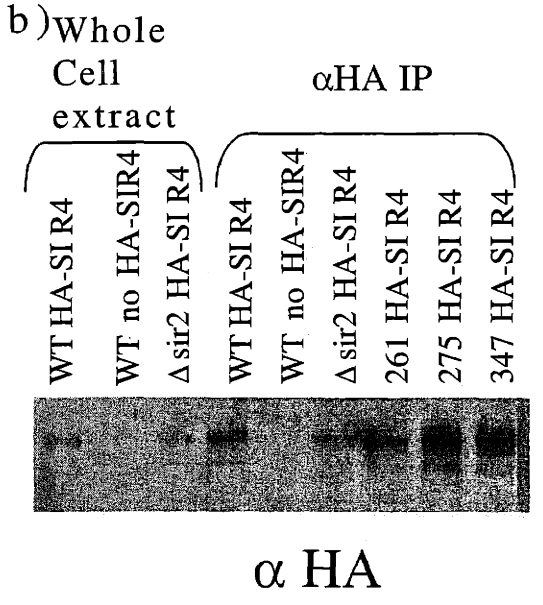

b)
wt
Δsir2
261
275
input
rDNA probe
Anti-acetylated Histone H3
Anti-acetylated Histone H4

Figure 5. Dominant negative effects of mutations and co-immuno-precipitation of SIR2 and SIR4. (A) URA3-Tel strains with a wild type and mutant copy of SIR2 present were spotted on 5-FOA media to test is the mutations effects on telomere silencing were dominant. (B) Whole cell protein extract from selected mutant strains (wild type, Δsir2, mutant 261, mutant 275, and mutant 347) with an HA tagged SIR4 gene was IPed with antibodies against the HA epitope. The IP extract along with WCE was run on a poly-acrylamide gel and blotted to a PVDF membrane so that Western Blotting analysis could be performed. The membrane was probed using antibody to the HA epitope to measure the efficiency of the pull down. (C) The PVDF membrane is re-probed with antibody recognizing Sir2p to measure the ability of the mutant Sir2p's to interact with SIR4 in vivo.

Figure 5


a)
control
FOA
wild type
Δsir2
wild type x 2
wt + 261
wt + 270
wt + 271
wt + 274
wt + 275
wt + 345
wt + 347
wt + 364


b)
Whole Cell extract
αHA IP
WT HA-SI R4
WT no HA-SIR4
Δsir2 HA-SI R4
WT HA-SI R4
WT no HA-SIR4
Δsir2 HA-SI R4
261 HA-SI R4
275 HA-SI R4
347 HA-SI R4
α HA

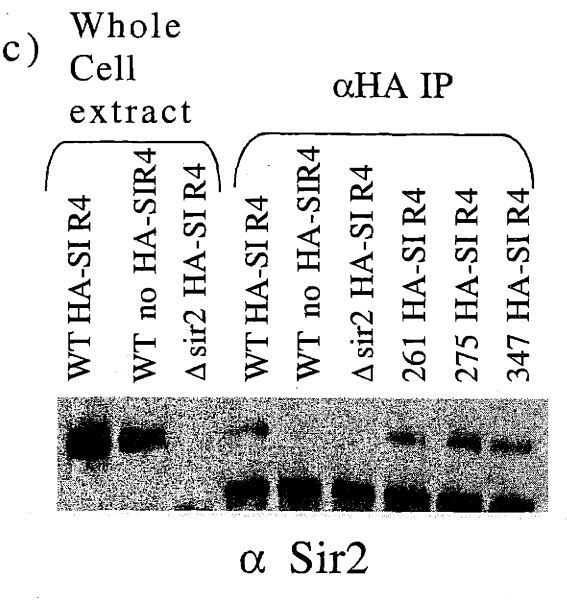
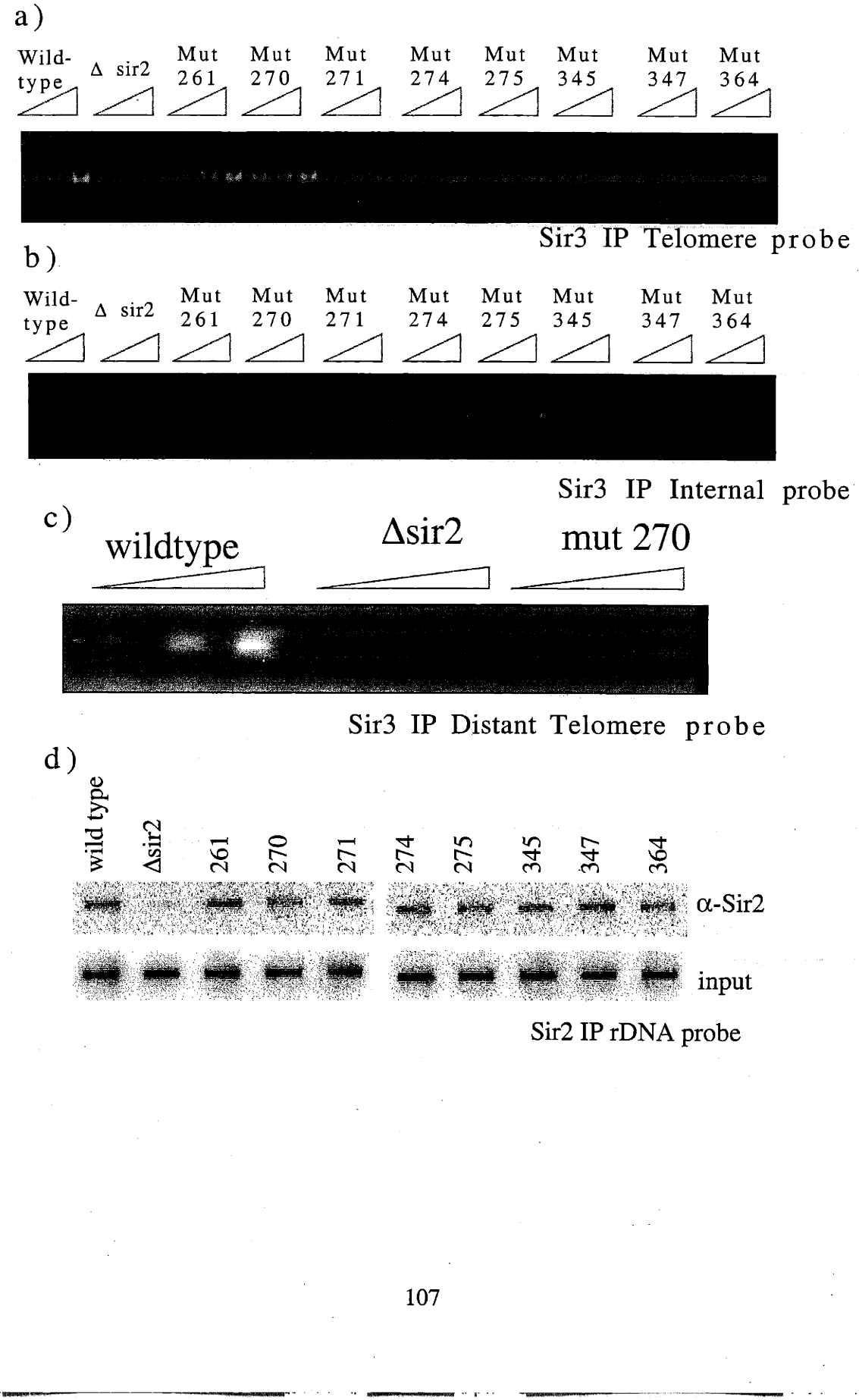

c)
Whole Cell extract
αHA IP
WT HA-SI R4
WT no HA-SIR4
Δsir2 HA-SI R4
WT HA-SI R4
WT no HA-SIR4
Δsir2 HA-SI R4
261 HA-SI R4
275 HA-SI R4
347 HA-SI R4
α Sir2

Figure 6. Chromatin IP of Sir complex. Chromatin Immuno-precipitation was used to investigate the ability of the Sir complex to localize to the telomeres and rDNA in the sir2 mutant strains. A) Cross-linked chromatin was IPed with anti-bodies to Sir3p. To test for localization of the Sir complex at the telomeres, the DNA pulled down was probed for telomere sequences using PCR primers corresponding to 300 bp from the end of the telomere at the right arm of chromosome VI. Three-fold dilutions of the IPed DNA were used in the PCR reaction. B) The pulled down DNA used above was probed for sequences corresponding to an internal non-silenced location on chromosome XII. C) The above pulled down DNA from wild type, Δsir2 and mutant 270 was probed for more distal telomere sequences using PCR primers that recognize sequences that are 3000 bp from the end of the telomere at the right arm of chromosome VI. D) Cross-linked chromatin pulled down with anti-Sir2p was blotted to a membrane using a slot blotting apparatus and probed for rDNA sequences.

Figure 6


a)
Wild-type
Δ sir2
Mut 261
Mut 270
Mut 271
Mut 274
Mut 275
Mut 345
Mut 347
Mut 364
Sir3 IP Telomere probe
b)
Wild-type
Δ sir2
Mut 261
Mut 270
Mut 271
Mut 274
Mut 275
Mut 345
Mut 347
Mut 364
Sir3 IP Internal probe
c)
wildtype
Δsir2
mut 270
Sir3 IP Distant Telomere probe
d)
wild type
Δsir2
261
270
271
274
275
345
347
364
α-Sir2
input
Sir2 IP rDNA probe

Chapter 4

Conclusion and Future Directions

SIR2 has generated a lot of interest because of its wide-ranging effects on transcription, recombination, and aging. Through this project and the work done by many others, SIR2 has moved from an amorphous gene that somehow affects silencing to an enzyme that has an activity that is closely related to its in vivo function. While these studies have answered important questions about SIR2 in the process they have posed many new ones.

Enzymatic Activity of Sir2p

The ADP-ribosylation of histones was the first identified enzymatic activity of yeast Sir2p (Tanny et al. 1999). It made sense that SIR2 would modify histones in someway given the in vivo correlation between silencing and histones already made. There were, however, some interesting quandaries. For one, the ADP-ribosylation of histones was rather inefficient. Far more robust was SIR2's hydrolysis of NAD. Why would SIR2 be so wasteful as to turn over most of the NAD in a pointless hydrolysis reaction? A second puzzle was why we and others never found an ADP-ribosylated histone product in a large enough quantity to prove by mass spec analysis that the histone really was getting modified (chapter 2 and (Landry et al. 2000; Tanner et al. 2000; Tanny and Moazed 2001). In our search for an ADP-ribosylated histone product, we found that Sir2p could deacetylate histone tails in the presence of NAD. This revealed a second enzymatic activity of Sir2p, but which was the relevant one? At first a mutation in the core domain appeared to

separate the activities (chapter 2) but further studies of mutants showed these activities were closely linked (chapter 3).
Despite this, the evidence suggests histone deacetylation is the more relevant activity in vivo. First, the deacetylation reaction is much stronger than the ADP-ribosylation reaction as witnessed by the fact that deacetylated products are fairly abundant in in vitro reactions while ADP-ribose products are rare. Second, there is a better correlation between the histone deacetylation activity of Sir2p and the state of silenced chromatin in the cell (Braunstein et al. 1993; Braunstein et al. 1996).

Even if deacetylation is the relevant reaction, why is ADP-ribosylation ocurring and what is its link to histone deacetylation? As mentioned above the NAD hydrolysis activity is much stronger than the ADP-ribosylation activity, which could mean that ADP-ribosylation is just a side reaction, and that NAD hydrolysis may be a much more important reaction. Recent papers investigating the mechanism of the deaceylation activity of Sir2 have supported this theory (Landry et al. 2000; Tanner et al. 2000; Tanny and Moazed 2001). They find a tight linkage between NAD hydrolysis and histone deacetylation. Moreover, they find the formation of an acetylated ADP-ribose product called 1-O-acetyl-ADP-ribose. The authors theorize that this linkage could be a coupling of the two reactions for energetic purposes or that the intermediate could be a signaling molecule itself.

The SIR2 Mutants

We hoped to cripple Sir2p's enzymatic activity by mutating well conserved residues in the core domain and then compare the biochemical activity of Sir2p in vitro with the activity of the mutants in vivo. Most of the mutations did indeed affect Sir2's ability to deacetylate histones. We expected the effect of these mutations to show a simple direct correlation between histone deacetylation activity and silencing. Many of the mutants that lost activity did lose the ability to silence; however, two of the mutants, Thr-261 and Arg-275, did not have this simple relationship. Instead, mutant 275 was unable to silence in vivo even though it could deacetylate histones in vitro while mutant 261 could silence in vivo even though it poorly deacetylated histones in vitro.

In chapter 3 we discussed potential reasons these mutants act as they do. As we described, these mutants raise the possibility that the histone deacetylation activity is an in vitro artifact, but this idea would ignore the correlation between SIR2 and the acetylation state of histones. Instead, the mutants could indicate complexities that we are only beginning to understand. It is possible that SIR2 could have an alternative substrate that is important for silencing or it is possible that the chromatin complex can affect these mutant's ability to deacetylate in vivo. It is also possible that these mutations affect other potential interactors that play a role in silencing.

The chromatin IP experiments could indicate how the Sir complex behaves at telomeres. The mutants Asn-345, Asp-347, and His-364 are especially interesting in this regard as they all lack enzymatic activity and fail to localize to the telomeres. If localization were independent of enzymatic activity then we might

expect some of these mutants to localize to the telomeres and yet be unable to silence because they have no ability to deacetylate the histones. Instead we see that enzymatic-dead mutants consistently fail to localize to the telomeres, suggesting that acetylation is an important step in the localization of the Sir complex to the telomeres. A model that ties this observation in with previous observations about the binding of various silencing factors to silenced regions is presented in figure 1. By this model, the SIR complex is initially recruited to the telomeres by a DNA binding factor like Rap1. Once there, Sir2p deacetylates the histones. After deacetylating the histone, Sir3p and Sir4p can now bind tightly to the histone tail. The binding of the SIR complex to the histone tail would allow for Sir2p to deacetylate neighboring histones and thus expand the region that the SIR complex can bind to. It is this binding of the complete SIR complex along with the deacetylation of histones that leads to the creation of silenced chromatin.

Future Directions

While much has been learned about SIR2, there is still much that the gene can tell us about various processes. The mutants 261 and 275 are mysteries to come from this work. They both lack a direct correlation between their in vitro and in vivo activities, yet understanding why could help us understand more about how SIR2 functions. As mentioned these mutants could presage another substrate for SIR2, another important interactor, or a more complicated relationship with chromatin. A genetic screen to

suppress the mutant 275 defect or to look for mutant 261-specific de-silencers could potentially tell us more about other genes involved in silencing. There is, as yet, no simple explanation for why these mutants behave as they do.

It is also important to more specifically define the relationship between NAD, SIR2, and deacetylation. As mentioned, people have already shown NAD hydrolysis and deacetylation are coupled, but this raises other questions. Why is the hydrolysis of NAD linked to deacetylation? If the hydrolysis of NAD is a source of energy for the deacetylation reaction as some have suggested then why do other histone deacetylases like Rpd3p not require cofactors like NAD? It is also possible that the 1-O-acetyl-ADP ribose product plays a role in the cell. It would be interesting to see what effects such a molecule has on the cell, but this is not necessarily an easy experiment as the molecule is newly discovered and cannot exactly be ordered from the Sigma catalogue.

Of course one of the big questions is what is the link between SIR2, NAD, and life span? There is already a link between caloric restriction, NAD levels, and SIR2, but it also raises new questions (Lin et al. 2000). Is SIR2 overexpression mimicking caloric restriction and is life span extension through caloric restriction a result of higher levels of SIR2 activity? Even if we know this, we still don't know why caloric restriction and SIR2 affect life span. We do know that SIR2 and caloric restriction affect the rate of rDNA circle accumulation in yeast (Kaeberlein et al. 1999; Lin et al. 2000), but we don't know how this is done and what other important roles SIR2 may play in aging. Perhaps life span analysis of locus specific alleles

of SIR2 may show us if all of the effects SIR2 has on life span are through its role at the rDNA. Knowing this, perhaps understanding the role of SIR2 and the RENT complex at the rDNA could also really enhance our knowledge of caloric restriction and aging.

To understand SIR2 will help us understand chromatin, DNA recombination, and aging. Are all of these processes connected or are they just three separate arenas that SIR2 happens to affect? Understanding the connection between these processes is probably the biggest thing that studies of SIR2 have to offer. It is, however, an arduous process. SIR2 was first identified as such over 15 years ago and we have only recently identified its enzymatic activities. Fortunately this means there are exciting things this gene can tell us in the coming years.

Acknowledgements. I would like to thank Brad Johnson for helpful comments on the writing of this chapter.

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

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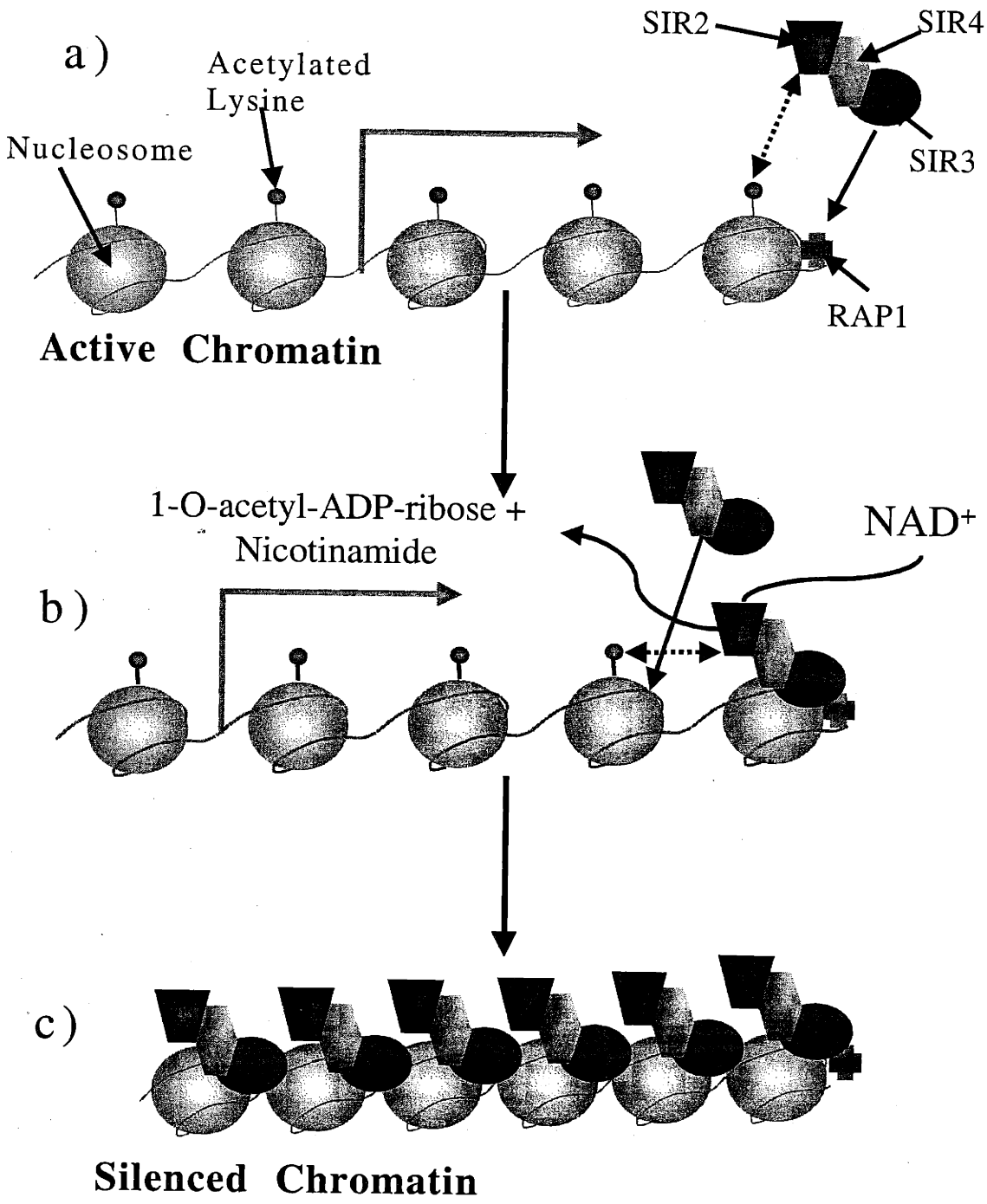
FIGURES

Figure 1. SIR complex assembly at the telomeres. a) Rap1p binds to the telomeres and recruits the SIR complex via Sir3p to the telomeres. b) The SIR complex binds to the histone tail. Sir2p deacetylates the histone tail and in the process breaks NAD down into nicotinamide and 1-O-acetyl-ADP-ribose. The proximity of SIR2 to the next nucleosome allows it to deacetylate the N-terminal tails of its histone H3 and H4 which allows another SIR complex to bind to it. c) As the process continues, more SIR complexes are recruited to the telomere creating a compact chromatin structure at the telomere.

Figure 1


a)
Acetylated
Lysine
Nucleosome
SIR2
SIR4
SIR3
RAP1
Active Chromatin
1-O-acetyl-ADP-ribose +
Nicotinamide
NAD+
b)
c)
Silenced Chromatin

Appendix

GCN5 effects on the ADE2 promoter are ADA1 independent

This Appendix summarizes the work I did in the Guarente lab on the ADA genes before I started working on SIR2.

Results and Discussion

GCN5 was the first gene to be identified as a histone acetyl transferase (HAT) and a transcriptional coactivator (Grant et al. 1997). This discovery demonstrated the importance of histone acetylation in controlling transcription at promoters. The state of histone acetylation is also important at silent loci like HM and the telomeres where the histones are hypoacetylated (Braunstein et al. 1993). We wanted to know if GCN5 and the ADAs, proteins that interact with GCN5 in a coactivator complex, might affect silencing in addition to their effects at promoters.

To determine whether the ADA/GCN5 complex plays a role in telomere silencing, we deleted ADA1, ADA2, ADA3, ADA5, and GCN5 in a strain with an ADE2 marker positioned at TELVR (PSY316AT). When ade2 mutant strains are grown in limiting concentrations of adenine, a red pigment is produced. In PSY316AT, a mixture of red (ADE2 off) and white (ADE2 on) colonies is observed, reflecting the variably silenced state at the telomere. As shown in Figure 1a, deletion of ADA2, ADA3, or GCN5 dramatically increased the ratio of red to white colonies to the point where almost all the colonies were red. This implies the deletion of these genes leads to a decrease in expression. Northern blots (figure 1b) confirmed these effects on ADE2 expression. Strikingly, when ADA1 or ADA5 was deleted, the ratio of red to white colonies decreased and expression of the ADE2 transcript increased indicating that these deletions actually desilence at the telomeres (figure 1a, b).

Because of the disparate effects on expression of the ada1 deletion versus the gcn5 deletion, we wanted to verify that they were in the same pathway. We constructed the following double mutants: gcn5/ada1, ada2/ada1, ada3/ada1 to test epistasis (Figure 2). Each double mutant gave rise to strong telomere silencing and uniformly red colonies indicating ADA2, ADA3, and GCN5 are epistatic to ADA1 (and presumably ADA5) when regulating ADE2 at the telomere. This is different from findings at normal promoters where deletion of ADA1 and ADA5 are more severe than deletions of GCN5 or the other ADA genes and in that case ADA1 and ADA5 are epistatic to GCN5 (Marcus et al. 1996; Roberts and Winston 1996; Horiuchi et al. 1997).

To verify that the GCN5 effect was a general silencing effect and not a promoter specific effect, we deleted ADA1 and GCN5 in a strain that has the URA3 marker at the telomere (figure 3a). This time both mutants failed to silence any better or worse than wild type. This suggests that the effect of GCN5 may not be a silencing effect but may be specific to the ADE2 promoter.

To test the possibility that the ADA genes control the ADE2 promoter we did northern blots on ADE2 wild type cells in strains deleted for ADA1, ADA2, ADA3, or ADA5. The expression levels in ada2 or ada3 deletion backgrounds is lower than wild type while the expression level in ada1 or ada5 deletion backgrounds is slightly higher than wild type (figure 3b). This indicates that GCN5 and the other ADA genes do not directly affect silencing but do have interesting affects on the ADE2 promoter.

Others have seen mutations in GCN5 increase silencing at the telomeres (Sun and Hampsey 1999). Our results suggest, however, that any affect that GCN5 may have on silencing is actually an affect on the promoter of the marker gene. The disparate effects of deletions in ADA1 versus GCN5 are, however, a novel observation. When the SAGA complex containing Gcn5p along with the other Ada proteins and many Spt proteins was purified, the authors also saw a smaller complex that contained Gcn5p, Ada2p, Ada3p, but not Ada1p or Ada5p (Grant et al. 1997). Because at that time all genes that were affected by GCN5 were also affected by ADA1 one line of thought was that the smaller complex may just be a pre-cursor complex to the SAGA complex. These results suggest that the smaller complex may also play a role as a transcriptional coactivator at some promoters. This is supported by the observation that the smaller complex has members not found in the SAGA complex suggesting that GCN5, ADA2, and ADA3 have a function in the cell separate from their role in the SAGA complex (Eberharter et al. 1999). The observation that deletions in ADA1 or ADA5 actually increase transcription of the ADE2 gene may be the result of releasing GCN5 and the other ADA genes from the SAGA complex so that more is present to act at promoters like ADE2.

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Eberharter, A., D.E. Sterner, D. Schieltz, A. Hassan, J.R. Yates, 3rd, S.L. Berger, and J.L. Workman. 1999. The ADA complex is a distinct histone acetyltransferase complex in Saccharomyces cerevisiae. *Mol Cell Biol* **19**: 6621-6631.

Grant, P.A., L. Duggan, J. Cote, S.M. Roberts, J.E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C.D. Allis, F. Winston, S.L. Berger, and J.L. Workman. 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev* **11**: 1640-1650.

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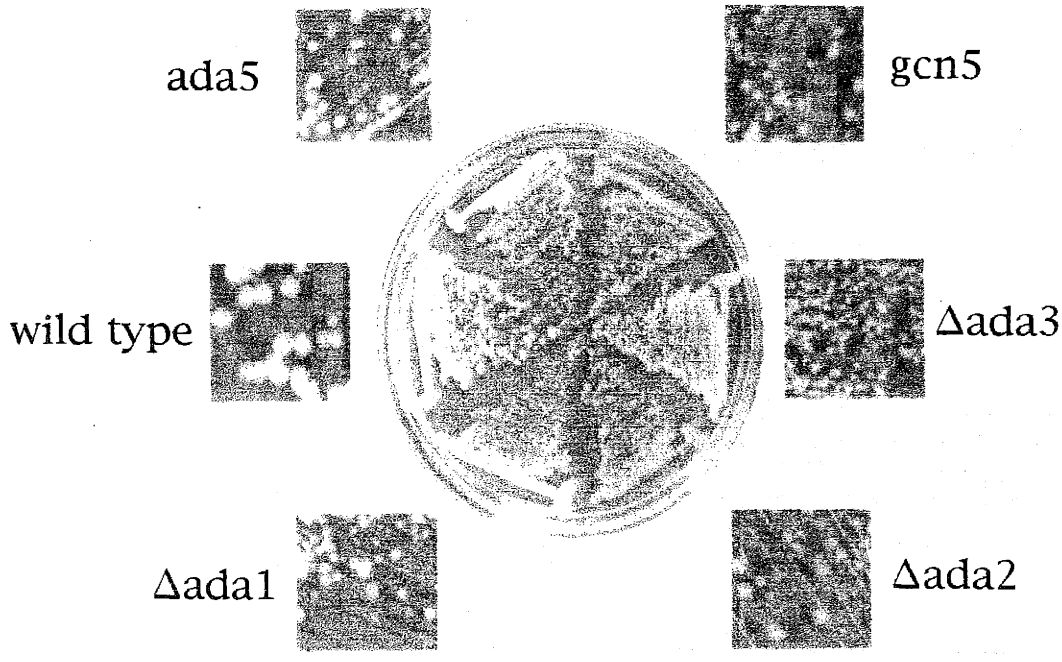
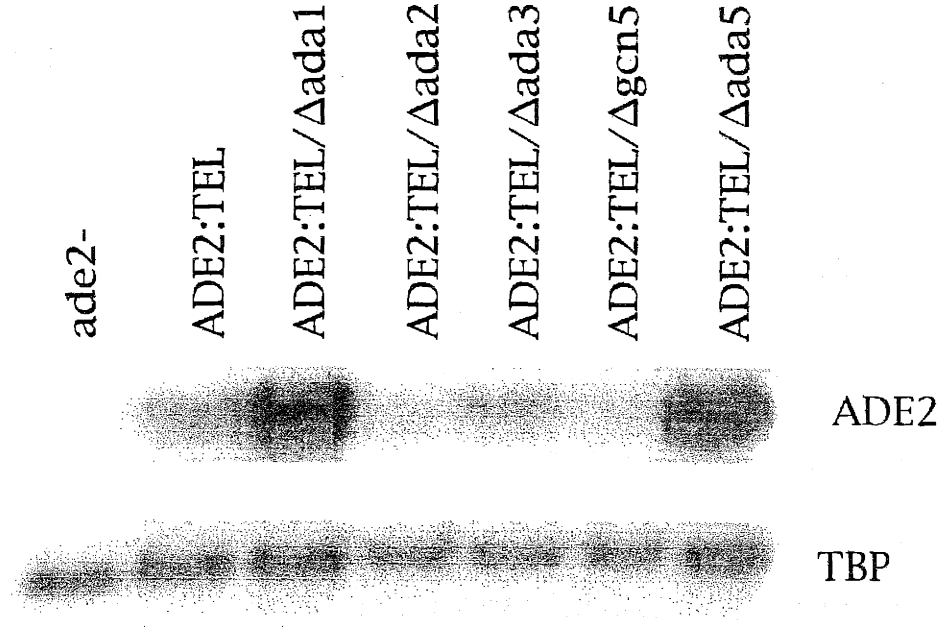
FIGURES

Figure 1. Deletion of the ADA genes affects ADE2 expression at the telomeres. Strain PSY316AT has the ADE2 gene integrated at the telomere on the right arm of chromosome 5. a) PSY316AT wt and this ADA1, ADA2, ADA3, GCN5, and ADA5 deleted were streaked onto plates with a low concentration of adenine. On such media cells that fail to express ADE2 produce a red pigment, while cells that express ADE2 are white. b) Northern blots were performed on 50 μg of total cellular RNA from the same strains. The blot was probed with ADE2 to measure expression of the marker and with TBP as a loading control.


ada5
gcn5
wild type
Δada3
Δada1
Δada2


ade2-
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ADE2:TEL/Δada1
ADE2:TEL/Δada2
ADE2:TEL/Δada3
ADE2:TEL/Δgcn5
ADE2:TEL/Δada5
ADE2
TBP

Figure 2. ada2, ada3, and gcn5 mutations are epistatic to an ada1 mutation. Double deletions of ADA1 and ADA2, ADA3, or GCN5 in PSY316-AT were made and streaked onto low adenine media.

Figure 2

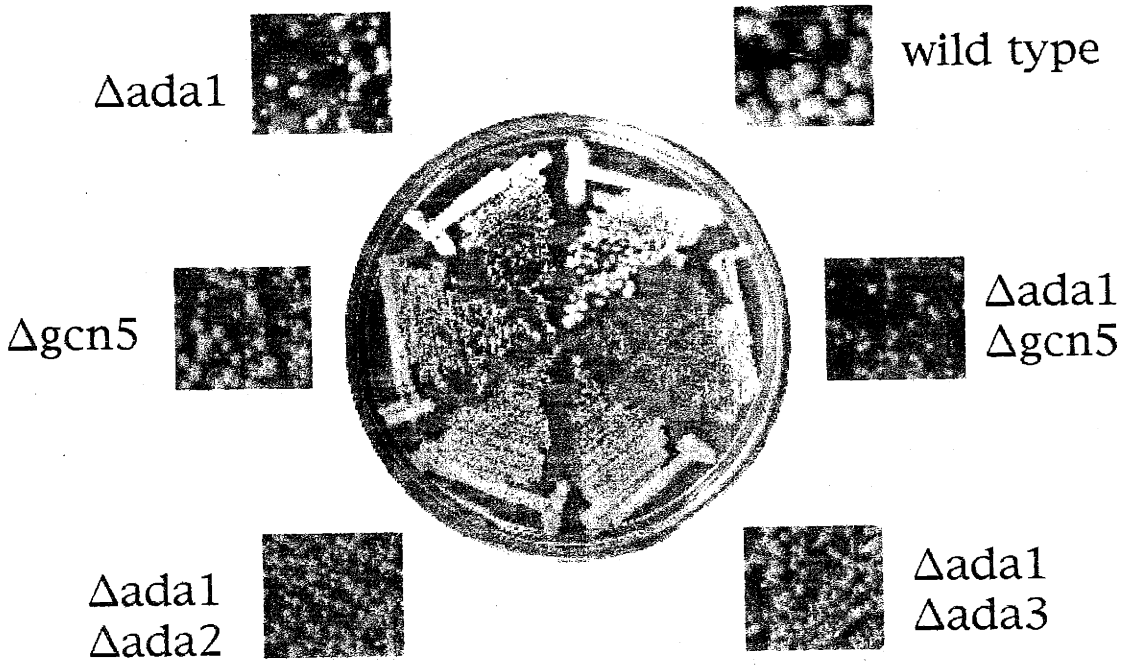
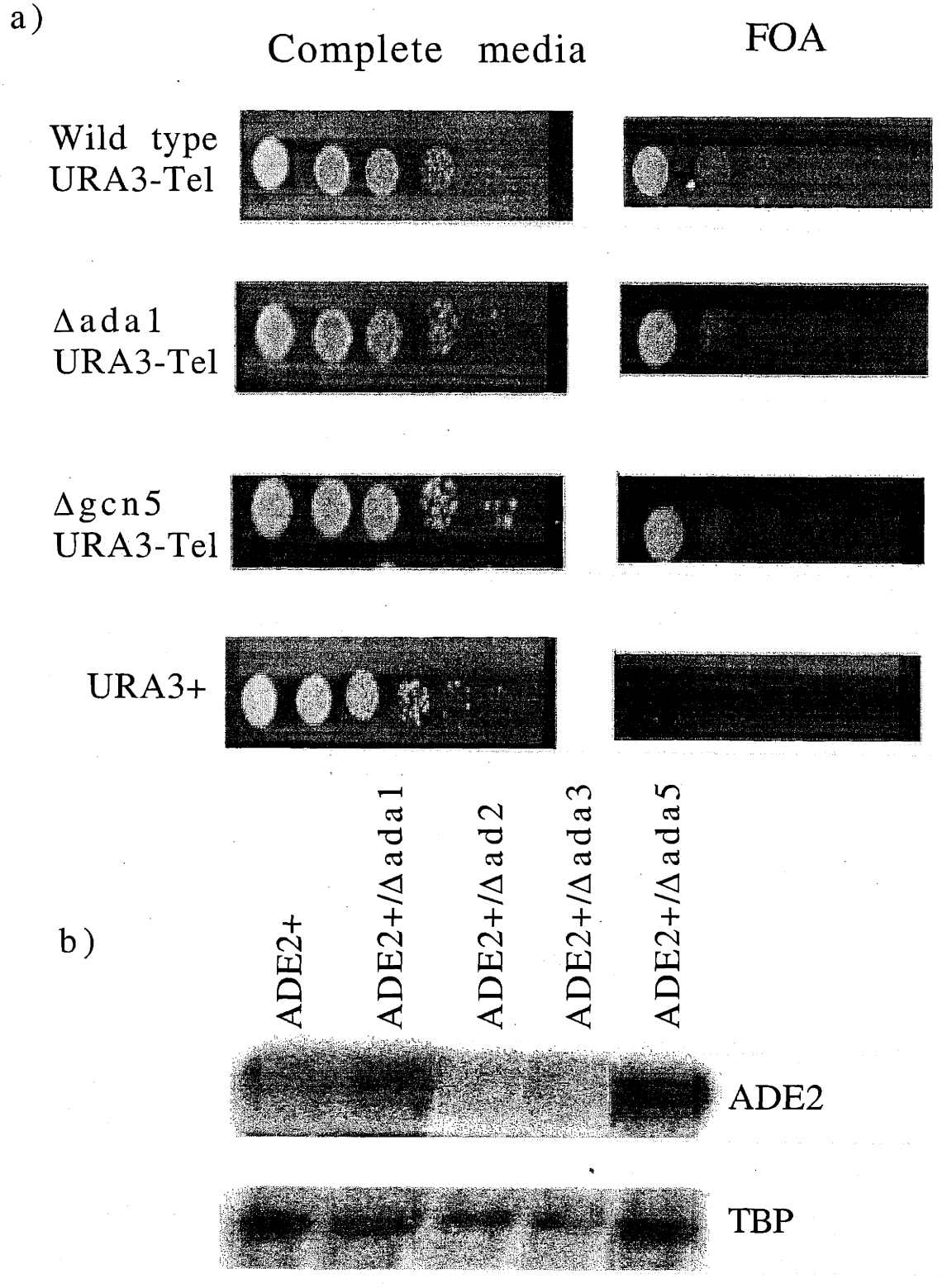


Figure 3. gcn5 mutation does not affect silencing. a) ADA 1 and GCN5 were mutated into PSY316UT a strain with the URA3 marker at the telomeres. PSY316UT wt, Δgcn5, and Δada1 along with PSY316 URA3+ was spotted onto 5-FOA plates to test for silencing of the URA3 gene at the telomere. They were also spotted on complete media plates to control for growth. b) Northern blots were performed on 50 μg of total cellular RNA from PSY316-ADE2+ strains with deletions in ADA1, ADA2, ADA3, or ADA5. The blot was probed with ADE2 to measure expression of the marker and TPB as a loading control.

Figure 3


a)
Complete media
FOA
Wild type URA3-Tel
Δada1 URA3-Tel
Δgcn5 URA3-Tel
URA3+
b)
ADE2+
ADE2+/Δada1
ADE2+/Δad2
ADE2+/Δada3
ADE2+/Δada5
ADE2
TBP