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Studies on Yeast Transcriptional Silencing:

I. A Sir2 Temperature-Sensitive Mutant

II. Regulation of Sir3 by Ris1

A Dissertation Presented

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Chia-Lin Wang

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The Graduate School

Chia-Lin Wang

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation

Dr. Rolf Sternglanz – Dissertation Advisor
Distinguished Professor, Department of Biochemistry and Cell Biology

Dr. Aaron Neiman – Chairperson of Defense
Associate Professor, Department of Biochemistry and Cell Biology

Dr. Neta Dean
Professor, Department of Biochemistry and Cell Biology

Dr. Bruce Futcher
Professor, Department of Molecular Genetics and Microbiology

Dr. Nancy C. Reich
Professor, Department of Molecular Genetics and Microbiology

Dr. Greg Prelich – Outside Member
Professor, Department of Genetics, Albert Einstein College of Medicine

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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Transcriptional silencing in *Saccharomyces cerevisiae* is processed by binding and spreading of silent information regulator (Sir) proteins to form a compacted chromatin structure, or heterochromatin. My research is mainly focused on two of these Sir proteins, Sir2 and Sir3 respectively.

A screen for yeast temperature-sensitive silencing mutants identified a strain with a point mutation in the *SIR2* gene. Haploid strains of either mating type carrying the

mutation were severely defective at mating at 37° but almost normal at 25°. A rapid loss of silencing at *HMR* was observed upon shifting the mutant from the low to the high temperature, but it took >8 hours to reestablish silencing after a shift back to 25°.

Silencing at the rDNA locus was also temperature sensitive, while telomeric silencing was totally defective at both temperatures. In enzymatic assays, the mutant exhibited defective deacetylase activity at both 37° and 25°. Interestingly, the mutant had much more NAD⁺-nicotinamide exchange activity than wild type, as did a mutation in the same region of the protein in the Sir2 homolog, Hst2. Thus, mutations in this region of the NAD⁺ binding pocket of the protein are able to carry out cleavage of NAD⁺ to nicotinamide but are defective at the subsequent deacetylation step of the reaction.

In the studies on Sir3, it was found that Sir3 is mono-sumoylated on K247 and Siz1 is the primary SUMO E3 ligase responsible. In a *ris1*Δ mutant, the level of sumoylated Sir3 increases, while the total Sir3 level is not affected. Mutation of the RING finger or of the ATPase motif of Ris1 also caused the accumulation of Sir3-SUMO. Overexpression of Sir3 causes it to spread from the silent chromatin into euchromatin and is somewhat toxic to yeast. This toxicity depends on Sir2 and Sir4. Abolishment of Sir3 sumoylation, deletion of *ris1*, or both, causes further spreading of Sir3 from the silent chromatin and leads to a more severe growth defect. We propose that down-regulation of sumoylated Sir3 by Ris1

provides a protection mechanism for the cell from inappropriate spreading of Sir3 and improper silencing of transcriptionally active regions.

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Publications

Wang CL, Prugar EJ and Sternglanz R (2009). Regulation of Sir3 by Ris1. (In Preparation)

Wang CL, Landry J and Sternglanz R (2008). A Yeast Sir2 Mutant Temperature- Sensitive for Silencing. *Genetics* 180(4):1955-62.

Schuetz A, Min J, Antoshenko T, Wang CL, Allali-Hassani A, Dong A, Loppnau P, Vedadi M, Bochkarev A, Sternglanz R and Plotnikov AN (2007). Structural basis of inhibition of the human NAD⁺-dependent deacetylase SIRT5 by suramin. *Structure* 15(3):377-89

Wang X, Connelly JJ, Wang CL and Sternglanz R (2004). Importance of the Sir3 N terminus and its acetylation for yeast transcriptional silencing. *Genetics* 168(1):547-5

CHAPTER ONE

Background and Significance

Transcriptional silencing in budding yeast and the roles of the Silent Information

Regulator (SIR) complex

Transcriptional silencing in yeast involves the formation of a specialized chromatin structure that represses transcription in a gene-independent way. Silencing in the budding yeast, *S. cerevisiae*, occurs at the mating-type loci, *HMR* and *HML*, and at telomeres.

This represents a well-studied model for studying heterochromatin formation in metazoans (Li et al, 2001). By forming a condensed chromatin structure, transcription at the yeast silent loci is prevented at either initiation or elongation; the exact mechanism remains unclear (Chen & Widom, 2005; Sekinger & Gross, 2001).

In *S. cerevisiae*, haploid cells exist as one of two mating types, **a** or α , determined by the allele at the mating type locus *MAT*. *MAT^a* and *MAT α* encode regulatory proteins that are responsible for the difference between mating type **a** and α . Yeast also have transcriptionally silenced copies of **a** and α alleles at two additional loci, *HML* (usually α)

and *HMR* (usually **a**). Loss of silencing at these loci as a result of mutation in a silencing factor causes haploid to cells express both **a** and α mating type information and lose the ability to mate (Loo & Rine, 1995). The *HM* loci are each flanked by silencers known as *E* (essential) and *I* (important). Each silencer contains several protein binding sites: ACS (**A**) site for origin replication complex (ORC) binding, RAP1 (**E**) site for Rap1 binding and ABF1 (**B**) site for Abf1 (Figure 1).

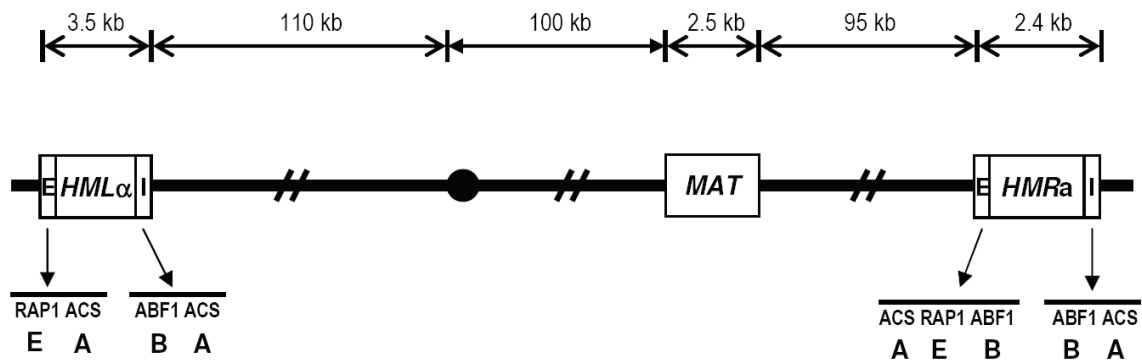


Figure 1: A schematic drawing of *Saccharomyces cerevisiae* chromosome III and the *HM* loci.

These silencer binding proteins recruit the silent information regulator (SIR) proteins to the silencers. There are four Sir proteins: Sir1, Sir2, Sir3 and Sir4 (Figure 2). Sir1 acts in establishing transcriptional silencing by enhancing the recruitment of the other Sir proteins to the silencer (Zhang et al, 2002). Sir1 localizes to silencers by binding to Orc1, one of the six subunits of ORC (Triolo & Sternglanz, 1996). Sir2 is an NAD^+ -dependent histone deacetylase that removes acetyl groups from lysines in the

N-terminal tails of histone H3 and H4, providing the high-affinity binding sites for Sir3 and Sir4 (Rusche et al, 2002). Sir proteins spread from the silencers throughout the target region and form silenced chromatin. Silencing at telomeres is similar to that at *HM* loci. Telomeres have multiple binding sites for Rap1. Rap1 can recruit Sir3 and a Sir4-Sir2 heterodimer and then these Sir proteins spread from telomeres into adjacent chromatin, causing the genes near telomeres to be silenced (Figure 2).

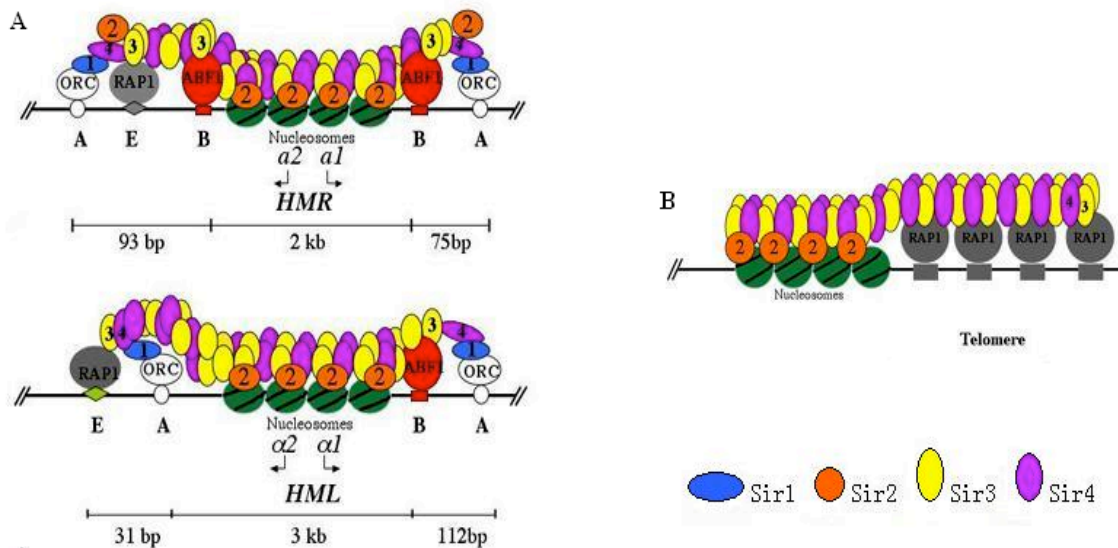


Figure 2: *HML*, *HMR* and telomere silencing. A: *HML* and *HMR* silencing. B: Telomeric silencing.

The enzymatic roles of Sir2, an NAD^+ -dependent deacetylase

Sir2 belongs to a large family of NAD^+ -dependent deacetylase called sirtuins. As shown in Figure 3, a sirtuin deacetylates proteins by cleaving NAD^+ when acetyl lysine is in the enzyme's active site, which then leads to the release of nicotinamide as well as

deacetylated lysine and formation of a novel compound, 2' (3')-O-acetyl-ADP-ribose (AAR) (Landry et al, 2000b; Tanner et al, 2000; Tanny & Moazed, 2001). Sir2 has specificity to deacetylate H4 lysine 16 *in vitro* and *in vivo*, although the enzyme also deacetylates H3 lysine 9 and lysine 14 to a lesser extent (Blander & Guarente, 2004). In Chapter 2, I will present my work on a novel *sir2* mutant called *sir2-276* that was identified in a screen for temperature-sensitive silencing mutants. This *sir2* mutant was further investigated in many different ways, including testing silencing defects at three different silent loci, measuring enzymatic activity at different steps of the reaction, and evaluating the loss of silencing and reestablishment of the silencing at both *HMR* and *HML* by doing temperature shift assays.

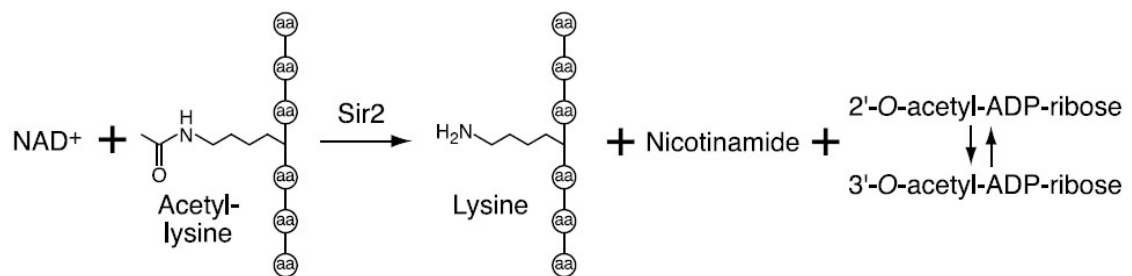


Figure 3: Sir2 is a NAD⁺-dependent deacetylase. Sir2 deacetylates proteins by cleaving NAD⁺ and leads to the release of nicotinamide as well as deacetylated lysine and formation of a novel compound, 2' (3')-O-acetyl-ADP-ribose (AAR). The figure is adapted from (Rusche et al, 2003).

Importance of post-translational modifications for SIR protein-dependent silencing

Many post-translational modifications have been found to be important for silencing. For example, as described in the previous section, deacetylation of histone tails, particularly H4 K16, is critical for the affinity and spreading of Sir proteins. Moreover, our previous lab member, X. Wang, had reported that the N-terminal acetylation of Sir3 is important for its silencing function at both *HM* loci and telomeres (Wang et al, 2004). Of note, Sir3 and Sir4 were also found to be modified by Small ubiquitin-related modifier (SUMO) (Denison et al, 2005). As a result, it would be important to understand if SUMO conjugation on Sir3 or Sir4 has any functional role.

SUMO functions by forming an isopeptide bond between its C-terminal carboxyl group and the ϵ -amino group of a lysine residue in the substrate (Johnson, 2004). Like ubiquitylation, the SUMO conjugation pathway begins with a SUMO E1 ATP-dependent activating enzyme called Aos1/Uba2 in budding yeast. Activated SUMO is then transferred to a SUMO E2 conjugating enzyme called Ubc9. Finally, SUMO is linked to the substrate with the help of one of the SUMO E3 ligases. Both E2 and the E3s contribute to substrate specificity (Figure 4). The lysine residue that can be SUMO-conjugated often resides in a short consensus sequence Ψ KXE (Ψ = I, L or V) (Sampson et al, 2001). This consensus motif is bound by the SUMO E2, Ubc9, directly.

E3s probably enhance specificity by interacting with other parts of the substrate.

However, for many proteins, the lysine sites for sumoylation are not located in the

consensus sequence (Denison et al, 2005). Sumoylation is a reversible modification, and

SUMO is removed from modified proteins by a family of isopeptidases called Ulp

(Figure 4). These SUMO-cleaving enzymes can rapidly desumoylate all conjugates upon

cell lysis, unless cells are lysed under denaturing environments or SUMO-cleaving

enzymes are inhibited.

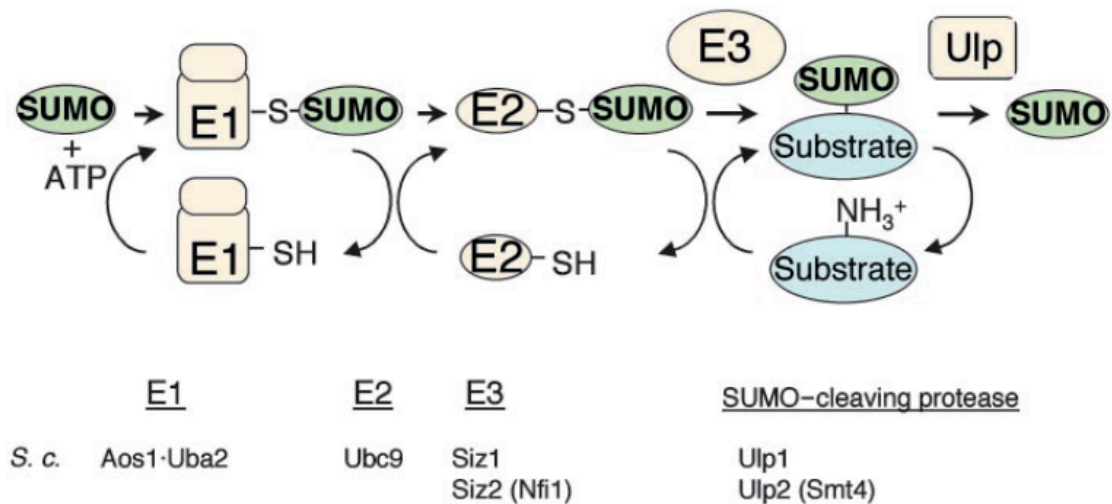


Figure 4: The SUMO conjugation pathway. (top) Enzymes and reactions of the SUMO pathway are described. (bottom) Enzymes present in *S. cerevisiae* (*S.c.*) are listed (Johnson, 2004).

Most SUMO-modified proteins that have been identified in mammalian systems are involved in transcriptional repression (Verger et al, 2003). Genetic studies in some model organisms have shown that SUMO plays a role in chromosome dynamics and higher

order chromatin structures. SUMO can enhance protein-protein interactions by promoting some multi-protein complex to assemble. SUMO was also found to prevent ubiquitylation of a protein by blocking the lysine where Ub would be conjugated (Hoegge et al, 2002).

As mentioned above, both Sir3 and Sir4 were reported to be SUMO conjugated by a global analysis of protein sumoylation in yeast. However, the actual functions and the specific E3 ligase(s) of Sir3/ Sir4 sumoylation are still not known. In Chapter 3, I will present my work on where and how Sir3 is sumoylated. I found that Sir3 is mono-sumoylated on lysine 247 and can be sumoylated at least by one of the yeast major SUMO E3 ligases, Siz1.

Ris1, a Sir4 associated protein, contains 3 distinctive motifs

RIS1, originally called *DIS1*, was isolated in a screen for genes that interfere with silencing when overexpressed (Zhang & Buchman, 1997). It was found that Ris1 participates in yeast mating type switching at a step subsequent to *HO* endonuclease cleavage at the *MAT* locus. The rate of mating type switching was found to be decreased in a *ris1Δ* strain. Two-hybrid studies have shown that the N-terminal half of Ris1 can interact with the C-terminal half of Sir4. In a series of one-hybrid screens, our lab also showed that Ris1 has targeted silencing ability that is Sir2- Sir3- and Sir4- dependent

(Andrulis et al, 2004), presumably because it can bind Sir4. The role of Ris1 in Sir protein-dependent silencing is still not understood.

Three distinctive motifs have been identified in Ris1 (Figure 5). First, Ris1 belongs to a family of the SWI2/SNF2-like DNA-dependent ATPases. Biochemical studies showed that *E. coli*-expressed Ris1 has DNA-stimulated ATPase activity (Zhang Zemin, PhD thesis, 1995). In addition, Ris1 contains a RING finger motif, which resembles ubiquitin E3 ligases. Lastly, it was reported that Ris1 can bind to SUMO non-covalently and potential SUMO-interacting motifs (SIMs) were determined (Hannich et al, 2005; Uzunova et al, 2007). Recently, it was also found that Ris1 can associate with a sumoylated nucleolar protein called Ebp2 through Ris1's own SUMO interacting motifs (Shirai & Mizuta, 2008).

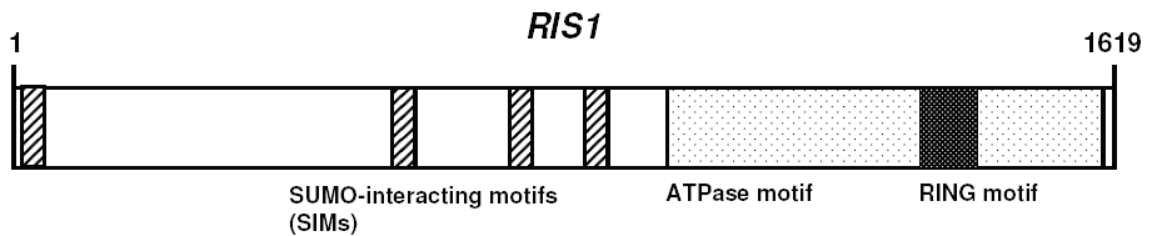


Figure 5: A schematic drawing of Ris1. The SUMO interacting motifs, the ATPase motif and the RING finger motif of Ris1 were labeled and indicated.

In a global mapping of yeast genetic interactions, a *RIS1* deletion displayed synthetic sickness with deletion alleles of either *SLX5* or *SLX8* (Pan et al, 2006). *SLX5*

and *SLX8* were first identified in a synthetic lethal screen. They are essential genes when the Sgs1/ Top3 pathway in yeast is abolished (Mullen et al, 2001). Slx5 and Slx8 were also found to form a heterodimer and play important roles in maintenance of genomic stability (Yang et al, 2006; Zhang et al, 2006). Of note, like Ris1, both Slx5 and Slx8 also contain SUMO-interacting motifs and a RING finger motif (Ii et al, 2007a; Uzunova et al, 2007; Xie et al, 2007). In recent studies, as shown in Figure 6, this new type of protein, named a SUMO-targeted ubiquitin ligase (STUbL), can specifically bind to SUMO-conjugated proteins by the SUMO interacting motifs and then poly-ubiquitylate and degrade its targets via the RING finger motif (Perry et al, 2008). The function of Slx5/Slx8 in targeting sumoylated proteins is known to be correlated with DNA damage pathway by targeting and degrading SUMO-conjugated Rad52 and Rad57 (Ii et al, 2007a; Xie et al, 2007). Very recently it was shown that Slx5/Slx8 heterodimer can also function as a quality control of a transcription regulator, Mot1, by down-regulating the level of the protein once it is sumoylated (Wang & Prelich, 2009b). For Ris1, it is only known that Ris1 can bind to a SUMO conjugated- Ebp2 (yeast homolog of human Epstein-Barr virus nuclear antigen 1-binding protein 2) through its SUMO interacting motif (Shirai & Mizuta, 2008). However, it is unclear if SUMO-Ebp2 degradation is regulated by Ris1. As a result, the role of Ris1 is still not as clear as it is for the Slx5/Slx8 heterodimer.

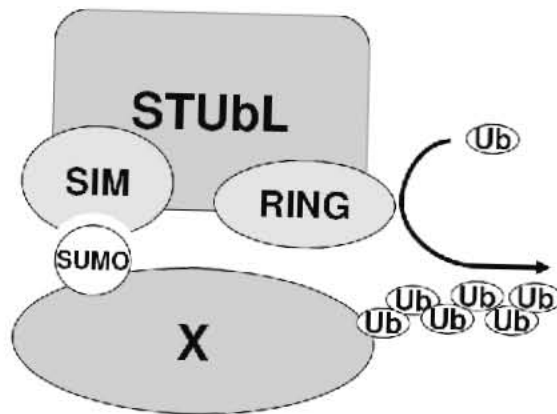


Figure 6: Schematic representation of a STUbL with its sumoylated substrate. A SUMO-targeted ubiquitin ligase, or STUbL, can specifically bind to SUMO-conjugated proteins by the SUMO interacting motifs (SIMs) and then poly-ubiquitylate and degrade its targets via the RING finger motif (RING).

In Chapter 3, I first present my work on where and how Sir3 is sumoylated. In the second half of Chapter 3, I will present my studies on sumoylated Sir3 and on the role of Ris1 in lowering the level of Sir3-SUMO. I demonstrate that Ris1 is important for regulating the spreading of Sir3 on chromatin. Moreover, I describe specific mutations of the SUMO interacting motif, the RING finger motif or the ATPase motif of Ris1. I show which of the motifs is important for targeting Sir3-SUMO and for genetic interactions with *slx5Δ*.

CHAPTER TWO

A Yeast Sir2 Mutant Temperature-Sensitive for Silencing

Most of the work in this chapter was published in *Genetics* 2008; 180(4):1955-62.

The results shown in this paper all were obtained by me except for the NAD⁺-exchange assays with Hst2 mutants, which were done by our former lab member, Dr. Joseph Landry.

Introduction

S. cerevisiae Sir2 protein is the founding member of a large family of NAD⁺-dependent deacetylases, so-called sirtuins, conserved from bacteria to mammals (Ramachandran et al, 2000). These enzymes deacetylate lysines on proteins in an unusual reaction in which NAD⁺ is cleaved, if and only if acetyl lysine is in the active site of the enzyme, releasing nicotinamide and subsequently deacetylating the lysine to form a novel a compound, 2'-O-acetyl-ADP- ribose (AAR, Chapter1, Figure 3) (Tanner et al, 2000; Tanny & Moazed, 2001). Nicotinamide is a noncompetitive inhibitor of these enzymes

(Landry et al, 2000a). The Sir2 family of NAD^+ -dependent deacetylases acts in two steps (Figure 7) (Jackson et al, 2003; Landry et al, 2000a; Sauve & Schramm, 2003). In the first reversible step, NAD is cleaved, releasing nicotinamide. In the second step, which is irreversible, the protein substrate is deacetylated, releasing 2'-*O*-acetyl-ADP ribose.

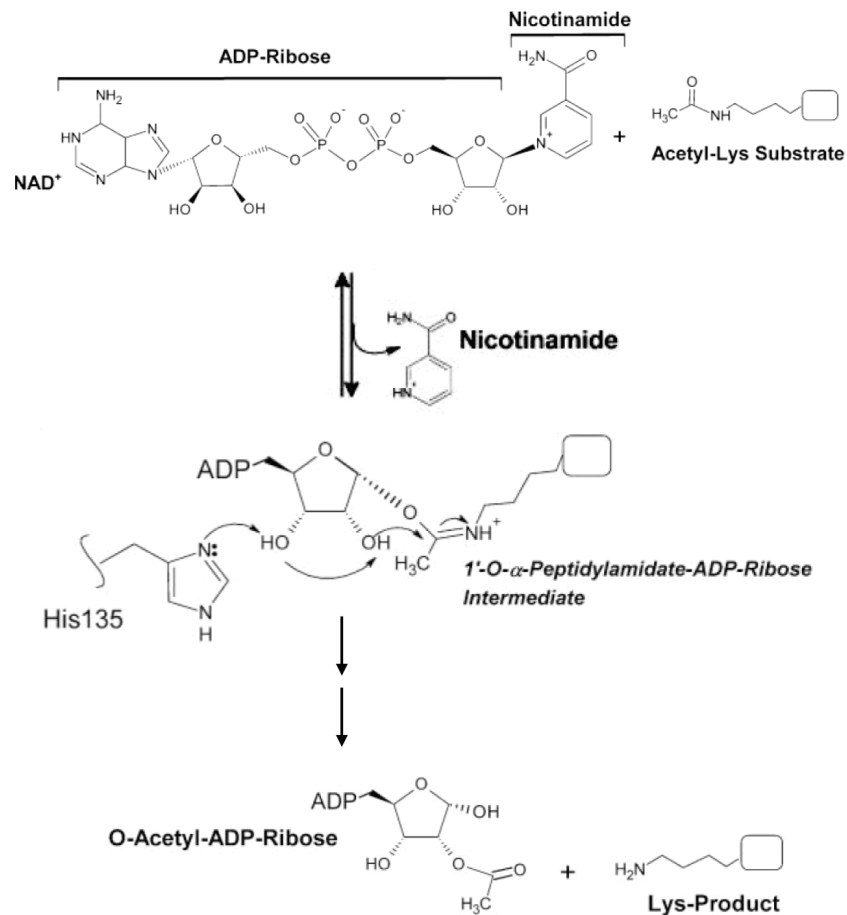


Figure 7: Schematic diagram of steps involved in the Sir2 reaction mechanism. (Khan & Lewis, 2006)

Several assays for sirtuins are available, including direct measurement of deacetylation, or the release of nicotinamide, NAD^+ hydrolysis during deacetylation, or

NAD⁺-nicotinamide exchange (Landry & Sternglanz, 2003). In the latter reaction, nonradioactive NAD⁺ is incubated with radioactive nicotinamide in the presence of enzyme and an acetylated substrate. Extent of the reaction is determined by the production of radioactive NAD⁺.

Yeast Sir2 is one of four Silent Information Regulator (SIR) proteins involved in transcriptional silencing of the silent mating-type loci, *HML* and *HMR*, as well as genes near telomeres (Figure 2) (Gasser & Cockell, 2001; Rusche et al, 2003). It facilitates silencing by deacetylating histone H4 lysine 16, thereby forming a binding site for a Sir2, Sir3, Sir4 complex on nucleosomes (Rudner et al, 2005). Sir2 is also present in a different protein complex at the rDNA (Shou et al, 1999; Straight et al, 1999); its role within the rDNA array is to suppress recombination and silence RNA polymerase II transcription (Gottlieb & Esposito, 1989; Smith & Boeke, 1997), presumably by deacetylating lysines on histones.

Studies on establishment and maintenance of silencing at the *HM* loci have been aided by the use of a *sir3* temperature-sensitive (ts) mutant, *sir3-8* (Lau et al, 2002; Miller & Nasmyth, 1984). These studies have shown that inactivating Sir3 by raising the temperature to 37° causes an immediate loss of silencing, whereas cells shifted from 37° to the permissive temperature, 25°, must pass through S phase and G₂/M of the cell cycle

to reestablish silencing. A similar conclusion was reached by workers who used special strains in which silencing was absolutely dependent on Sir1 (Kirchmaier & Rine, 2001; Li et al, 2001). In those cases, reestablishment of silencing by turning on expression of Sir1 also required passage through the cell cycle and could not occur in cells held in G₁. Interestingly, a recent study using a galactose-regulated promoter to control Sir3 expression suggested that reestablishment of the *fully* silent state required several generations (Katan-Khaykovich & Struhl, 2005). Here I identified a *sir2* temperature-sensitive point mutation that changes a single highly conserved residue in the active site of the enzyme. I characterized its effect on silencing at all four loci where it acts, and its enzymatic properties *in vitro*.

Material and methods

Strains and plasmids

Strains and plasmids used in this chapter are listed in Table 1 and Table 2, respectively. Strains used for the complementation test with strain TS1 were KN207, KN877, RS9 and KN9b. Yeast deletion mutants were constructed using standard methods (Longtine et al, 1998). *SIR2* and *sir2-276* fragments, containing 300 bp both upstream and downstream of the open reading frame, were amplified from genomic DNA of yeast

strains RS1 and TS1, respectively, using primers CLP46 (5'-CCGCTCGAGTTTCTTTG-ACCCAACGCCTT-3') and CLP47 (5'-CCGGAATTCATCTAGCACTCCTTCCAACCA-3'). Amplified products were digested with *EcoRI* and *XhoI* and cloned into the vector pRS316 (*URA3*, *CEN6*) to make pCLW18 and pCLW19, respectively. The same fragments were cloned into the vector pRS313 (*HIS3*, *CEN6*) to make pCLW21 and pCLW22, respectively. The entire *SIR2* and *sir2-276* coding regions were sequenced to confirm that there were no amplification-induced mutations. The GST-tagged *SIR2* *E. coli* expression plasmid, pDM111a, was obtained from D. Moazed (Tanny et al, 1999). The *sir2-276* coding sequence was amplified from the genomic DNA of yeast strain TS1 by using primers CLP44 (5'-CCGGAATTCATGACCATCCCACATATG-3') and CLP45 (5'-CCGCTCGAGTTAGAGGGTTTTGGGATG-3'). Amplified product was digested with *ClaI* and *XhoI*. The *ClaI-XhoI* fragment, which contains the S276C mutation, was used to replace the *ClaI-XhoI* fragment of pDM111a to create pCLW20. The *HST2* *E. coli* expression plasmid pJWL03 was constructed as described (Landry et al, 2000b). *hst2* R45A, H135A, Q115A and E128A mutant expression plasmids were made from pJWL03 with a site-directed mutagenesis kit (Stratagene) to create pJWL41, pJWL43, pJWL46 and pJWL47, respectively, as described (Min et al, 2001).

Mating assays

For patch mating assays, cells transformed with the indicated plasmids were patched onto the appropriate synthetic complete selective medium and grown at 25° or 37° for 1 day. Patches were transferred onto yeast-peptone-dextrose (YPD) medium by replica plating along with the appropriate mating tester strain (DC16 or DC17). After 6 hours, these cells were transferred by replica plating onto synthetic dextrose (SD) plates to select for diploids. Diploids were allowed to grow at 25° or 37° for 2 days. For quantitative mating assays, cells were grown in liquid YPD medium at 25° or 37° to exponential phase. Cells were diluted in YPD and plated onto YPD to count the total number of cells. Appropriate dilutions were also plated onto SD with 10^7 cells of an exponentially growing mating tester strain (DC16 or DC17) at 25° or 37°. Mating efficiency was calculated as the fraction of cells that mated. All values are the means of data from three independent assays.

Quantitative RNA measurements

Cells were grown overnight in YPD and used to inoculate 30 ml of fresh YPD. Cells were then grown to an $A_{600} \sim 0.8$, harvested, washed with diethyl pyrocarbonate-treated water and total RNA purified according to the protocol described (Ambion). Before cDNA synthesis, PCR controls were performed to confirm the absence of chromosomal DNA. To generate cDNA, 2 μ g of total RNA was used in a 20- μ l

reaction with reverse transcriptase (Invitrogen). Two microliters of each cDNA reaction was amplified with a thermal cycler set of 94° for 4 minutes and then 23 cycles (for detection of *a1* or *PMAl* transcript in *MATα* strains) or 33 cycles (for detection of $\alpha 1$ or *PMAl* transcript in *MATa* strains). *a1* transcript was detected from cDNA using primers as described previously (Smeal et al, 1996). $\alpha 1$ transcript was detected using CLP52 (5'-TCCAGATTCCTGTTCCCTTCCT-3') and CLP53 (5'- CATTCTTCAGCGAGCAG-AGAA-3'). *PMAl* transcript was detected using PMA1-2018 (5'-CTATTATTGAT-GCTTTGAAGACCTCCAG -3') and PMA1-2290 (5'-TTATGGGGTATGTCTATTAT-TTTGGGCA-3'). PCR products were resolved on 2% agarose gel stained with ethidium bromide. For radioactive PCR, the same amounts of cDNAs were analyzed by standard reaction mixtures to which 1 μ Ci of [³²P]dATP (3 Ci/ μ mol) had been added. Products were resolved on a 2% agarose gel and quantified using a Storm840 scanner and ImageQuant software (Molecular Dynamics).

For temperature-shift experiments overnight cultures were grown at 25° or 37° for 2hr and then shifted to 37° or 25°, respectively. Cells were collected at different time points in 3 (25°-37 ° shift) to 12hr (37°-25 ° shift) and total RNA isolated for RT-PCR by using primers that can specifically amplify *HMRal* transcripts. Primers that can amplify *PMAl* transcripts were also used as a control.

Silencing assays

Strains CCF100, RS2350 and RS2351 were used to measure telomeric silencing.

Strains were grown in YPD for 1 day at 25 °C or 37 °C and then plated in five-fold serial dilutions on YPD medium and synthetic complete (SC) medium containing 0.1%

5-fluoroorotic acid (5-FOA). For rDNA silencing assays, strains RS2364 and RS2365

transformed with plasmids pCLW21 (*SIR2*), pCLW22 (*sir2-276*) or pRS313 (vector)

were grown in SC-His-Leu medium at 25° or 37° for 1 day. Cells were spotted in fivefold

serial dilutions on SC-His and SC-His-Leu + 5-FOA medium. Plates were incubated at 25

°C and 37 °C respectively for 2 to 4 days, depending on medium and temperatures.

Protein expression in *E. coli*

Sir2 and Sir2 S276C were expressed in *E. coli* BL21(DE3) from pDM111a and

pCLW20 after a 3-hour induction with 0.5 mM isopropyl β -D-thiogalactoside (IPTG) at

room temperature. Proteins were purified using Glutathione Sepharose 4 Fast Flow

Affinity Resin (Amersham) according to the manufacturer's instructions. Hst2 and

mutants were expressed from pJWL03 (wild type), pJWL41 (R45A), pJWL43 (H135A),

pJWL46 (Q115A) and pJWL47 (E128A) in *E. coli* BL21(DE3) and purified as described

(Landry et al, 2000b). Purified proteins were dialyzed against 50 mM sodium phosphate

(pH 7.2) and frozen at -80° in 50 mM sodium phosphate (pH 7.2), 0.5 mM DTT and 10%

glycerol. Protein concentrations were estimated by comparing Coomassie brilliant blue staining of samples with BSA standards, analyzed by SDS/PAGE.

NAD⁺ hydrolysis assays

Reactions were carried out as previously described (Landry & Sternglanz, 2003), using 50 μ M H4 peptide or H4 lysine 16-acetylated peptide, 1 μ Ci [4-³H]NAD (0.37 Ci/mmol), and 50 - 150 ng purified Sir2 or Sir2 S276C. Reactions were incubated at 25° or 37° for 1 hour and terminated by adding 13.5 μ l 0.5 M sodium borate (pH 8.0), and quenching on ice. Released nicotinamide was extracted by the addition of 0.5 ml of water-saturated ethyl acetate. After vortexing, samples were centrifuged at 16,000 x g for 2 min. 0.35 ml of the ethyl acetate fraction was transferred to 3 ml of Ecoscint scintillation fluid and the released nicotinamide quantified in a liquid scintillation counter.

Deacetylation assays

Reactions were carried out as previously described (Landry & Sternglanz, 2003), using 50 μ M H4 peptide or H4 lysine 16-acetylated peptide, 125 μ M NAD, and 100 ng Sir2 or Sir2 S276C. Reactions were incubated at 25° or 37° for 1 hour and terminated by adding 13.5 μ l 0.5 M sodium borate (pH 8.0), and quenching on ice. 1 μ l of each reaction

was spotted to a nitrocellulose membrane and histone deacetylation was detected by western blotting with an anti-histone H4 acetyl K16 antibody (Serotec).

NAD⁺-nicotinamide exchange assays

Assays were performed as described previously (Landry et al, 2000b) at 30° for 1 hr in 20 µl with 0.5 mM NAD (Sigma N-1511), enzymes (200 ng to 600 ng for Sir2 or Sir2 S276C; 1.3 ng for Hst2 mutants), 0.1 mM [¹⁴C]nicotinamide (Sigma N-2142, 53.1 mCi/mmol), 50 mM sodium phosphate (pH 7.2), 0.5 mM DTT and 0.2 mg/ml Chicken erythrocyte histones. After incubation, 8 µl of each reaction was spotted to Whatman HPKF Silica Gel 60 A thin layer chromatography plates. The plates were developed in a preequilibrated chamber with 80:20 ethanol: 2.5 M ammonium acetate. After chromatography, the plates were air dried, sprayed with EN³HANCE (NEN NEF981) and exposed to film (Kodak X-Omat AR) at -80 °C for 24 hr.

Table 1: Strains used in Chapter 2

Strain	Genotype
KN207	<i>MATa sir1-1 ade6 arg4 leu2 trp1</i>
KN877	<i>HMLa MATa HMRa sir2-275 leu2-1 can1-100 trp1-1 his3 his4 ade2-1 Met⁻</i>
RS9	<i>HMLa MATa HMRa sir3::LEU2 leu2-1 can1-100 trp1-1 his3 his4 ade2-1 Met⁻</i>
KN9b	<i>HMLa MATa HMRa sir4 ade2 his4 leu2 tyr can1 trp1 ura3</i>
W303-1a	<i>MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1</i>
W303-1b	<i>MATα ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1</i>
DC16	<i>MATa his1</i>
DC17	<i>MATα his1</i>
RS869	W303-1a <i>sir2Δ::URA3</i>
RS1717	W303-1b <i>sir2Δ::his5⁺</i>
RS1	W303-1b <i>HMR/TRP1</i>
TS1	W303-1b <i>HMR/TRP1 sir2-276</i>
AMR1	W303-1a <i>nat1-5::LEU2</i>
RS2291	W303-1b <i>sir2-276</i> (Segregant of TS1 x AMR1)
RS2292	W303-1a <i>sir2-276</i> (Segregant of TS1 x AMR1)
CCF100	W303-1a <i>HMRΔE::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>
RS2350	<i>MATα HMRAE::TRP1 rDNA::ADE2-CAN1 TELVR::URA3 sir2-276</i> (Segregant of CCF100 x RS2291)
RS2351	<i>MATα HMRAE::TRP1 rDNA::ADE2-CAN1 TELVR::URA3 sir2-276</i> (Segregant of CCF100 x RS2291)
DS2804	W303-1a <i>NTS1::mURA3-LEU2</i>
DS2805	W303-1b <i>NTS1::mURA3-LEU2</i>
RS2364	DS2804 <i>sir2Δ::kanMX6</i>
RS2365	DS2805 <i>sir2Δ::kanMX6</i>

Table 2: Plasmids used in Chapter 2

Name	Description
pCLW18	pRS316 <i>SIR2</i> (<i>URA3</i> , <i>CEN6</i> , its own promoter)
pCLW19	pRS316 <i>sir2-276</i> (<i>URA3</i> , <i>CEN6</i> , its own promoter)
pCLW21	pRS313 <i>SIR2</i> (<i>HIS3</i> , <i>CEN6</i> , its own promoter)
pCLW22	pRS316 <i>sir2-276</i> (<i>HIS3</i> , <i>CEN6</i> , its own promoter)
pDM111a	pGEX-5X-1 <i>SIR2</i> (<i>GST-SIR2</i>)
pCLW20	pGEX-5X-1 <i>sir2-276</i> (<i>GST-sir2-276</i>)
pJWL03	pET28b <i>HST2</i> (<i>His₆-HST2</i>)
pJWL41	pET28b <i>hst2 R45A</i> (<i>His₆-hst2 R45A</i>)
pJWL43	pET28b <i>hst2 H135A</i> (<i>His₆-hst2 H135A</i>)
pJWL46	pET28b <i>hst2 Q115A</i> (<i>His₆-hst2 Q115A</i>)
pJWL47	pET28b <i>hst2 E128A</i> (<i>His₆-hst2 E128A</i>)

Results

Identification and characterization of a *sir2* ts mutant

In order to identify temperature-sensitive (ts) silencing mutants, a yeast strain, RS1, with a *TRP1* reporter gene at *HMR* was used (Brand et al, 1985). This strain could not grow without tryptophan in the medium due to silencing of the *TRP1* gene (a Trp⁻ phenotype). Spontaneous mutants were selected that were Trp⁺ at 37° but Trp⁻ at 25°. One such mutant, TS1, was analyzed further by crossing it with *sir1*, *sir2*, *sir3* or *sir4* mutants of opposite mating type (see Material and methods and Table 2 for a description of these strains). Analysis of the Trp phenotype of the resultant diploids demonstrated that the mutation in TS1 failed to complement the *sir2* mutation and thus suggested the silencing defect in TS1 was due to a mutation in the *SIR2* gene. Further evidence that the mutation was in the *SIR2* gene came from analysis of a cross of strain TS1 with a *nat1::LEU2* mutant (Mullen et al, 1989). *NAT1* and *SIR2* are adjacent genes and therefore recombination between them should be extremely rare. Eleven tetrads resulting from this cross were analyzed. All were Parental; i.e., all the Leu⁻ segregants exhibited ts silencing and/or a ts Trp phenotype while none of the Leu⁺ segregants did. This demonstrated that the ts silencing mutation was tightly linked to *NAT1* and thus consistent with a *sir2* mutation.

Therefore the *SIR2* gene was amplified from strain TS1 by PCR and sequenced. A single nucleotide change was found when the sequence was compared to that of the parental wild-type strain. It was predicted to change Ser276 to Cys. The amplified *SIR2* fragment from both the mutant and the wild-type parent was cloned into a yeast shuttle vector. The two resulting plasmids were transformed into *sir2* Δ strains and mating of the transformants tested. As seen in Figure 8, mating is normal at both 25° and 37° for wild-type *SIR2* but is ts for the mutant. This demonstrates conclusively that mutation causing the ts silencing defect is in *SIR2*.

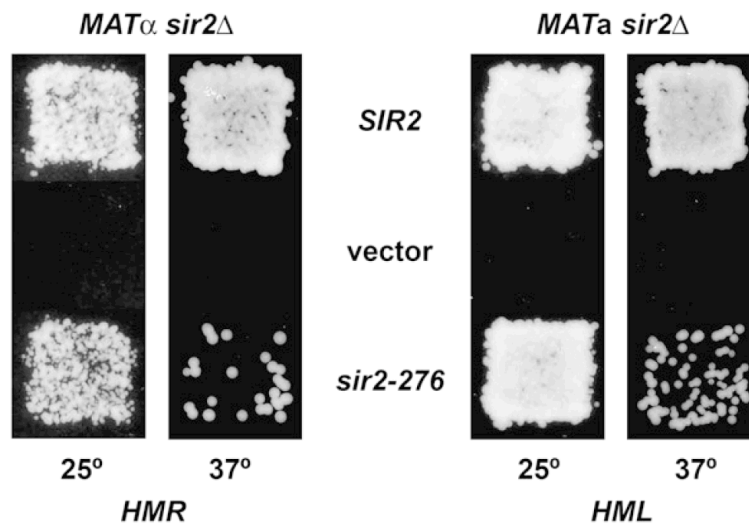


Figure 8: The *sir2-276* mutation confers a temperature-sensitive mating defect.

The indicated strains were transformed with a plasmid expressing *SIR2*, a vector or a plasmid expressing *sir2-276* (pCLW18, pRS316, and pCLW19 for the *MAT α sir2* strain, RS1717; pCLW20, pRS313, and pCLW21 for the *MATa sir2* strain, RS869). Mating was measured at 25° or 37° as indicated.

This allele was named *sir2-276*. The mutation was also crossed into strains of both α and α mating type. Again mating of these strains was seen to be ts, as judged by both patch mating (not shown) and quantitative mating (Table 3). Note that mating was about 10-fold worse at 37° in the *MAT α* strain than in the *MAT α* strain, suggesting that silencing was more defective at *HMR* than at *HML*.

Table 3: Quantitative mating results of *SIR2* and *sir2-276* strains

	<i>MATα</i>		<i>MATα</i>	
	25 °C	37 °C	25 °C	37 °C
<i>SIR2</i>	1	1	1	1
<i>sir2-276</i>	7.0×10^{-1}	3.9×10^{-5}	1.3	1.6×10^{-4}

Quantitative RT-PCR was used to measure $\alpha 1$ RNA from *HMR* in the *MAT α* strain and $\alpha 1$ RNA from *HML* in the *MAT α* strain (Figure 9, A and B). This confirmed that the mutation conferred a silencing defect and it was greater at *HMR* than at *HML*. The data also showed that some RNA from *HMR* was detected even at 25°, showing that the Sir2 protein from the mutant is somewhat defective even at a lower temperature.

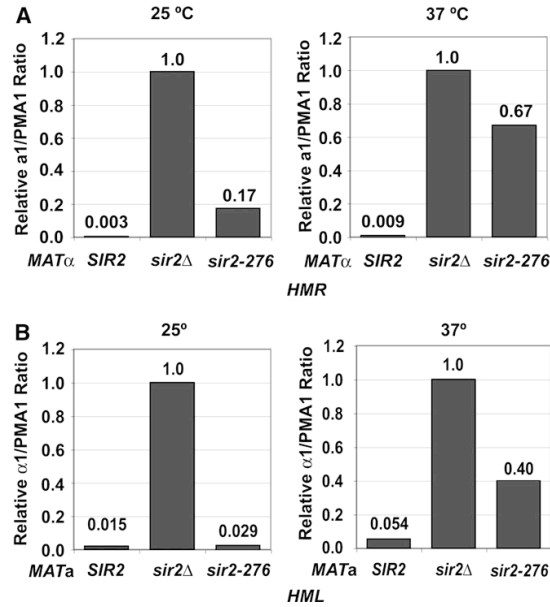


Figure 9: The *sir2-276* mutation has a temperature-sensitive silencing defect at the *HM* loci. Expression from the *HM* loci of *SIR2*, *sir2Δ*, or *sir2-276* strains was determined by RT-PCR. (A) cDNA derived from RNA from *MATα* strains grown at either 25° or 37° was amplified with primers to *a1* and *PMA1*. The a1/PMA1 ratios were calculated and shown relative to the value for the *sir2Δ* strain, which was set to 1. (B) cDNA from *MATa* strains cultured at either 25° or 37° was amplified with primers to *α1* and *PMA1*. The α1/PMA1 ratios were calculated and shown as in A.

The *sir2-276* mutation was also introduced into a strain with a *URA3* reporter gene near a telomere. As can be seen in Figure 10, the mutation led to a complete loss of telomeric silencing at both 25° and 37°. To assess rDNA silencing, plasmids containing either wild-type *SIR2* or *sir2-276* were transformed in two *sir2Δ* strains with a *URA3* reporter gene in the rDNA array. The rDNA silencing of the transformants were then tested. As shown in Figure 11, silencing at rDNA was normal at both 25° and 37° for wild-type *SIR2* but was somewhat defective in the mutant at 37° for each of the two

strains tested. In summary, *sir2-276* causes a temperature-sensitive silencing defect at both *HM* loci and rDNA and a complete silencing defect at telomeres.

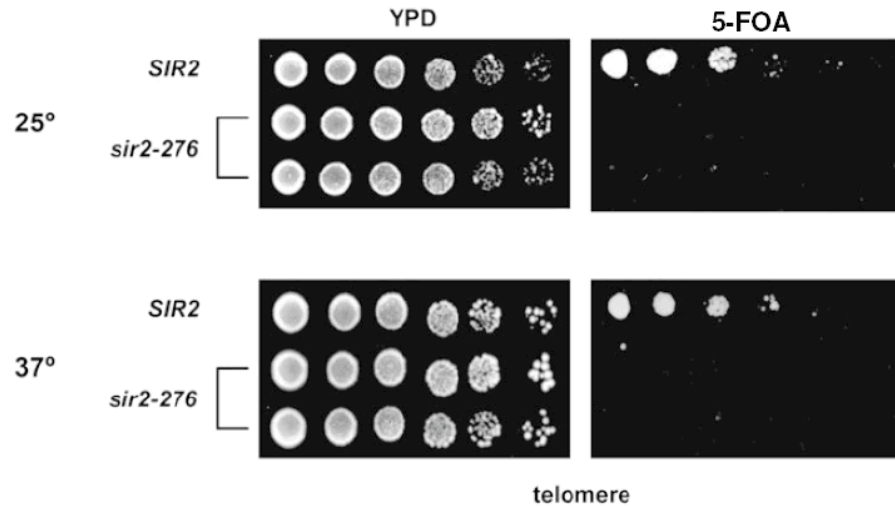


Figure 10: The *sir2-276* mutation confers a complete silencing defect at telomeres.

Fivefold serial dilution of overnight cultures grown in YPD medium of *SIR2* and *sir2-276* strains containing a *URA3* reporter gene near telomere VR were spotted onto YPD plates and 5-FOA plates at either 25° or 37°. Good growth on 5-FOA plates means good silencing.

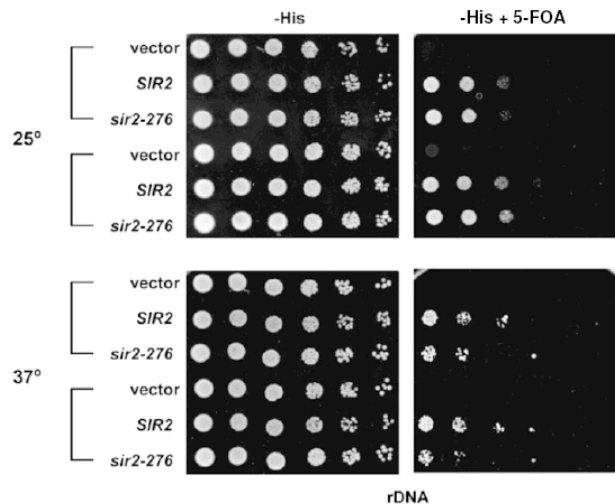


Figure 11: The *sir2-276* mutation confers a temperature-sensitive silencing defect at rDNA.

Two *sir2Δ* strains containing a *URA3* reporter gene within the rDNA, were transformed with a *SIR2* plasmid, a plasmid expressing *sir2-276*, or vector. Transformed strains were grown overnight in SC -His -Leu medium at 25° or 37° and fivefold serial dilutions spotted on selective medium without and with 5-FOA.

To determine if the mutant Sir2 protein was degraded at 37°, Westerns were performed on extracts from cells grown at 25° and 37°. As seen in Figure 12, the mutant protein is present in amounts similar to that of the wild-type strain at both temperatures. Thus, the observed silencing defect of the mutant is due to loss of function of the mutant protein, not to its degradation.

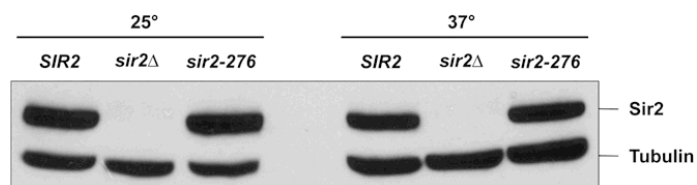


Figure 12: The Sir2-276 mutant protein is stable at 37°. A Western with an antibody to Sir2 showed that similar amounts of Sir2 were present in wild-type cells and the *sir2-276* mutant grown at 25° or at 37°. No signal was seen for a *sir2Δ* strain. An antibody to tubulin was used to show that similar amounts of total protein were present in all lanes.

Studying reestablishment of silencing at *HMR* and *HML* by temperature shifts using *sir2-276*

Previous experiments with a *sir3* ts mutant showed that mutant cells shifted from a low to a high temperature lose silencing rapidly while cells shifted from a high temperature to a low temperature require many hours and passage through the cell cycle to regain full silencing (Lau et al, 2002; Miller & Nasmyth, 1984). I wanted to determine whether the same behavior would be seen with the *sir2-276* mutant. As seen in Figure 13, when cells were shifted from 25° to 37° (left), silencing at *HMR* was lost rapidly. On the other hand, when cells were shifted from 37° to 25° (Figure 13, right), it took more than 8

hours to see significant silencing of *HMR*. These results suggest that *sir2-276*, similar to *sir3-8*, also needs to pass through the cell cycle to reestablish complete silencing.

A similar experiment was done to observe the reestablishment of silencing at *HML*. Surprisingly, in contrast with what I observed at *HMR*, when cells were shifted from 37° to 25°, silencing at *HML* was reestablished within a relatively shorter time in 4 hours (Figure 14, right). This unusual result implies that the kinetics for reestablishing silencing differ for *HMR* and *HML*. Possible mechanisms and further experiments to distinguish silencing reestablishment at these 2 silent mating type loci will be described in the Discussion and Future plans sections of this chapter.

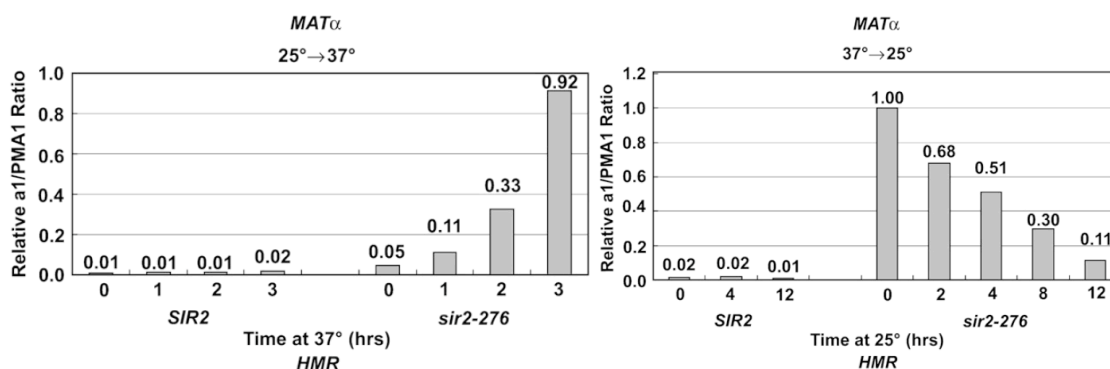


Figure 13: Measurements of $a1$ mRNA from *HMR* after temperature shifts of *MAT α* *SIR2* and *sir2-276* strains. Cells were grown at 25° for 2 hr and raised to 37° for 0, 1, 2, and 3 hr (left), or grown at 37° for 2 hr and dropped to 25° for 0, 2, 4, 8, and 12 hr (right). cDNAs collected at different time points were amplified with primers to *a1* and *PMA1* and RNA was quantified as in Figure 8.

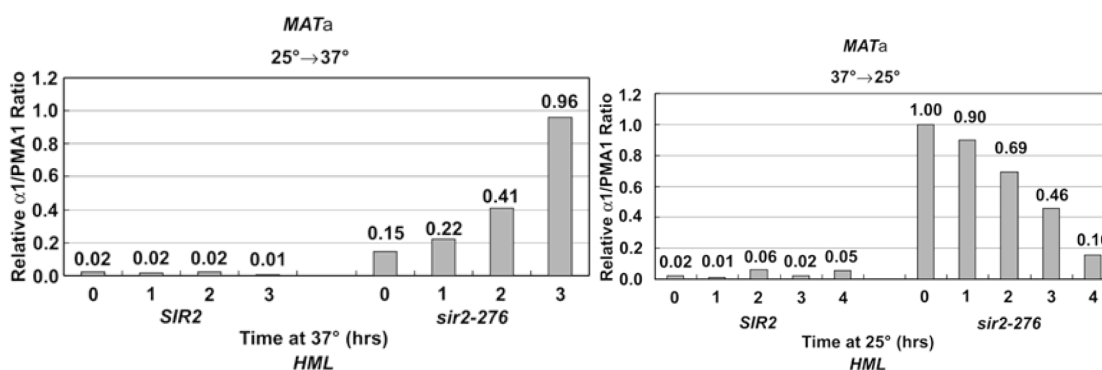


Figure 14: Measurements of $\alpha1$ mRNA from *HML* after temperature shifts of *MAT α* *SIR2* and *sir2-276* strains. Cells were grown at 25° for 2 hr and raised to 37° for 0, 1, 2, and 3 hr (left), or grown at 37° for 2 hr and dropped to 25° for 0, 1, 2, 3, and 4 hr (right). cDNAs collected at different time points were amplified with primers to $\alpha1$ and *PMA1* and RNA was quantified as in Figure 8.

***In vitro* enzymatic assays of recombinant Sir2-276 mutant protein**

The Ser276Cys mutation was introduced into a Sir2 plasmid used to express the recombinant protein in *E. coli*. Both the wild-type and mutant recombinant proteins were purified using glutathione beads and then assayed for enzymatic activity. First, an H4 peptide acetylated on Lys16 was assayed by an NAD⁺ hydrolysis assay (Landry & Sternglanz, 2003). As seen in Figure 15, the mutant protein had less activity than the wild-type protein at both 25° and 37° but retained some activity at both temperatures. Note that no activity for either protein was seen with an unacetylated H4 peptide, as expected.

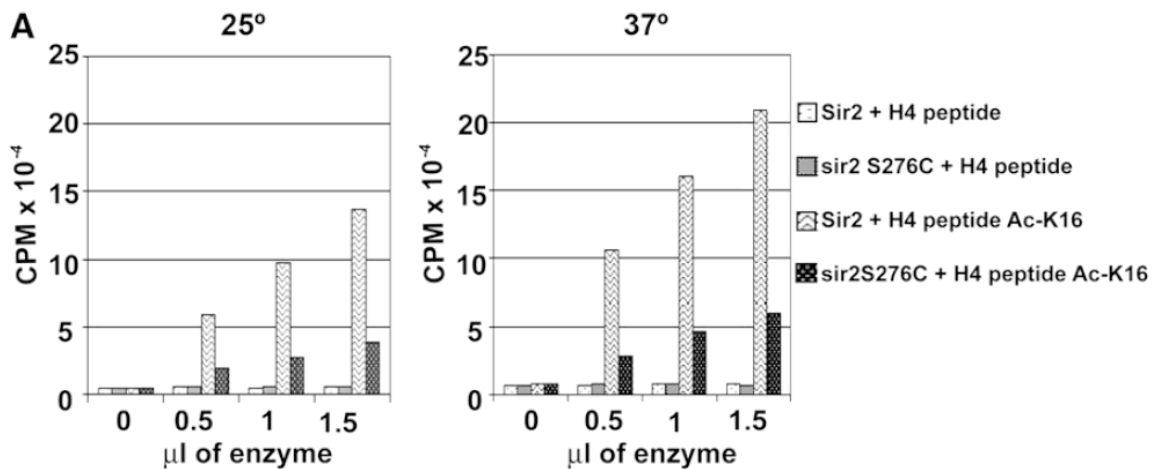


Figure 15: NAD⁺ hydrolysis assays with recombinant Sir2 and Sir2 S276C. Assays were performed by incubating enzyme (0–1.5 µl of Sir2 or Sir2 S276C, 100 ng/µl), radioactive NAD⁺ labeled on the nicotinamide moiety, and H4 peptide (unacetylated or acetyl-K16) at 25° or 37° for 1 hr. Activity was determined by measuring the amount of [³H]nicotinamide released. The Sir2 S276C mutant protein shows defective NAD⁺ hydrolysis activity at both temperatures.

In another assay, deacetylation of the acetylated H4 peptide was measured directly by use of an antibody specific to H4 acetyl-Lys16. A Western blot of the reactions demonstrated good NAD⁺-dependent deacetylation activity for the wild type protein but little or no activity for the mutant at both temperatures (Figure 16).

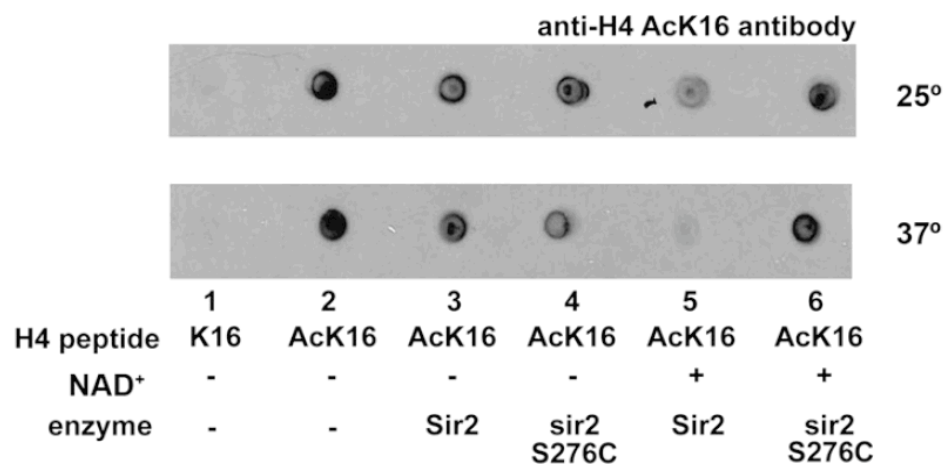


Figure 16: Deacetylase assays with recombinant Sir2 and Sir2 S276C. The assays were performed by incubating enzyme (100 ng of Sir2 or Sir2 S276C), +/- NAD⁺, and H4 peptide (unacetylated or acetyl-K16) at 25° or 37° for 1 hr. The reaction mixture was spotted onto paper and activity was determined by probing with an anti-H4 acetyl-K16 antibody. Deacetylase activity is shown by a weaker response to the H4 acetyl-K16 antibody. As in Figure 14, Sir2 S276C shows defective deacetylase activity at both temperatures.

Next I assayed both proteins using the NAD⁺-nicotinamide exchange assay to observe the reverse activity in the first step of Sir2 enzymatic reaction. As mentioned, the reaction was determined by the production of radioactive NAD⁺ when nonradioactive

NAD⁺ was incubated with radioactive nicotinamide in the presence of enzyme and an acetylated substrate (Landry & Sternglanz, 2003). A surprising result was obtained. The mutant reproducibly showed greater exchange activity than wild type (Figure 17). The same protein preparations were used in the two deacetylase assays described above as in this exchange assay. Since the mutant protein had less activity in both deacetylase assays, the greater NAD⁺-nicotinamide exchange activity seen is a real effect and cannot be due to a higher concentration of functional mutant protein. This result was reminiscent of an unpublished result our former lab member, Dr. Joseph Landry, had obtained previously with a particular Hst2 mutant. As shown in Figure 18, an Hst2 Arg45Ala mutant also had greater exchange activity than wild type, whereas three other point mutants in conserved residues have lower activity than wild type. The Arg45 residue of Hst2 corresponds to Arg275 of Sir2 and thus is right next to Ser276. Why mutations in this conserved region of Sir2 have greater NAD⁺-nicotinamide exchange activity but less deacetylation activity will be discussed below.

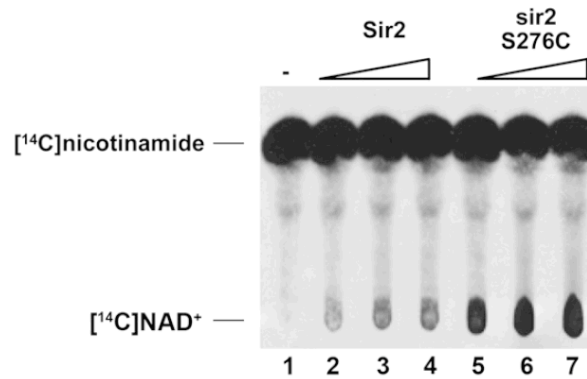


Figure 17: NAD⁺–nicotinamide exchange assays with recombinant Sir2 and Sir2 S276C. The assays were performed by incubating enzyme (2–6 μ l of Sir2 or Sir2 S276C, 100 ng/ μ l), NAD⁺, radioactive nicotinamide and chicken histones at 30° for 1 hr. Activity was determined by the amount of [¹⁴C]NAD⁺ formed. Sir2 S276C protein shows enhanced NAD⁺–nicotinamide exchange activity compared with Sir2.

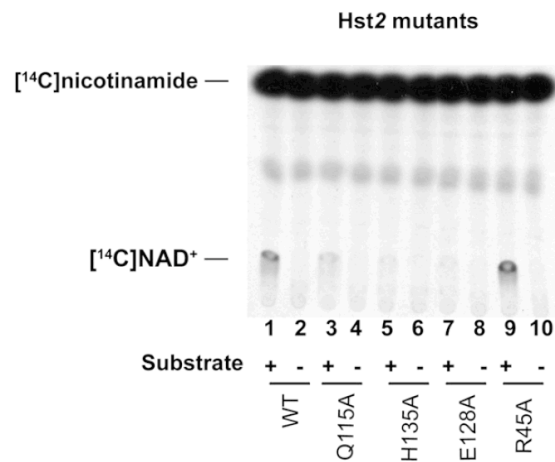


Figure 18: NAD⁺–nicotinamide exchange assays with Hst2 mutants. Recombinant wild-type and mutant Hst2 proteins were assayed by NAD⁺–nicotinamide exchange assays. The assays were done by incubating 1.3 ng of enzyme, NAD⁺, radioactive nicotinamide, and chicken histones (substrate, with or without) at 30° for 1 hr. The Q115A, H135A, and E128A mutants show reduced exchange activity while the R45A mutant shows enhanced activity. (This work is done by Dr. Joseph Landry)

Discussion

As mentioned, *sir2-276* was identified as a spontaneous mutation that led to a temperature-sensitive silencing defect at *HMR*. This mutation changes only one residue in Sir2: Ser276 to Cys. Interestingly, such a minor change of a side chain from -CH₂-OH to -CH₂-SH caused a significant enzymatic defect *in vitro* and significant silencing defects *in vivo*, showing the importance of Ser276 in Sir2. This amino acid is in a conserved disordered loop of the NAD⁺ binding pocket of the enzyme, termed the C site in the Sir2 superfamily (Figure 19) (Min et al, 2001).

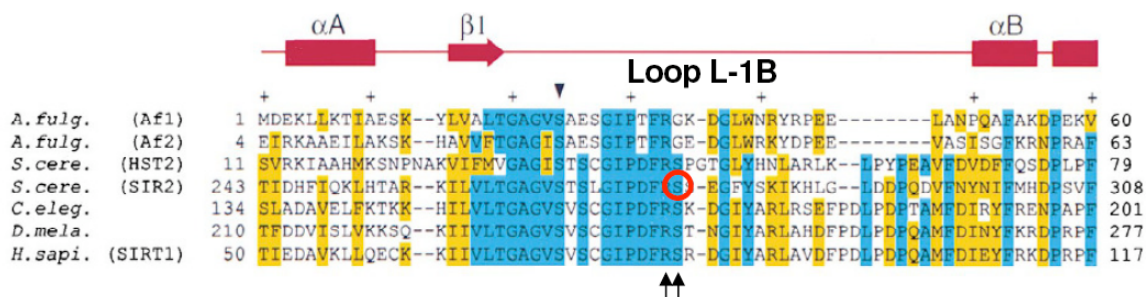


Figure 19: Sequence alignment of SIR2 homologs. Positions at which five or more sequences are identical are highlighted cyan. Positions at which five or more are similar are highlighted yellow. The positions of α helices and β strands as well as the disordered loop of NAD⁺ binding pocket, loop L-1B, are indicated above the sequence. The Ser 276 in Sir2 is circled in red. The conserved Arg and Ser residues within loop L-1B are indicated by arrows. (Min et al, 2001)

The cocrystal structure of the yeast Sir2 homologue Hst2 with the product of NAD hydrolysis, 2'-O-Acetyl-ADP ribose (AAR), has been resolved (Zhao et al, 2003).

The structure indicates that Ser46, which is the homologous residue of Sir2 Ser276, is adjacent to Phe44 and Arg45 that form hydrogen bonds with the phosphate group of AAR (Figure 20). These results support the idea that Ser276 in Sir2 is important for its enzymatic activity as discussed below.

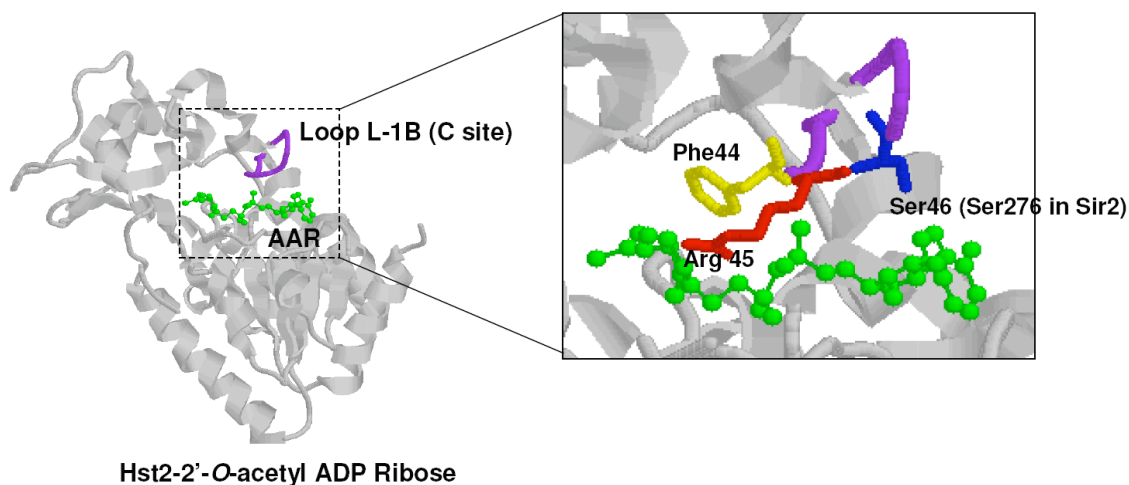


Figure 20: The co-crystal structure of the yeast Sir2 homologue Hst2 with the product of NAD hydrolysis, 2'-O-Acetyl-ADP ribose (AAR). The disordered loop of NAD⁺ binding pocket (C site), loop L-1B, is labeled in purple while AAR is in green. Three conserved residues in this loop, including Phe44, Arg45 and Ser46, are colored in yellow, red and blue respectively. The Ser46 of Hst2 is the homologous residue of Sir2 Ser276. (Zhao et al, 2003)

As described, the Sir2 family of NAD⁺-dependent deacetylases acts in two steps (Figure 7) (Jackson et al, 2003; Landry et al, 2000a; Sauve & Schramm, 2003). In the first step, nicotinamide is released via the cleavage of NAD. This step is reversible in the presence of excess nicotinamide and makes the exchange reaction possible *in vitro*. In the second step, which is irreversible, the protein substrate is deacetylated, releasing

2'-*O*-acetyl-ADP ribose. In our enzymatic assays, I observed that Sir2 S276C protein is defective in deacetylation as determined by two assays, NAD⁺ hydrolysis and western blotting. In these two assays the activities were measured by detecting the final products of the whole two-step reaction, nicotinamide and unacetylated lysine, respectively. In the NAD⁺-nicotinamide exchange assay, which measures the reversal of the first step in the reaction, both Sir2 S276C and Hst2 R45A showed more activity than wild-type protein. I think this is because Sir2 S276C mutation and the adjacent mutation in Hst2 inhibit the second step but not the first. This increases the lifetime of an acetyl-lysine-ADP ribose 1'-*O*-alkylamide intermediate and thus leads to increased NAD⁺-nicotinamide exchange (Denu, 2005). Recently, Khan and Lewis (2006) determined kinetic parameters for several different *hst2* mutants, including the R45A mutant our lab has used. They found that the R45A mutant has a very similar binding constant (K_m) as wild type for either NAD⁺ or an acetylated lysine substrate but the rate constant (k_{cat}) is about 6 times slower than wild type. They therefore proposed, as I do, that the function of Arg45 in Hst2 was to stabilize the acetyl-lysine-ADP ribose intermediate formed between step one and step two of the reaction, supporting the idea that the adjacent Arg and Ser residues in the conserved loop play an important role in the second step of the deacetylation reaction.

Two silencing assays, mating (Figure 8 and Table 3) and quantitative RNA

measurements (Figure 9), both showed that the *sir2-276* mutation had a greater silencing defect at *HMR* than at *HML*. This result was somewhat surprising. Generally mutations that do not completely abolish silencing, such as *nat1/ard1*, *sas2*, or leaky *sir3* mutations, abolish silencing at telomeres, weaken it at *HML*, and hardly affect *HMR*. In other words, silencing is considered strongest at *HMR*, perhaps because the *HMR-E* silencer is the only one of the four *HM* silencers with binding sites for three proteins, ORC, Rap1, and Abf1. Thus, it is not clear why the *sir2-276* mutant affects *HMR* more than *HML*.

My *in vitro* enzymatic assays have shown that Sir2 S276C is equally defective at both 25° and 37° (Figures 14 and 15). On the other hand, the mutant exhibited a temperature-sensitive mating and silencing defect (Figures 8 and 9) and an rDNA silencing defect (Figure 11). However, the mutant had a complete loss of telomeric silencing at both temperatures (Figure 10). Moreover, in the quantitative RT-PCR assays, some RNA from *HMR* was detected even at 25° (Figure 9), showing that the mutant is somewhat defective at a lower temperature. It is not clear why the *sir2-276* strain has a strong temperature-sensitive silencing defect at the *HM* loci and rDNA when the mutant protein is enzymatically defective even at a lower temperature. Apparently, more Sir2 activity is required *in vivo* at 37° than at 25°.

In the studies of the *sir3* ts mutant, *sir3-8*, it was found that inactivating Sir3 at

the nonpermissive temperature led to a rapid loss of silencing when observing both *HML* and *HMR* (Miller & Nasmyth, 1984) or *HMR* alone (Lau et al, 2002). Holmes and colleagues recently described several *sir2* ts mutants (Hickman et al, 2007; Matecic et al, 2006). Their mutants fell into two classes. One group had defects in deacetylase activity and the other had defects in binding to Sir4 (Matecic et al, 2006). Consistent with previous results, they found that the deacetylase-defective mutants lost silencing rapidly at *HML* when raised to the nonpermissive temperature. By using *sir2-276* mutant, I also found that silencing at *HMR* was lost rapidly in 4 hours when shifting the temperature from 25° to 37° (Figure 13 and 14).

For studies on the reestablishment of silencing, it previously was shown that silencing observed at both *HML* and *HMR* (Miller & Nasmyth, 1984) or *HMR* alone (Lau et al, 2002) must pass through S phase and G₂/M phase. Another study in which silencing was reestablished by galactose induction of Sir3 showed that restoration of complete silencing of *HMR* took more than three generations (Katan-Khaykovich & Struhl, 2005). Similarly, I found that it took more than 8 hours after lowering the temperature of the *sir2* ts mutant to observe significant silencing of *HMR* (Figure 13), clearly more than one generation.

Surprisingly, different from my work and other published results on *HMR*

silencing, I found that silencing at *HML* was rapidly reestablished in 4 hours when shifting a *MATa sir2* ts mutant from 37° to 25°, which is about more than 4 hours faster than restoring silencing at *HMR* (Figure 14). However, since these temperature shifting assays were performed in unsynchronized cells, it is still unclear whether reestablishment of silencing *HML* can bypass some of the cell cycle states that are required at *HMR*. Since *HML* differs from *HMR* in both the content of silencers and the **a**/ α genes within the silencers, it is important to distinguish whether the silencers or the **a1**/ α 1 promoters causes this kinetic difference for reestablishing silencing. It will be also interesting to study the cell cycle dependence of silencing reestablishment at both silent loci with this *sir2-276* mutant and compare it with previous studies in which the controlled production of Sir3 or Sir1 was used to examine reestablishment of silencing as a function of the cell cycle.

Conclusions

A screen for *Saccharomyces cerevisiae* temperature-sensitive silencing mutants identified a strain with a point mutation in the *SIR2* gene. The mutation changed Ser276 to Cys. This amino acid is in the highly conserved NAD⁺ binding pocket of the Sir2 family of proteins. Haploid strains of either mating type carrying the mutation were

severely defective at mating at 37° but almost normal at 25°. Silencing at the rDNA locus was also temperature-sensitive. On the other hand, telomeric silencing was totally defective at both temperatures.

Measurements of RNA from the *HMR* locus demonstrated that silencing was lost rapidly upon shifting the mutant from the low to the high temperature, while it took >8 hours to reestablish silencing after a shift back to 25°. However, silencing at *HML* was reestablished in 4 hours, which is much faster than at *HMR*.

Enzymatic activity of the recombinant wild-type and mutant Sir2 protein was compared by three different assays. The mutant exhibited less deacetylase activity than the wild-type protein at both 37° and 25°. Interestingly, the mutant had much more NAD⁺-nicotinamide exchange activity than wild type, as did a mutation in the same region of the protein in the Sir2 homolog, Hst2. Thus, mutations in this region of the NAD⁺ binding pocket of the protein are able to carry out cleavage of NAD⁺ to nicotinamide but are defective at the subsequent deacetylation step of the reaction.

Future Plans

As shown in Figure 13 and 14, I had a surprising finding on the different kinetics of reestablishment of silencing for *HMR* versus *HML*. Previous studies have shown that

restoring silencing at *HMR* must pass through S phase and G₂/M of the cell cycle to reestablish silencing (Lau et al, 2002). It will be interesting to determine if reestablishing silencing at *HML* also requires passage through the cell cycle by using the well studied *sir3* ts mutant, *sir3-8*, as well as my *sir2* ts mutant, *sir2-276*.

Of note, one significant difficulty with studying cell cycle dependent-reestablishment of silencing in common yeast strains that have an *HML* α locus is that we can't use α factor to arrest cells at G₁ phase, because of the expression of α genes from *HML* when silencing is derepressed. One way to overcome this is to create an *HML* α *MAT* Δ *HMRA* Δ strains in which no α genes are expressed. This will allow us to use α factor to arrest cells in G₁ phase and test if silencing can be re-established at *HML* under this condition. In another way, we can still use common yeast strains that do have an *HML* α locus by treating cells with HU or nocodazole to block them at the beginning of S phase or at G₂ phase. Using these two approaches, we can test if restoring silencing at *HML* is dependent on passage through the cell cycle, as it is at *HMR* (Lau et al, 2002). Another student in our lab, Jie Ren, is currently working on this project.

CHAPTER THREE

Regulation of Sir3 by Ris1

Introduction

Sir3 is known to have many different post-translational modifications, some of which are correlated with its functions. For example, stress-induced hyperphosphorylation of Sir3 affects silencing at telomeres and is involved in regulating yeast lifespan (Ai et al, 2002; Ray et al, 2003; Stone & Pillus, 1996). The N-terminal acetylation of Sir3, which has been shown in our lab to be important for silencing at *HM* loci and telomeres, was recently reported to be required for recognizing the core region of nucleosomes (Onishi et al, 2007; Sampath et al, 2009; van Welsem et al, 2008). As mentioned in Chapter 1, both Sir3 and Sir4 were found to be SUMO-conjugated in a proteomic study, while the actual function of this modification remains unclear (Denison et al, 2005).

SUMO is known to form an isopeptide bond with a lysine residue that is often in a short consensus sequence Ψ KXE (Ψ = I, L or V) (Johnson, 2004; Sampson et al, 2001),

although the sumoylated lysine sites of many proteins were not found in the consensus sequence (Denison et al, 2005). SUMO-conjugation is known to promote the assembly of protein complexes by enhancing protein-protein interactions that leads to various biological functions, one of which includes the regulation of the dynamics of higher order chromatin structures (Mahajan et al, 1997; Tanaka et al, 1999). As mentioned in Chapter 1, a group of proteins that contains both SUMO interacting motifs (SIMs) and an Ub-E3 like RING finger motif were found to down-regulate many sumoylated proteins. This new group of SUMO-related proteins, named SUMO-targeted ubiquitin ligases (STUbLs) was proposed to bind specifically to SUMO-conjugated proteins by SUMO-interacting motifs, and the targeted proteins were then poly-ubiquitylated and degraded through the RING finger motif (Figure 6) (Perry et al, 2008). In budding yeast, Slx5, Slx8 and Ris1 were identified as the members of this new protein family (Ii et al, 2007a; Uzunova et al, 2007; Xie et al, 2007).

Ris1, a Sir4-associated protein, participates in yeast mating type switching at the *MAT* locus and has Sir protein dependent-targeted silencing ability (Andrulis et al, 2004; Zhang & Buchman, 1997). In addition to SIMs and a RING finger motif, Ris1 also has a SWI2/ SNF2-like DNA-dependent ATPases and exhibits DNA-stimulated ATPase activity (Zhang & Buchman, 1997). Of note, a *RIS1* deletion displayed synthetic sickness

with deletion alleles of either *SLX5* or *SLX8*, the other two STUbL members, in a genome-wide study in yeast (Pan et al, 2006). *SLX5* and *SLX8* are essential genes when the Sgs1/ Top3 pathway in yeast is abolished (Mullen et al, 2001). Slx5 and Slx8 form a heterodimer that can bind to double-stranded DNA; they are required for repairing damaged DNA and repressing genomic instability (Yang et al, 2006; Zhang et al, 2006). The Slx5/Slx8 heterodimer was also shown to have ubiquitin E3 ligase activity *in vitro*. SUMO-conjugated Rad52 and Rad57, two proteins required for homologous recombination of damaged DNA, were found to be poly-ubiquitylated by Slx5/Slx8 (Denison et al, 2005; Xie et al, 2007). It was proposed that once Rad52 and Rad57 finish their work on repairing damaged DNA, these two proteins are sumoylated. The sumoylated Rad52 and Rad57 are then recognized and degraded by Slx5/Slx8. This process seems to be important for maintaining the stability of genome. Very recently, Slx5/Slx8 was found to have a quality control function for a transcriptional regulator called Mot1. Mot1 tends to be SUMO-conjugated to a much greater extent when the protein is defective or misfolded, and this allows Slx5/Slx8 to down-regulate the amount of this sumoylated protein (Wang & Prelich, 2009a). In contrast to Slx5 and Slx8, the actual substrate and biological role of Ris1 is unknown. Although it is known that Ris1 can bind to a SUMO-conjugated Ebp2 (yeast homolog of human Epstein-Barr virus

nuclear antigen 1-binding protein 2) through its SUMO-interacting motif (Shirai & Mizuta, 2008), it is not clear if SUMO-Ebp2 degradation is controlled by Ris1.

In this chapter I present evidence that Sir3 is mono-sumoylated at lysine 247. This residue is the only lysine within a SUMO consensus sequence found in the whole protein. Siz1 is the primary SUMO E3 ligase responsible for Sir3 sumoylation. I found that the level of SUMO-conjugated Sir3 is increased in a *ris1*Δ strain but not in an *slx5*Δ strain. I also observed that the RING finger motif and the ATPase motif of Ris1 are required for this function. A synthetic sickness phenotype was also observed in an *slx5*Δ background when either of these two motifs in Ris1 was abolished. I also found that spreading of Sir3 on silent chromatin is regulated by both sumoylation and Ris1. These findings suggest that Ris1 can down-regulate the level of SUMO-conjugated Sir3. This provides a protection mechanism to avoid inappropriate spreading of Sir3, and thus prevents inappropriate silencing of transcriptionally active regions.

Materials and Methods

Strains and plasmids

Strains and plasmids used in this study are listed in Table 4 and Table 5, respectively.

Yeast deletion mutants were constructed using standard methods (Longtine et al, 1998).

The *SIR3* point mutant (K247R) or *SIR3*¹⁻³⁸⁰ point mutants (I245G, K247R, E249A) were introduced by site-directed mutagenesis. A *RIS1* fragment, containing 300 bp both upstream and downstream of the open reading frame, was amplified from genomic DNA from the yeast strain W303-1a. Amplified product was digested with *Bam*HI and cloned into the cloning vector pCR-Blunt (Invitrogen). The *ris1* mutants that abolish the activities of the SUMO interacting motif (376DEDE379→AAAA), the RING finger motif (C1330A), or the ATPase motif (K975A) were designed based on previous studies (Bottomley et al, 2005; Hannich et al, 2005; Richmond & Peterson, 1996) and made by site-directed mutagenesis.

To integrate *ris1* motif mutations into the genome of strain W303-1a for generation of yeast strains RS2609, RS2610 and RS2611, each of the mutagenized *ris1* fragments was first cloned into the yeast integration vector pRS306 (*CEN*, *URA3*) and then digested within the 5' UTR of *ris1* with *Psh*AI. The digested fragments were transformed into strain RS2640 (W303-1a *ris1*Δ::*kanMX6*) and Ura⁺ transformants were selected. These transformants were then grown on synthetic complete (SC) medium containing 0.1% 5-fluoroorotic acid (5-FOA) to delete the *URA3* cassette from the *ris1* mutant allele. Candidates that were both 5-FOA resistant and G418-sensitive were selected. The

genotypes and the mutations of *risI* on the genome of the resulting strains were confirmed by PCR and sequencing.

Purification of SUMO-conjugates

A protocol similar to that used by Johnson *et al.* was employed with minor modifications (Denison et al, 2005). Briefly, a strain expressing (His)₆-FLAG-SUMO under the control of a *GALI* promoter (p315-P_{GAL}-HFSMT3) was grown in 500 ml of SC medium containing 2% galactose to an OD₆₀₀ of approximately 1.50. These cells were harvested by centrifugation, resuspended in lysis buffer (8M Urea, 50 mM Tris, pH 8.0) containing 10 µg/ml each of aprotinin, leupeptin, and pepstatin A and 1 mM PMSF, and lysed with beads beating. The resulting mixture was centrifuged at 4° for 30 minutes at 13,200 rpm. The supernatant was cleared with a 0.45 µm filter and the protein concentration was measured. 70 µl of the filtered extract was saved as the input control while the rest incubated with 1ml slurry of Ni-NTA resin (Qiagen) for two hours at 4° and then loaded onto a column. Bound proteins were washed with 10 ml of wash buffer (lysis buffer containing 5 mM imidazole and 300 mM NaCl) and eluted in buffer containing 300 mM imidazole, 1 M urea, and 50 mM Tris, pH 8.0. Fractions containing protein were combined and diluted 10-fold with RIPA buffer containing 0.1% SDS, 1mM PMSF, and 1 mM β-mercaptoethanol. The mixture was then incubated with 80 µl of anti-FLAG

sepharose (Sigma) at 4° overnight. The next day, the beads were pelleted by centrifugation, transferred to 1.5 ml centrifuge tubes, and washed 6 times with 1 ml aliquots of RIPA buffer containing 0.1 % SDS. Proteins were eluted with buffer containing 100 mM glycine, pH 2.2, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS.

Chromatin immunoprecipitation

The experiments were performed and modified as previously described (Connelly et al, 2006). Briefly, 200 OD₆₀₀ of cells were cross-linked with formaldehyde for 30 min. Cells were pelleted and washed in PBS. Pellets were lysed in IP lysis buffer (50 mM HEPES-KOH pH 7.5, 10 mM magnesium chloride, 150 mM potassium chloride, 0.1 mM EDTA pH 8.0, 10% glycerol, 0.1% NP-40, and protease inhibitors). After lysis, the insoluble debris was resuspended in the supernatant and sonicated 20 seconds for 12 times at level 3 with an Ultrasonics, Inc. sonicator. The extract was then cleared by centrifugation at 13,200 rpm for 10 min and the protein concentration of the supernatant was measure. 2.5 mg of protein was added to a total volume of 350 µl of IP buffer. 20 µl of the mixture was saved as the input control. 1 µg of anti-Sir3 antibody was added and incubated at 4° overnight for Sir3 immunoprecipitation. 50 µl of protein-G agarose was equilibrated in IP buffer, added to precipitate antibody-bound protein complex and rotated at 4° for 2 hours. Supernatant was removed from the protein-G beads and the beads were washed 4 x 15 min

in cold IP buffer. The immunoprecipitated chromatin was eluted in 100 μ l of IP elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) by incubation at 65° for 30 min. Eluted material was removed from beads to a new tube and the beads were eluted again as described with 50 μ l IP elution buffer. 7 μ l of saved input sample was also mixed with 130 μ l elution buffer to make a 2% input control. The inputs and combined eluates were incubated overnight at 65° to reverse crosslinks. DNA was isolated from the eluant by purification over a QiaQuick PCR column (Qiagen). The DNA was eluted from the column in 25 μ l of water. The input and immunoprecipitated materials were 1:25 and 1:10 diluted, respectively. PCR was performed to enrich the fragments at chromosome VI-R telomere and *PMAI* with the primer pairs as described previously (Hoppe et al, 2002; Wang et al, 2008; Xu et al, 2007) and PCR products were resolved on 2% agarose gel. For radioactive PCR, the same amounts of DNAs were analyzed by standard reaction mixtures to which 1 μ Ci of [³²P]dATP (3 Ci/ μ mol) had been added. Products were resolved on a 2% agarose gel and quantified using a Storm840 scanner and ImageQuant software (Molecular Dynamics). Fold enrichment was calculated by dividing the enrichment for each IP by the enrichment for the input. The fold enrichment was calculated for two or three independent experiments and the average and standard error calculated.

Synthetic sickness assays

To test if the *ris1* motif mutants can cause the synthetic sickness when *SLX5* is deleted, a *URA3* plasmid that expresses *SLX5* (pZW15) was transformed into the *ris1* mutant strains, RS2609 (SUMO interacting motif mutant), RS2610 (RING finger mutant) and RS2611 (ATPase mutant) and the chromosomal *SLX5* gene was in turn deleted to create the resulting strains, RS2624, RS2625 and RS2626, respectively. These strains were grown in yeast-peptone-dextrose (YPD) medium overnight. These cultures were fivefold serially diluted and spotted onto either a YPD plate, to see a permissive growth, or a YPD + 5-FOA plate, to force out the Slx5-expressing plasmid and hence observe the synthetic phenotype of *ris1* mutants in combination with *slx5* Δ .

Recombinant protein purification and *in vitro* ubiquitylation assays

GST- fused Ris1 or Rkr1 C-terminal fragment containing its RING finger motif were expressed in *E. coli* BL21 (DE3) from pCLW33 and pCLW53 after a 3 hour induction with 0.5 mM isopropyl β -D-thiogalactoside (IPTG) at room temperature. The two proteins were purified using Glutathione Sepharose 4 Fast Flow Affinity Resin (Amersham) according to the manufacturer's instructions and the method previously described (Braun et al, 2007). Purified proteins were dialyzed against 50 mM sodium phosphate (pH 7.2) and frozen at -80° in 50 mM sodium phosphate (pH 7.2), 0.5 mM

DTT and 10% glycerol. Protein concentrations were estimated by comparing Coomassie brilliant blue staining of samples with BSA standards, analyzed by SDS/PAGE.

Ubiquitylation assays were performed as described (Braun et al, 2007). Ubiquitin activating enzyme E1 (yeast recombinant Uba1), conjugating enzyme E2 (human recombinant UbcH5a) and ubiquitin (human recombinant) were commercially obtained (Boston Biochem). Uba1 (100 ng), UbcH5a (200 ng) and GST fusion proteins (about 500 ng) were mixed in reaction buffer (50 mM Tris-Cl pH 7.5, 2 mM ATP, 2.5 mM MgCl₂, and 0.5 mM DTT) with 2.5 µg of ubiquitin in a final volume of 30 µl. Samples were then reacted at 30° for 90 min. Proteins were separated on a SDS-polyacrylamide gel and a Western was performed with anti-ubiquitin antibody to detect ubiquitin-conjugated substrates and anti-GST antibody (Abcam) to detect the levels of GST fusion proteins.

Mating assays

The *MATa* strains of interest were grown in the appropriate synthetic complete selective medium and grown at 25°, 30° or 37° for 1 day. Cells were fivefold serially diluted and spotted onto a yeast-peptone-dextrose (YPD) medium without or with a replica plated mating tester strain, DC17. After 6 hours of incubation at respective temperatures, these cells were transferred by replica plating onto synthetic dextrose (SD) plates to select for diploids. Diploids were allowed to grow at 25°, 30° or 37° for 2 days.

Table 4: Strains used in Chapter 3

Strain	Genotype	Source
W303-1a	<i>MATα ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1</i>	R. Rothstein
W303-1b	<i>MATα ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1</i>	R. Rothstein
JCY3	W303-1a <i>sir3Δ::kanMX6</i>	This laboratory
RS2160	W303-1a <i>ris1Δ::kanMX6</i>	This study
RS2146	W303-1b <i>sir3Δ::kanMX6 ris1Δ::HIS3</i>	This study
MHY501	<i>MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2</i>	M. Hochstrasser
MHY3495	MHY501 <i>ris1Δ::HIS3</i>	M. Hochstrasser
MHY3858	MHY501 <i>slx5Δ::kanMX6</i>	M. Hochstrasser
MHY3944	MHY501 <i>ris1Δ::HIS3 slx5Δ::kanMX6</i>	M. Hochstrasser
MHY2870	<i>MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 siz1Δ::HIS3</i>	M. Hochstrasser
RS2609	W303-1a <i>ris1 376D to E379 into AAAA</i>	This study
RS2610	W303-1a <i>ris1 C1330A</i>	This study
RS2611	W303-1a <i>ris1 K975A</i>	This study
RS2570	W303-1a <i>slx5-11::hphMX4</i> + pRS416 <i>SLX5 (CEN, URA3, own promoter)</i>	This study
RS2571	W303-1a <i>ris1Δ::kanMX6 slx5-11::hphMX4</i> + pRS416 <i>SLX5 (CEN, URA3, own promoter)</i>	This study
RS2624	W303-1a <i>ris1 376D to E379 into AAAA slx5::kanMX6</i> + pRS416 <i>SLX5 (CEN, URA3, own promoter)</i>	This study
RS2625	W303-1a <i>ris1 C1330A slx5::kanMX6</i> + pRS416 <i>SLX5 (CEN, URA3, own promoter)</i>	This study
RS2626	W303-1a <i>ris1 K975A slx5::kanMX6</i> + pRS416 <i>SLX5 (CEN, URA3, own promoter)</i>	This study
RS2095	W303-1a <i>SIR4:Myc₁₃ kanMX6 san1::HIS3</i>	This study
RS2104	W303-1a <i>SIR4:Myc₁₃ kanMX6 san1::HIS3 ris1::TRP1</i>	This study

RS1232	<i>MATα sir3-8^{ts} ade2 his3 his4 leu2 lys2 ura3 trp1-1</i>	This laboratory
DC17	<i>MATα his1</i>	This laboratory
RS2517	RS1232 <i>ris1::kanMX6</i>	This laboratory
RS2532	RS1232 <i>slx5::TRP1</i>	This study
RS2533	RS1232 <i>ris1::kanMX6 slx5::TRP1</i>	This study

Table 5: Plasmids used in Chapter 3

Name	Description	Source
p315-P _{GAL} -HFSMT3	pRS315 <i>His6-FLAG-SMT3</i> (<i>CEN</i> , <i>LEU2</i> , <i>GAL10</i> promoter)	G. Blobel
pCLW58	pRS315 <i>His6-FLAG-smt3 K11R, K15R, K19R</i> (<i>CEN</i> , <i>LEU2</i> , <i>GAL10</i> promoter)	M. Hochstrasser
pXR58	pRS314 <i>SIR3</i> (<i>CEN</i> , <i>TRP1</i> , own promoter)	This laboratory
pEP6	pRS314 <i>sir3 K247R</i> (<i>CEN</i> , <i>TRP1</i> , own promoter)	This study
pCLW38	pRS426 <i>SIR3</i> (<i>2μ</i> , <i>URA3</i> , <i>ADHI</i> promoter)	This study
pCLW39	p426 <i>sir3 K247R</i> (<i>2μ</i> , <i>URA3</i> , <i>ADHI</i> promoter)	This study
pCLW42	pRS424 <i>SIR3</i> (<i>2μ</i> , <i>TRP1</i> , <i>GAL1</i> promoter)	This study
pCLW43	pRS424 <i>sir3 K247R</i> (<i>2μ</i> , <i>TRP1</i> , <i>GAL1</i> promoter)	This study
pJC59	pFBL23 <i>SIR3</i> ¹⁻³⁸⁰ (<i>2μ</i> , <i>TRP1</i> , <i>ADHI</i> promoter)	This laboratory
pCLW11	pFBL23 <i>SIR3</i> ¹⁻³⁰⁰ (<i>2μ</i> , <i>TRP1</i> , <i>ADHI</i> promoter)	This study
pJC150	pFBL23 <i>SIR3</i> ¹⁻²¹⁴ (<i>2μ</i> , <i>TRP1</i> , <i>ADHI</i> promoter)	This laboratory
pCLW14	pFBL23 <i>SIR3</i> ¹⁻³⁸⁰ <i>K247R</i> (<i>2μ</i> , <i>TRP1</i> , <i>ADHI</i> promoter)	This study
pCLW23	pFBL23 <i>SIR3</i> ¹⁻³⁸⁰ <i>I246G</i> (<i>2μ</i> , <i>TRP1</i> , <i>ADHI</i> promoter)	This study
pCLW24	pFBL23 <i>SIR3</i> ¹⁻³⁸⁰ <i>E249A</i> (<i>2μ</i> , <i>TRP1</i> , <i>ADHI</i> promoter)	This study
pCLW71	pRS414 <i>RIS1</i> (<i>CEN</i> , <i>TRP1</i> , <i>CYC1</i> promoter)	This study
pCLW72	pRS414 <i>ris1 376D to E379 into AAAA</i> (<i>CEN</i> , <i>TRP1</i> , <i>CYC1</i> promoter)	This study
pCLW73	pRS414 <i>ris1 C1330A</i> (<i>CEN</i> , <i>TRP1</i> , <i>CYC1</i> promoter)	This study
pCLW74	pRS414 <i>ris1 K975A</i> (<i>CEN</i> , <i>TRP1</i> , <i>CYC1</i> promoter)	This study
pZW15	pRS416 <i>SLX5</i> (<i>CEN</i> , <i>URA3</i> , own promoter)	G. Prelich
pCLW33	pGEX-2T <i>RIS1</i> ¹²⁸³⁻¹⁶¹⁹ (<i>GST-RIS1 RING finger</i>)	This study

motif
pCLW53 pGEX-2T *RKR1*¹²⁵¹⁻¹⁵⁶² (*GST-RIS1 RING*
finger motif) This study

Results

Sir3 is mono-sumoylated on K247

Previous studies have shown that both Sir3 and Sir4 can be conjugated with SUMO. Single or multiple slower migrated proteins were observed after purification of sumoylated proteins, using antibodies against Sir3 or Sir4, respectively. The results suggested that Sir3 is sumoylated on a single lysine residue, while Sir4 is either sumoylated on many different lysines or has poly-SUMO chains (Denison et al, 2005). To narrow down the region of Sir3 that is sumoylated, different Sir3 N-terminal fragments together with His-FLAG-tagged SUMO were expressed in a *sir3Δ* strain. The sumoylated proteins were purified by both a Ni⁺⁺ column and anti-FLAG beads. I usually call this process SUMO purification. After purifying sumoylated proteins, an antibody against the Sir3 N-terminus was used to detect Sir3 and SUMO-conjugated Sir3 fragments before or after purification. As shown in Figure 21, after SUMO purification, a single slower migrating protein can be detected in a full-length Sir3, a Sir3¹⁻³⁸⁰ and a Sir3¹⁻³⁰⁰ fragment (lane 4, 8 and 12, respectively), but not in a Sir3¹⁻²¹⁴ fragment (lane 16), suggesting that Sir3 is sumoylated between amino acid 215 and 300.

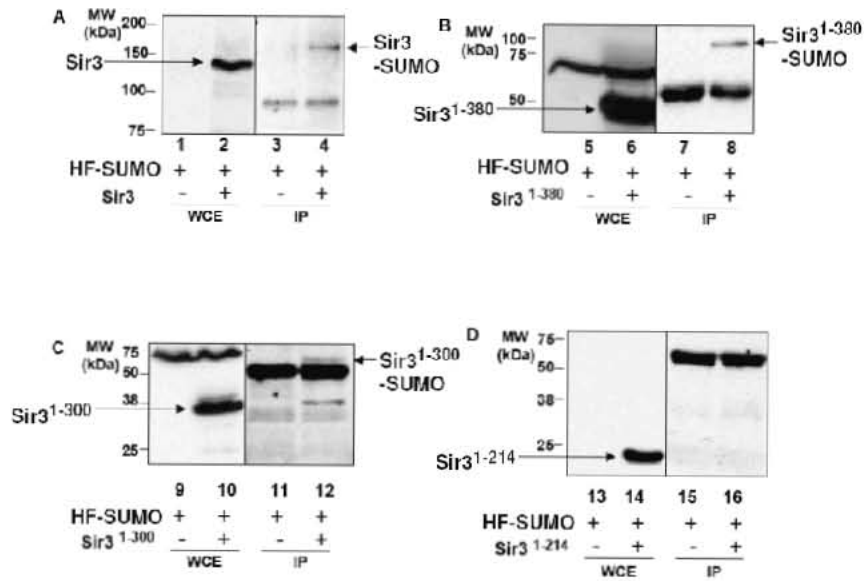


Figure 21: Sir3 is sumoylated between amino acids 215 and 300. His-FLAG-tagged SUMO (HF-SUMO) alone or with a full-length Sir3 (A), truncated Sir3¹⁻³⁸⁰ (B), Sir3¹⁻³⁰⁰ (C) or Sir3¹⁻²¹⁴ (D) were ectopically expressed in a *sir3Δ* strain. The sumoylated proteins were purified by both a Ni⁺⁺ column and anti-FLAG beads. A Western with an antibody to the Sir3 N-terminus was done for the samples before and after purifying the SUMO-conjugated proteins (WCE and IP, respectively). The arrows indicate the positions of unsumoylated and sumoylated Sir3 fragments.

I further analyzed the protein sequence of Sir3 and noticed that Sir3 has only one SUMO consensus sequence, IKIE, among the whole 978 amino acids of the protein. The lysine within this sequence is residue 247, which is within the region we demonstrated with the sumoylation region (215-300) we had resolved in Figure 21. To determine whether Sir3 is sumoylated at lysine 247, a Sir3 K247R mutant was used to analyze if sumoylation is abolished in this mutant. As shown in Figure 22A, this K247R mutant protein was not sumoylated (lane 6), demonstrating that Sir3 is sumoylated on K247. Of

note, the protein level of Sir3 in the whole cell extract doesn't change when Sir3 K247R mutant can't be sumoylated (compare lane 3 with lane 2 in Figure 22A). This result suggests that the fraction of sumoylated Sir3 is very small and that the total amount of Sir3 is unaffected even when Sir3 can't be sumoylated.

To determine if the SUMO consensus sequence is required for Sir3 sumoylation, the first, second or fourth position of the SUMO consensus sequence in a Sir3¹⁻³⁸⁰ fragment was mutagenized and used to test if these mutated proteins can be sumoylated. As shown in Figure 22B, Sir3¹⁻³⁸⁰ was not sumoylated when either of the SUMO consensus sequence was mutated (lane 7, 6 and 8, respectively). This strongly suggests that the consensus sequence is necessary for Sir3 sumoylation and that K247 is the actual site of SUMO conjugation. In order to exclude the possibility that Sir3 can form a multi-SUMO chain on K247, a SUMO mutant, *smt3* K11, 15, 19R, that was reported to strongly reduce SUMO chain-formation (Bylebyl et al, 2003), was used to see if preventing poly-sumoylation would affect the migration of a SUMO-conjugated Sir3¹⁻³⁸⁰ fragment. As shown in Figure 22C, the mobility of wild-type-SUMO- with the mutant-SUMO-conjugated Sir3¹⁻³⁸⁰ (lane 5 with 6) was similar, indicating that poly-SUMO chain formation on Sir3¹⁻³⁸⁰ is unlikely. In summary, I conclude that Sir3 is mono-sumoylated on K247, at the only SUMO consensus sequence found in the protein.

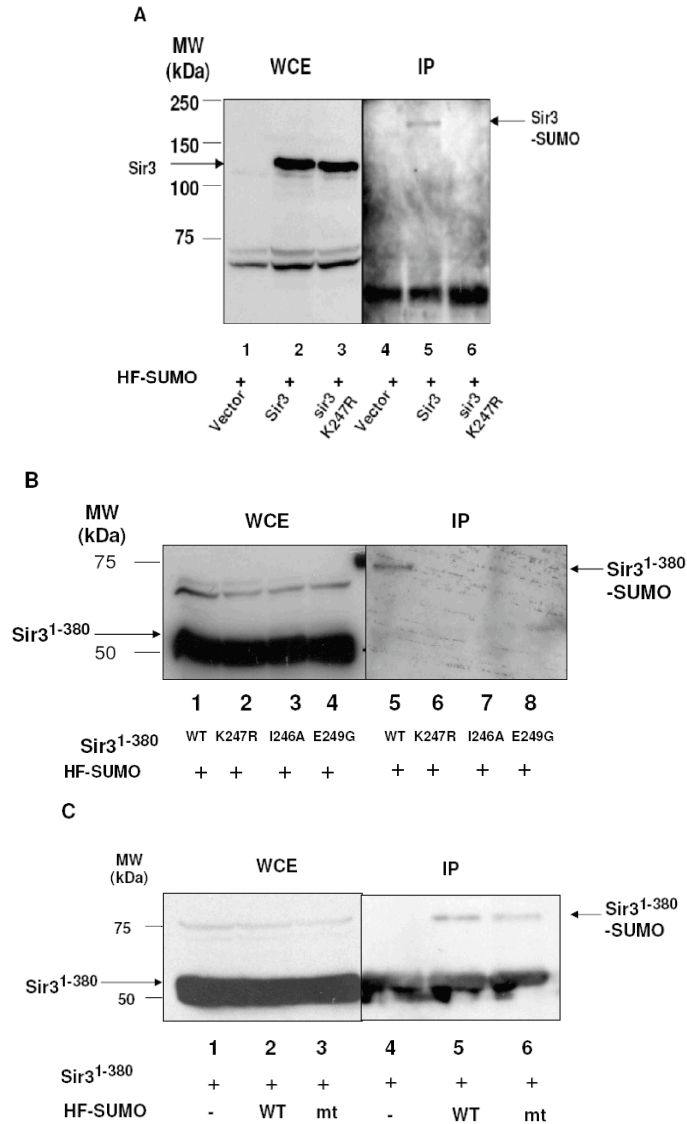


Figure 22: Sir3 is mono-sumoylated on residue K247. (A) Sir3 is sumoylated on K247. A plasmid expressing His-FLAG-tagged SUMO and a plasmid expressing Sir3 or Sir3 K247R or a vector were transformed into a *sir3Δ* strain. (B) Sir3 sumoylation requires the intact SUMO consensus sequence, 246 IKIE 249. Both His-FLAG-tagged SUMO and different Sir3¹⁻³⁸⁰ fragments (wild type, K247R, I246A or E249G) were ectopically expressed in JCY3. (C) Sir3 is mono-sumoylated. A plasmid expressing Sir3¹⁻³⁸⁰ fragment and a plasmid expressing His-FLAG-tagged SUMO wild type or poly-SUMO-abolished mutant (HF-Sumo K11, 15, 19R) or a vector were transformed into JCY3. The sumoylated proteins were purified and a Western with an antibody to the Sir3 N-terminus was done for the samples before and after purifying the SUMO-conjugated proteins (WCE and IP, respectively). The arrows indicate the positions of unsumoylated and sumoylated Sir3 fragments.

Siz1 is a SUMO E3 ligase for Sir3

In budding yeast, Siz1 and Siz2 are the major SUMO E3 ligases responsible for most of the SUMO-conjugation *in vivo* (Johnson & Gupta, 2001; Takahashi et al, 2001). To determine if Sir3 is a substrate for Siz1, a Sir3¹⁻³⁸⁰ fragment and His-FLAG-tagged SUMO was ectopically expressed in a *siz1Δ* strain and a SUMO purification was performed. As shown in Figure 23, the amount of SUMO-conjugated Sir3¹⁻³⁸⁰ was greatly reduced in the *siz1Δ* cells (lane 6), suggesting that Siz1 is a SUMO-E3 for Sir3. Of note, SUMO-conjugated Sir3¹⁻³⁸⁰ in *siz1Δ* was still detectable, in comparison with no detectable signal in the SUMO consensus sequence-abolished mutants in Figure 22A and 22B, indicating that there are still other SUMO E3s that contribute to Sir3 sumoylation. In the same experiment, I found that Siz2 plays very little or no role as a SUMO E3 for Sir3 (lane 7 and lane 8).

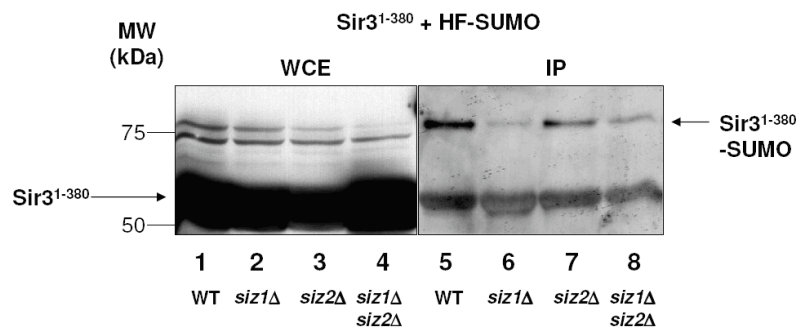


Figure 23: Siz1 is one of the SUMO ligases of Sir3. Both His-FLAG-tagged SUMO and a Sir3¹⁻³⁸⁰ fragment were ectopically expressed either in a wild type, a *siz1Δ*, a *siz2Δ*, or a *siz1Δ siz2Δ* strain. A Western with an antibody to the Sir3 N-terminus was done for the inputs (WCE) and the SUMO-purified samples (IP). The arrows indicate the positions of unsumoylated and sumoylated Sir3 fragments.

The protein level of sumoylated Sir3 is increased when *RIS1*, not *SLX5*, is deleted

As described in Chapter 1, Ris1 has been considered to be a member of SUMO-targeted ubiquitin ligases (STUbLs) that may recognize many sumoylated substrates through a SUMO interacting motif (SIM) and poly-ubiquitylate the targeted proteins by a RING finger motif, thus leading to degradation of the targeted proteins (Perry et al, 2008; Uzunova et al, 2007). To test if Ris1 can act like a STUbL, His-FLAG-tagged SUMO was ectopically expressed in a *ris1Δ* strain and an antibody against FLAG was used to detect the global sumoylated proteins. If Ris1 behaves like a STUbL, deletion of *RIS1* should lead to the accumulation of many SUMO-conjugated proteins. As shown in Figure 24A, SUMO-conjugated proteins accumulated significantly when *RIS1* was deleted. This confirmed a previous result (Uzunova et al, 2007). Since Ris1 is known to associate with Sir4 (Andrulis et al, 2004; Zhang & Buchman, 1997) and it was also reported that both Sir3 and Sir4 can be sumoylated (Denison et al, 2005), one possible role for Ris1 is to lower the level Sir3-SUMO or Sir4-SUMO. To test this idea, His-FLAG-tagged SUMO was ectopically expressed in a *ris1Δ* strain and a SUMO purification assay was performed to see if Sir3-SUMO accumulates when *RIS1* is deleted. As shown in Figure 24B, this experiment demonstrated that an increased amount of

Sir3-SUMO was observed in *ris1Δ* cells, supporting the idea that Ris1 can decrease the level of Sir3-SUMO.

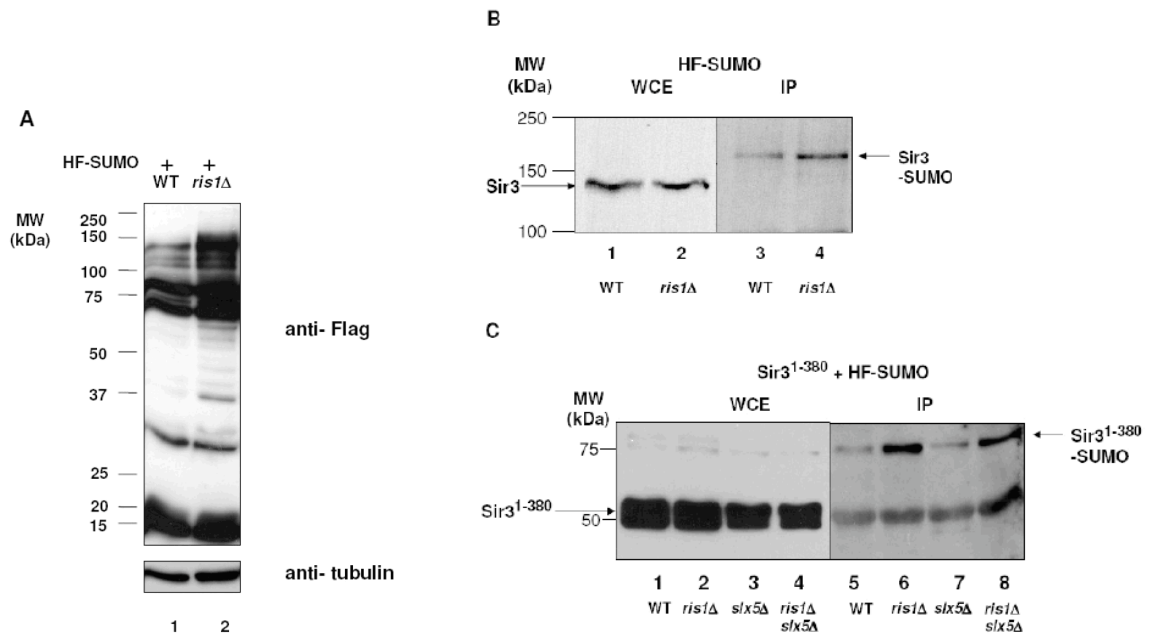


Figure 24: Deletion of *RIS1* causes accumulation of SUMO-conjugated proteins, including Sir3-SUMO. (A) SUMO-conjugated proteins accumulated in a *ris1Δ* strain. His-FLAG-tagged SUMO was ectopically expressed in either a wild type or a *ris1Δ* strain. An immunoblotting with an anti-FLAG antibody showed the SUMO-conjugated proteins. An antibody to tubulin showed similar amounts of total proteins loaded in each lane. (B) The protein level of sumoylated Sir3 is increased when *RIS1* is deleted. The sumoylated proteins in a wild type or a *ris1Δ* strain were purified and a Western with an antibody to the Sir3 N-terminus was done for the samples before and after purifying the SUMO-conjugated proteins (WCE and IP, respectively). (C). Sir3-SUMO does not accumulate when *SLX5* is deleted. His-FLAG-tagged SUMO and Sir3¹⁻³⁸⁰ fragment were ectopically expressed in a wild type, a *ris1Δ*, an *slx5Δ*, or a *ris1Δ slx5Δ* strain. The sumoylated proteins in each sample were purified and a Western with an antibody to the Sir3 N-terminus was done for the inputs (WCE) and the SUMO-purified samples (IP). The arrows indicate the positions of unsumoylated and sumoylated Sir3 fragments.

Of note, the level of Sir3 in the whole cell extract is the same in the wild-type and *ris1Δ* strains (compare lane 2 with lane 1 in Figure 24B). Also the signal of Sir3-SUMO cannot be detected directly in the whole cell extract. Similar to the data shown in Figure 22A, these results suggest that the fraction of total Sir3 that is sumoylated is small and that the total amount of Sir3 is unaffected either when Sir3 can't be sumoylated (Figure 22A) or when Sir3-SUMO accumulates (Figure 24B).

As mentioned, budding yeast also has another identified STUbL member, the Slx5/Slx8 heterodimer. Since a *ris1Δ slx5Δ* double mutant grows much more poorly than either single mutant and Sir3-SUMO accumulates when *RIS1* is deleted, it was of interest to determine if deletion of *SLX5* also causes accumulation of sumoylated Sir3. To test this, a Sir3¹⁻³⁸⁰ fragment and His-FLAG-tagged SUMO were ectopically expressed in a wild-type, a *ris1Δ*, an *slx5Δ* and a *ris1Δ slx5Δ* strain and the level of SUMO-conjugated Sir3¹⁻³⁸⁰ was detected by using an anti-Sir3 antibody. As shown in Figure 24C, the protein level of SUMO-conjugated Sir3¹⁻³⁸⁰ in *slx5Δ* and *ris1Δ slx5Δ* strains is almost the same as that in wild type and in *ris1Δ*, respectively, showing that sumoylated Sir3 accumulated when *RIS1* but not *SLX5* was deleted.

Overexpression of Sir3 is toxic and this toxicity is exacerbated by loss of sumoylation or *RIS1*

During some experiments that examined silencing regulated by Sir3, I noticed that overexpressing Sir3 by using a 2μ plasmid with an *ADHI* promoter was toxic to yeast (compare lane 2 with lane 1 in Figure 25). Further tests indicated that this toxicity was Sir2- and Sir4-dependent (compare lane 4 with lane 3 and lane 6 with lane 5 in Figure 25). One explanation for this toxicity is that overexpression causes further spreading of Sir3 from the silent region into euchromatin, which in turn leads to inappropriate silencing of transcriptionally active regions. Since this toxicity is Sir2- and Sir4-dependent, we think both Sir2 and Sir4 are required to form a complex with Sir3 at the beginning as a sort of nucleation process, which then allows the spreading of Sir3 later from the silent regions.

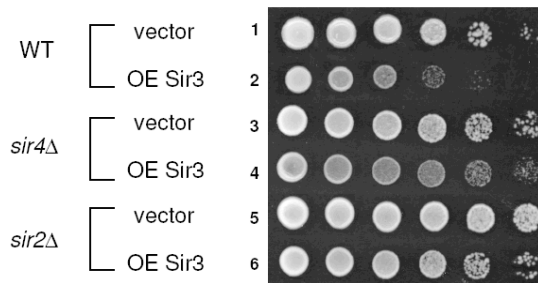


Figure 25: The toxicity caused by overproducing Sir3 is Sir2- and Sir4-dependent. A plasmid overexpressing wild type Sir3 or a vector was transformed into a wild-type, a *sir4*Δ or a *sir2*Δ strain. The cells were fivefold serially diluted, spotted on the selective medium (SC-Trp) and grown at 30° for 2 days. Part of this work was done by Evelyn Prugar.

Based on these observations, I hypothesized that the mislocalization of Sir3 caused by its overexpression may be regulated by sumoylation. In this model, Ris1 can decrease the protein level of Sir3-SUMO and in turn prevent further spreading from the silent

region. If this hypothesis is correct, either abolishment of Sir3 sumoylation by using a Sir3 K247R mutant or deletion of *RIS1* would increase more mislocalized Sir3 or Sir3-SUMO, respectively, that causes more inappropriate silencing and make cells even sicker. To test this, either wild-type Sir3 or Sir3 K247R mutant protein was overexpressed by a 2μ plasmid with an *ADHI* promoter in a *sir3* Δ mutant or *sir3* Δ *ris1* Δ double mutant, and the growth of cells was observed. As shown in Figure 26, increased toxicity was observed when overexpressing the Sir3 K247R SUMO-abolished mutant protein (lane 3), or overexpressing either type of Sir3 protein in the *ris1* Δ background (lane 5 and 6). Our interpretation of this result is that overexpression of Sir3 K247R mutant protein or deletion of *RIS1* does lead to greater growth defect than overexpression of Sir3 in a *RIS1*⁺ strain.

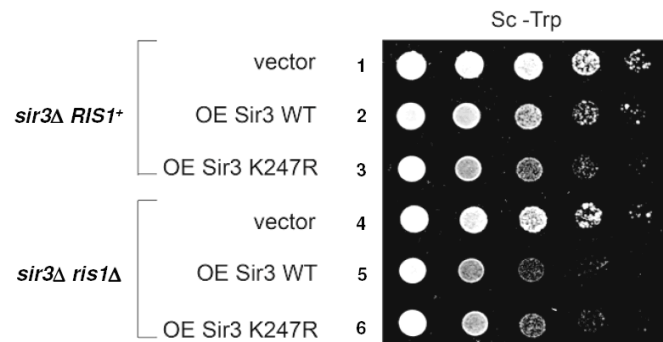


Figure 26: The toxicity caused by overproducing Sir3 is increased when sumoylation or *RIS1* is abolished. A *TRP1* plasmid overexpressing wild type Sir3 or Sir3 K247R mutant or a vector was transformed into a *sir3* Δ or a *sir3* Δ *ris1* Δ strain. The cells were fivefold serially diluted, spotted on selective medium and grown at 30° for 2 days.

This result was somewhat surprising. As mentioned earlier, no significant change in the total amount of Sir3 was observed in the whole cell extract when Sir3 sumoylation was abolished (Figure 22A) or when *RIS1* was deleted (Figure 24B). The result in Figure 26 indicates that even though only a tiny fraction of Sir3 is affected by sumoylation and Ris1, decreasing this small amount of Sir3 can still prevent the cells from getting sicker when Sir3 is overexpressed.

The spreading of Sir3 on silent chromatin is affected by sumoylation and Ris1

To obtain additional evidence that preventing Sir3 sumoylation or decreasing the level of sumoylated Sir3 does lead to further spreading of Sir3 from silent chromatin and hence increased toxicity in yeast, a series of chromatin immunoprecipitation (ChIP) assays were performed and Sir3 occupancy was observed at the silent region or a transcriptionally active region of the chromatin.

In these experiments, I overproduced wild-type Sir3 or Sir3 K247R mutant by using a 2 μ plasmid containing a *GALI* promoter. Based on our model, our prediction is that if the spreading of Sir3 is regulated by sumoylation and Ris1 in turn decreases the amount of Sir3-SUMO, Sir3 should spread further when Sir3 K247R mutant protein is expressed or when *RIS1* is deleted. To test this, both *RIS1*⁺ and *ris1* Δ strains were grown in a galactose medium to overexpress either wild type Sir3 or the Sir3 K247R mutant for 4.5

hours and the ChIP samples were collected for quantitative PCR to see the enrichment at the telomeric region of chromosome VI-R and at the *PMAI* gene, a transcriptionally active region close to the centromere of chromosome VII within 15 kb. The spreading of Sir3 when produced at physiological levels (no overexpression) on chromosome VI-R telomere and *PMAI* was also measured and is shown in Figure 27A and 28A. Both Figure 27A and 28A showed that the occupancy of normally expressed Sir3 dropped sharply from the telomere and we think that the detected occupancy of Sir3 at *PMAI* was simply background. As shown in Figure 27B and 27C, when wild-type Sir3 was overexpressed (*RIS1*⁺ OE *SIR3*), the occupancy on chromosome VI-R was significantly higher than normal at a distance of 10 kb from the telomere and even at *PMAI*, a locus within the region normally unoccupied by Sir proteins. This indicates that overproduced Sir3 does spread further from the silent chromatin even to some transcriptional active regions close to the centromere, leading to inappropriate silencing and growth defect in cells. Strikingly, when the Sir3 K247R mutant was overexpressed (*RIS1*⁺ OE *sir3* K247R), or when either type of Sir3 was overexpressed in the *ris1*Δ strain (*ris1*Δ OE *SIR3*, *ris1*Δ OE *sir3* K247R), the amount of Sir3 was even greater, especially at 20 kb from VI-R telomere and at *PMAI*. This result agrees with our model that Sir3 can spread further when sumoylation is abolished or *RIS1* is deleted.

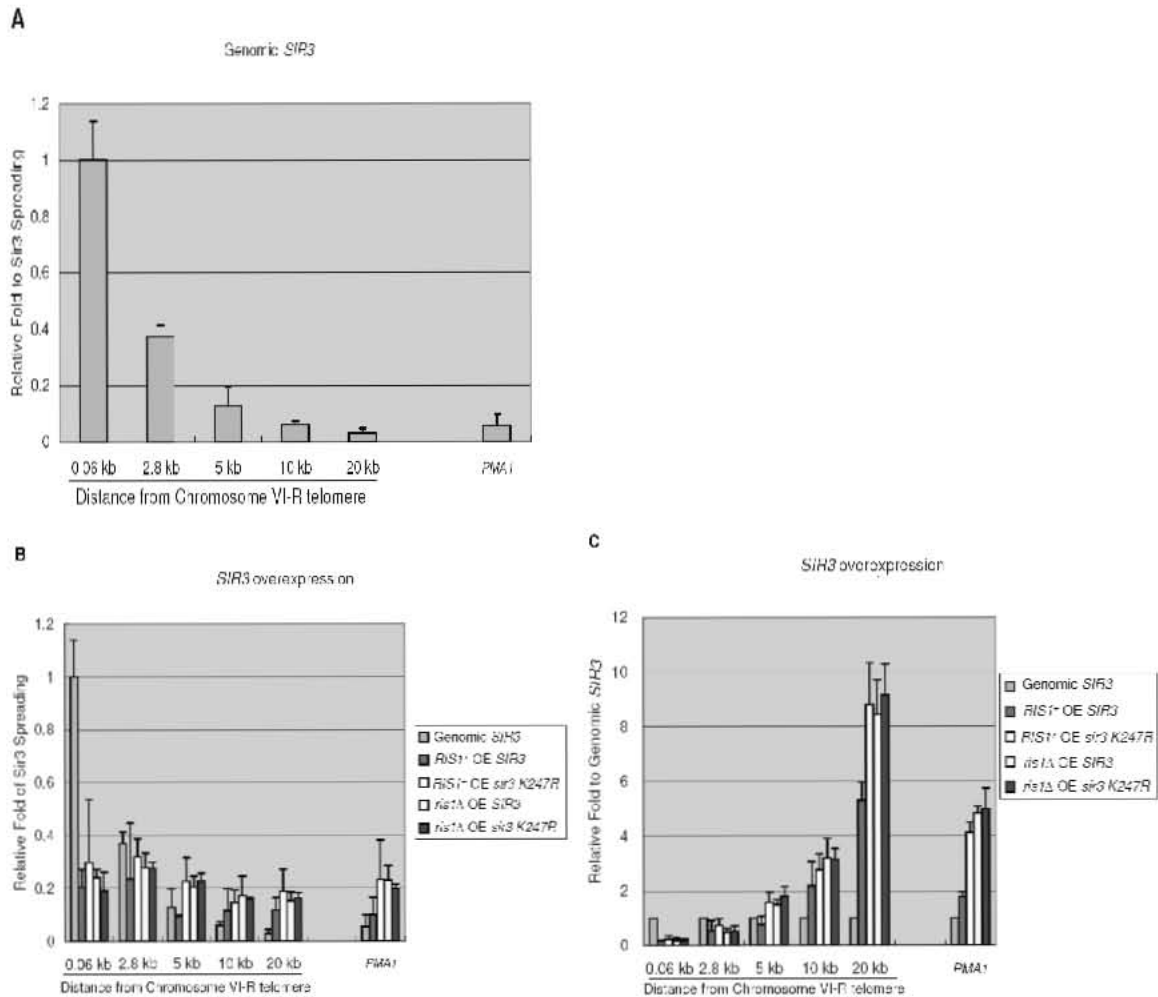


Figure 27: Overexpressed *Sir3* spreads further when sumoylation or *RIS1* is abolished. (A) The spreading pattern of normally expressed *Sir3* from its single genomic copy on telomere VI-R and at *PMA1*. The occupancies of *Sir3* at different distances from chromosome VI-R telomere and at *PMA1* are displayed in blue bars. The amount closest to the telomere is adjusted to 1. (B) The spreading patterns of normally expressed and overexpressed *Sir3* under different conditions on telomere VI-R and at *PMA1*. The occupancies of normal expressed or overexpressed *Sir3* under different conditions (overexpressing wild-type *Sir3* or *Sir3* K247R mutant by a high-copy plasmid driven by a *GAL1* promoter in a *RIS1*⁺ or a *ris1Δ* strain) at different distances from telomere VI-R and at *PMA1* are displayed. The amount of normally expressed *Sir3* (Genomic *Sir3*) closest to the telomere is adjusted to 1. (C) The relative ratios of overexpressed *Sir3* under different conditions to normally expressed *Sir3* from its single genomic copy at different distances from chromosome VI-R telomere and at *PMA1*. All the blue bars at different locations shown in (A) and (B) are adjusted to 1 in (C) to be compared with the occupancies of overexpressed *Sir3* under different conditions.

In this experiment, I also noticed that when either type of Sir3 was overexpressed, the occupancy near the telomere (Figure 27B and 27C, 0.06 kb and 2.8 kb from the VI-R telomere) actually dropped. I think this was because the overproduced Sir3 was spreading to so many different places away from the silent region that it was titrated away from the telomeres. In order to see spreading of Sir3 in a more natural way, I repeated this experiment by only moderately increasing (MI) the amount of wild-type Sir3 or Sir3 K247R mutant, and again using ChIP to monitor Sir3 occupancy. To do this, I used a *SIR3*⁺ strain carrying a *CEN* plasmid with *SIR3*'s own promoter expressing *SIR3* or *sir3* K247R. As shown in Figure 28B and 28C, no significant drop of Sir3 near the telomere was observed when moderately increasing Sir3 or Sir3 K247R. In this case (*RIS1*⁺ MI *SIR3*) the Sir3 occupancy of moderately increased wild-type Sir3 looks almost the same as the normally expressed Sir3 (genomic *SIR3*). Impressively, under this situation, moderately increasing the *sir3* mutant protein in a *RIS1*⁺ strain (*RIS1*⁺ MI *sir3* K247R), or either type of Sir3 in the *ris1Δ* strain (*ris1Δ* MI *SIR3*, *ris1Δ* MI *sir3* K247R) still shows more spreading from telomere VI-R, especially at a distance of 10 kb from the end. Therefore, based on the toxicity assay and the ChIP data, I conclude that the spreading of Sir3 from the silent region is regulated both by sumoylation and Ris1.

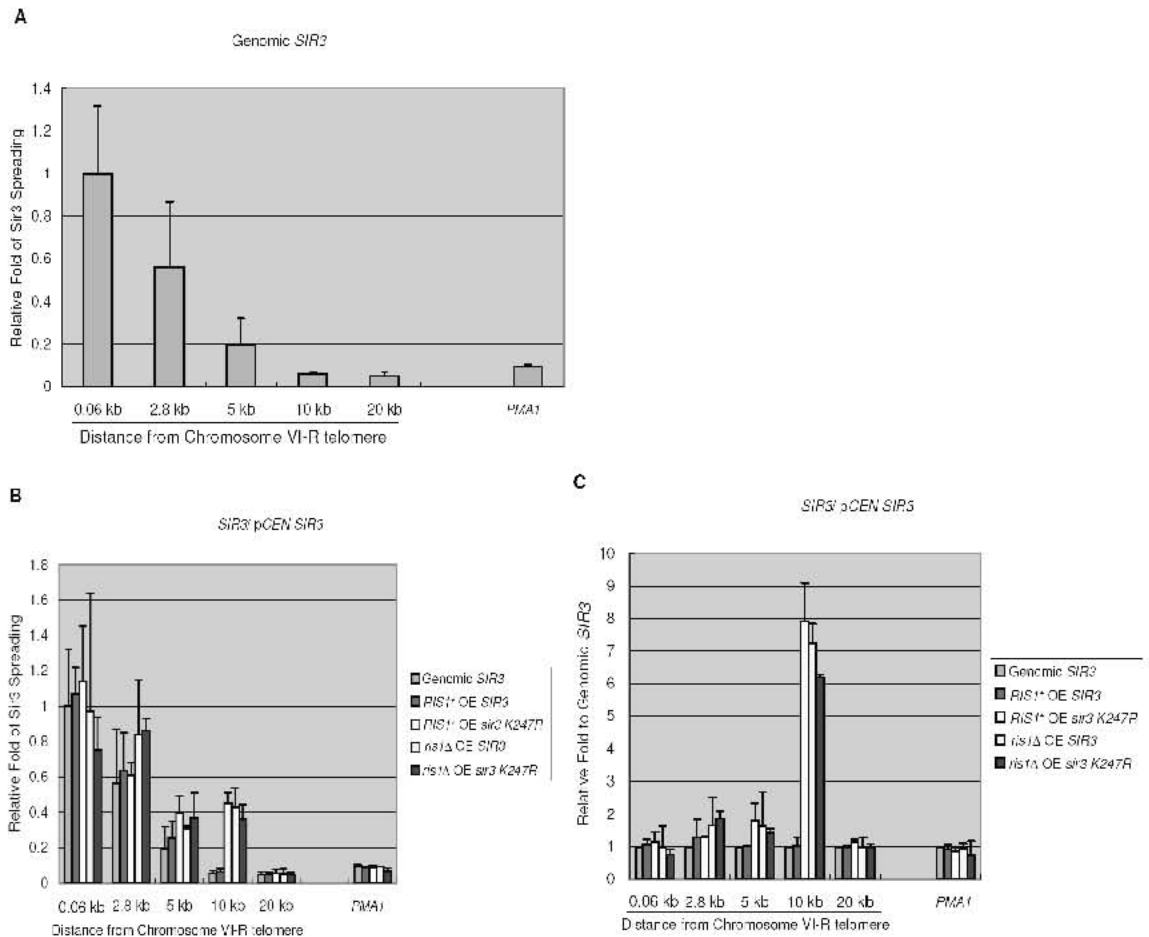


Figure 28: Moderately increased Sir3 also spreads further when sumoylation or *RIS1* is abolished. (A) The spreading pattern of normally expressed Sir3 from its single genomic copy on telomere VI-R and at *PMA1*. The occupancies of Sir3 at different distances from chromosome VI-R telomere and at *PMA1* are displayed in blue bars. The amount closest to the telomere is adjusted to 1. (B) The spreading patterns of normally expressed and moderately increased Sir3 under different conditions (moderately increasing wild-type Sir3 or Sir3 K247R mutant by a *CEN* plasmid driven by *SIR3*'s own promoter with the existence of one genomic copy of *SIR3* in a *RIS1*⁺ or a *ris1Δ* strain) at different distances from telomere VI-R and at *PMA1* are displayed. The amount of normal expressed Sir3 (Genomic Sir3) closest to the telomere is adjusted to 1. (C) The relative ratios of moderately increased Sir3 under different conditions to normally expressed Sir3 from its single genomic copy at different distances from chromosome VI-R telomere and at *PMA1*. All the blue bars at different locations shown in (A) and (B) are adjusted to 1 in (C) to be compared with the occupancies of moderately increased Sir3 under different conditions.

The roles RING finger and ATPase motif of Ris1 for down-regulation of

Sir3-SUMO and genetic interaction with *slx5Δ*

As a member of the STUbL family, Ris1 is known to have both SUMO-interacting motifs and a RING finger motif that are thought to play a role in decreasing the amount of many SUMO-conjugated proteins through poly-ubiquitylation and proteasomal degradation. In addition to these two motifs, Ris1 also has a SWI/SNF-like ATPase motif that is not found in any of the other known STUbL members (Perry et al, 2008; Zhang & Buchman, 1997). In order to determine if these three motifs contribute to the functions of Ris1, *ris1* mutant strains were constructed that abolish the SUMO interaction motif (*ris1* 376DEDE379 into AAAA), the RING finger motif (*ris1* C1330A), or the ATPase motif (*ris1* K975A). These mutations were designed and introduced based on published work (Figure 29) (Hannich et al, 2005; Lorick et al, 1999; Richmond & Peterson, 1996). All the *ris1* motif mutations were integrated at the endogenous *RIS1* locus.

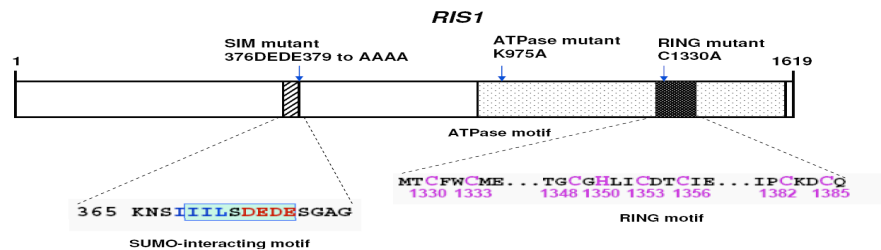


Figure 29: A schematic drawing of *ris1* motif mutations. The regions of the SUMO-interacting motif (SIM), ATPase motif and RING finger motif within Ris1 are labeled. The conserved sequence of the SUMO-interacting motif and RING finger motif are also shown. The arrows indicate the locations and mutagenized amino acid(s) of each of the three motifs.

To test if any of these motifs plays a role in decreasing the level of sumoylated Sir3, a Sir3¹⁻³⁸⁰ fragment and His-FLAG-tagged SUMO were ectopically expressed in a wild-type or one of the three *ris1* mutant strains described above and the level of SUMO-conjugated Sir3¹⁻³⁸⁰ in these strains was measured by using an anti-Sir3 antibody. As shown in Figure 30, SUMO-conjugated Sir3¹⁻³⁸⁰ significantly accumulated in the *ris1* RING finger mutant and the ATPase mutant (lane 7 and 8), indicating that both the RING finger motif and the ATPase motif of Ris1 are required for decreasing the level of sumoylated Sir3. On the other hand, the SIM motif mutation had no effect; it had the same level of SUMO-conjugated Sir3¹⁻³⁸⁰ as wild-type Ris1.

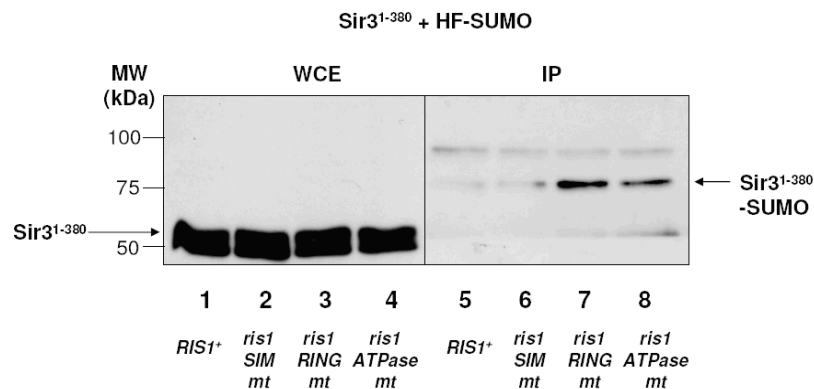


Figure 30: The RING finger motif mutation and ATPase motif mutation of Ris1 cause the accumulation of Sir3-SUMO. His-FLAG-tagged SUMO and Sir3¹⁻³⁸⁰ fragment were ectopically expressed in a wild type, a *ris1* SUMO-interacting motif (SIM) mutant, a *ris1* RING finger mutant, or a *ris1* ATPase mutant strain. A Western with an antibody to Sir3 N-terminus was done for the inputs (WCE) and the SUMO-purified samples (IP). The arrows indicate the positions of unsumoylated and sumoylated Sir3 fragments.

In a genome-wide study, *ris1*Δ was found to be synthetically sick with *slx5*Δ and *slx8*Δ (Pan et al, 2006). To determine whether the three motifs of Ris1 contributed to the genetic interaction with *SLX5*, the *SLX5* gene was deleted in a wild-type strain, in a *ris1*Δ mutant, or in the three *ris1* mutant strains and growth measured with or without the existence of ectopically expressed Slx5. As shown in Figure 31A, no growth defect can be observed in any of the 5 strains when Slx5 was ectopically expressed. When no Slx5 was present in these strains (Figure 31B), a synthetic sickness phenotype was observed in the *slx5*Δ *ris1*Δ strain (lane 2), agreeing with previous work (Pan et al, 2006). The growth of the *slx5*Δ *ris1* SUMO-interacting motif mutant (lane 3) was as good as the *slx5*Δ *RIS1*⁺ strain (lane 1). Of note, the *slx5*Δ *ris1* RING finger mutant (lane 4) showed some growth defect but it was not as great as for the *slx5*Δ *ris1*Δ strain, while the growth defect of the *slx5*Δ *ris1* ATPase mutant (lane 5) was as severe as *slx5*Δ *ris1*Δ. Therefore, both the RING finger motif and ATPase motif of Ris1, to different degrees, have a genetic interaction with *slx5*Δ.

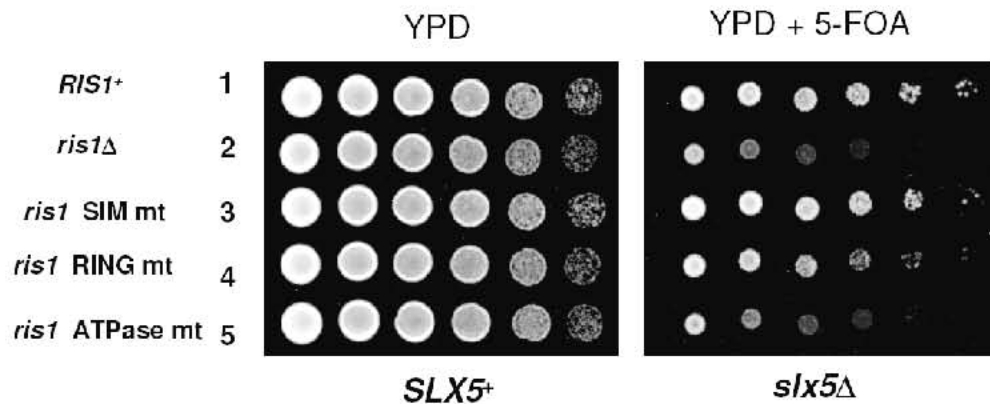


Figure 31: The RING finger motif and ATPase motif of Ris1 are required for genetic interaction with *slx5*Δ. An *slx5*Δ *RIS1*⁺ strain (lane 1), an *slx5*Δ *ris1*Δ strain (lane 2), or three *slx5*Δ *ris1* motif mutant strains (lane 3-5) carrying a *URA3* plasmid expressing Slx5 were grown overnight in YPD medium. Fivefold serial dilutions were spotted on YPD medium without (Left) and with (Right) 5-FOA. Growth on 5-FOA can only occur if the *SLX5 URA3* plasmid is not present.

Interestingly, when performing a similar experiment by using a plasmid to express the Ris1 motif mutants driven by a *CYCI* promoter in a *ris1*Δ *slx5*Δ strain, I noticed that the ATPase mutant had a very severe growth defect that was even greater than that of the double deletion (Figure 32A, compare lane 5 with lane 1). I also found that this mutant was partially dominant when it was expressed in a *RIS1*⁺ *slx5*Δ strain (Figure 32B, lane 2). In summary, I conclude that the RING finger motif and especially the ATPase motif of Ris1 play a role in decreasing the level of sumoylated Sir3 and in the genetic interaction with *slx5*Δ.

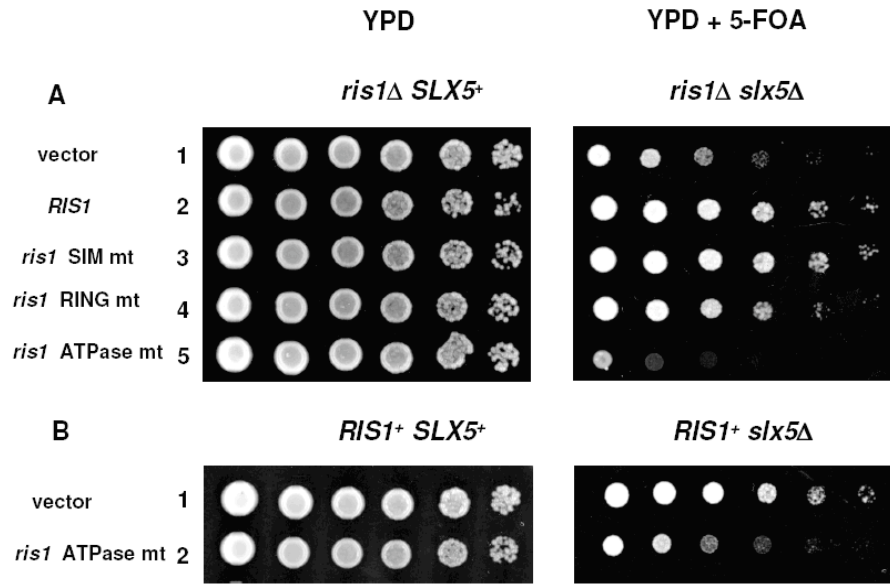


Figure 32: Plasmid-expressed Ris1 ATPase mutant has a very severe growth defect when *SLX5* is deleted. (A) Plasmid-expressed Ris1 ATPase mutant caused a more severe synthetic sickness with *slx5Δ* than *ris1Δ slx5Δ*. A *URA3* plasmid expressing Slx5 with a *CYC1* promoter-containing plasmid expressing wild-type Ris1 (lane 2) or three Ris1 motif mutants (lane 3-5), or a vector (lane 1) were transformed into an *slx5Δ ris1Δ* strain. (B) Plasmid-expressed Ris1 ATPase mutant was partially dominant in a *RIS1⁺ slx5Δ* strain. A *URA3* plasmid expressing Slx5 with a plasmid expressing the Ris1 ATPase mutant (lane 2) or a vector (lane 1) were transformed into an *slx5Δ* strain. Cells grown overnight in YPD medium were fivefold serially diluted and spotted on YPD medium without (Left) and with (Right) 5-FOA. Growth on 5-FOA can only occur if the *SLX5 URA3* plasmid is not present.

Deletion of *SIR4* doesn't affect the ability of Ris1 to lower the level of Sir3-SUMO

As mentioned, Ris1 was known to associate with Sir4 (Zhang & Buchman, 1997), which is known to be multi-sumoylated (Denison et al, 2005). Based on these observations, I tested if sumoylated Sir4 also accumulated in a *ris1Δ* strain. To detect Sir4 sumoylation, I ectopically expressed His-FLAG-tagged SUMO in a *RIS1⁺* or *ris1Δ* strain in which *SIR4* in the genome was C-terminally tagged with Myc. A SUMO purification assay was performed. As shown in Figure 33, I found that unlike Sir3, the level of sumoylated Sir4 was not increased in a *ris1Δ* strain (compare lane 4 with lane 3), suggesting that Ris1 is not involved in down-regulating Sir4-SUMO as it is for Sir3-SUMO.

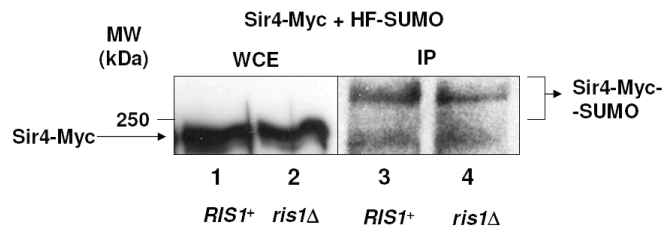


Figure 33: The level of sumoylated Myc-tagged Sir4 is not increased when *RIS1* is deleted. His-FLAG-tagged SUMO was ectopically expressed in a C-terminal Myc-tagged Sir4 strain without or with the deletion of *RIS1* (RS2095 and RS2014, respectively). A Western with an antibody to Myc was done for the inputs (WCE) and the SUMO-purified samples (IP). The arrows indicate the positions of unsumoylated and sumoylated Sir4-Myc.

In spite of this result, it was still possible that Sir4 would be required to recruit Ris1 for decreasing the level of Sir3-SUMO. If this is true, it would be expected that deletion of *SIR4* or *SIR2*, which interacts with Sir4, would prevent the recruitment of Ris1 via Sir4 to down-regulate Sir3-SUMO. As a result, the level of Sir3-SUMO should be increased in a *sir4*Δ and a *sir2*Δ strains.

To test this, I ectopically expressed His-FLAG-tagged SUMO and a Sir3¹⁻³⁸⁰ fragment in a wild type strain, a *sir4*Δ strain or a *sir2*Δ strain and performed a SUMO purification assay. As shown in Figure 34, the level of SUMO-conjugated Sir3¹⁻³⁸⁰ was increased in the *sir4*Δ and in the *sir2*Δ strains, compared with the level in the wild-type strain (compare lane 5 and lane 6 with lane 4). On the other hand, the amount of total Sir3¹⁻³⁸⁰ in the wild-type strain was also lower than in the *sir4*Δ and *sir2*Δ strains (compare lane 2 and lane 3 with lane 1). Therefore, the increased level of sumoylated Sir3¹⁻³⁸⁰ found in the *sir4*Δ and *sir2*Δ strains after purifying sumoylated proteins was not judged to be significant. Therefore, I tentatively conclude that the down-regulation of Sir3-SUMO by Ris1 is not Sir4- or Sir2-dependent. This experiment should be repeated to verify the conclusion.

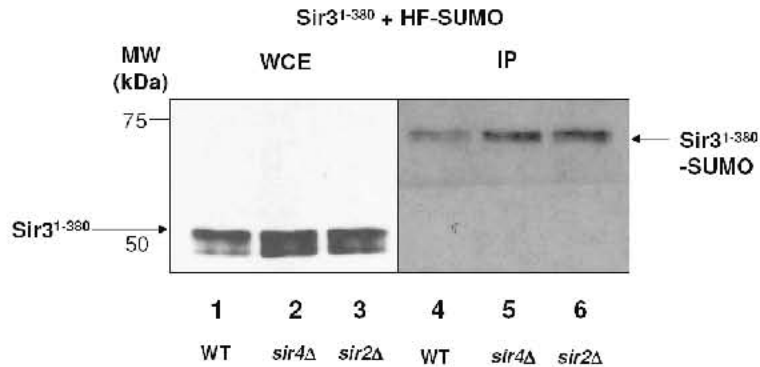


Figure 34: The level of SUMO-conjugated Sir3¹⁻³⁸⁰ is not significantly increased when *SIR4* or *SIR2* is deleted. His-FLAG-tagged SUMO and Sir3¹⁻³⁸⁰ fragment were ectopically expressed in wild type (W303-1a) or a *sir4Δ* or a *sir2Δ* strain. A Western with an antibody to the Sir3 N-terminus was done for the inputs (WCE) and the SUMO-purified samples (IP). The arrows indicate the positions of unsumoylated and sumoylated Sir3 fragments. Note that the protein level of Sir3¹⁻³⁸⁰ in the whole cell extract from wild-type yeast (lane 1) is significantly lower than in the *sir4Δ* (lane 2) or *sir2Δ* sample (lane 3).

The RING finger motif of Ris1 doesn't have *in vitro* auto-ubiquitylation activity

As mentioned, recent studies have shown that the Slx5/Slx8 heterodimer has ubiquitin E3 activity *in vitro* (Uzunova et al, 2007; Xie et al, 2007). Moreover, it was also shown that Ris1 protein in a yeast extract can be pulled down by bacterially expressed recombinant GST-fused Ubc4, one of the ubiquitin E2s (Uzunova et al, 2007), suggesting that Ris1 can bind to ubiquitin E2 and poly-ubiquitylate its protein substrates. In order to test if the RING finger motif of Ris1 has ubiquitin ligase activity, I purified the bacterially expressed recombinant GST-fused C-terminal Ris1 (amino acid 1283-1619) that contains its RING finger motif and used this fusion protein to perform *in vitro* ubiquitylation assays. According to the literature, GST itself is a good substrate for

poly-ubiquitylation (Braun et al, 2007). As a result, the reaction only contained ubiquitin, E1, E2 and GST-RING of Ris1. The RING finger of Rkr1 was used as a positive control (Braun et al, 2007). The auto-ubiquitylation of GST-RING was detected using an anti-Ub or anti-GST antibody. As shown in Figure 35, GST-Rkr1 RING fusion protein shows strong *in vitro* ubiquitylation activity (lane 4), indicating the assay system is working. However, no ubiquitylation activity was seen when the GST-Ris1-RING fusion protein was used (lane 3), showing that the RING finger of Ris1 fused to GST doesn't have auto-ubiquitylation activity *in vitro*.

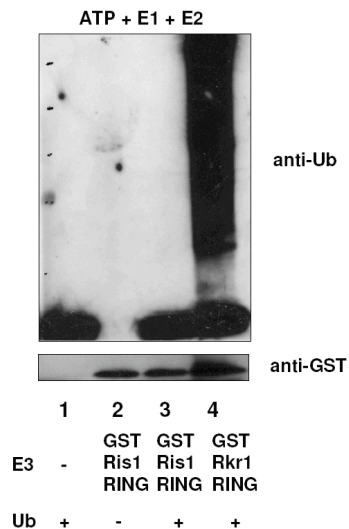


Figure 35: The RING finger motif of Ris1 doesn't have auto-ubiquitylation activity *in vitro*. Purified recombinant ubiquitin E1, E2 and ATP were mixed without (lane 2) or with ubiquitin (lane 1, 3 and 4) and no E3 (lane 1), GST-Ris1-RING finger motif fusion protein (lane 2 and 3) or GST-Rkr1-RING finger motif (lane 4). Proteins were separated by SDS-PAGE and a Western was performed with anti-ubiquitin antibody to detect ubiquitylated substrates or an anti-GST antibody to detect GST fusion proteins. The GST-Rkr1 RING finger motif fusion protein used in lane 4 was previously shown to have a strong auto-ubiquitylation activity (Braun et al, 2007).

Neither Ris1 nor Slx5 is involved in quality control of a Sir3 temperature-sensitive mutant protein, Sir3-8

As described, the yeast transcription regulator Mot1 tends to be sumoylated when the protein is defective or misfolded. This SUMO-conjugated protein is then targeted and degraded by the Slx5/Slx8 heterodimer that plays a role in quality control of this transcription factor (Wang & Prelich, 2009a). Interestingly, a Sir3 temperature-sensitive silencing mutant protein, Sir3-8, has been shown to be degraded at 37°, and that leads to the loss of silencing at the non-permissive temperature (Rine & Herskowitz, 1987; Stone et al, 2000). Based on the findings from Mot1 studies, it would be possible that the stability of Sir3-8 might be controlled by Ris1 or the Slx5/Slx8 heterodimer.

To test this, I deleted *RIS1*, *SLX5* or both in a *sir3-8* strain. Cells were grown at the permissive (25°), intermediate (30°) or nonpermissive (37°) temperature and harvested for a Western using an anti-Sir3 antibody to see if the protein level of Sir3-8 is increased when *RIS1*, *SLX5* or both are deleted. As shown in Figure 36, compared with wild-type Sir3, the protein level of Sir3-8 is very low even at 25° (compare lane 5 with lane 1 in Figure 36A and lane 2 with lane 1 in Figure 36B), and Sir3-8 can hardly be detected at 37° and 30° (lane 3 in Figure 36A and lane 5 in Figure 36B). Moreover, no significant increase of Sir3-8 level was observed at 25° or 37° in a *ris1*Δ mutant (compare lane 6

with lane 5 and lane 4 with lane 3 in Figure 36A) or at both 25° and 30° in a *slx5Δ* mutant or in a *slx5Δ ris1Δ* double mutant (compare lane 3 and 4 with lane 2; lane 6 and 7 with lane 5 in Figure 36B). These results indicate that the protein stability of Sir3-8 is not regulated by either Ris1 or Slx5.

Even though no significant increase of Sir3-8 level can be observed when *RIS1*, *SLX5* or both were deleted, it is still possible that a very small fraction of Sir3-8 may be up-regulated that is sufficient enough to partially repress the temperature-sensitive silencing defect. In order to test this, I performed mating assays to see if deletion of either of these two genes can partially rescue the temperature-sensitive silencing defect caused by *sir3-8*. As shown in Figure 37A, in a *sir3-8* strain, silencing at *HML* was as good as the wild type at 25° (lane 2 versus lane 1, 25°), much worse than the wild type at 30° (lane 2 to lane 1, 30°) and completely defective at 37° (lane 2, 37°). When *RIS1* was deleted, no significant improvement of silencing at *HML* was observed at 30° (lane 7, 30°) and silencing was still completely defective at 37° (lane 7, 37°), demonstrating that the temperature-sensitive silencing defect can't be suppressed when *RIS1* is deleted. In Figure 37B, a similar result was observed. Deletion of either *SLX5* or both *SLX5* and *RIS1* couldn't improve the *sir3-8* silencing ability at 30° (compare lane 9 and 10 with lane 8,

30°). Based on the results in Figure 36 and 37, I conclude that neither *Ris1* nor *Slx5* function as a quality control of the *Sir3* temperature-sensitive mutant protein, *Sir3-8*.

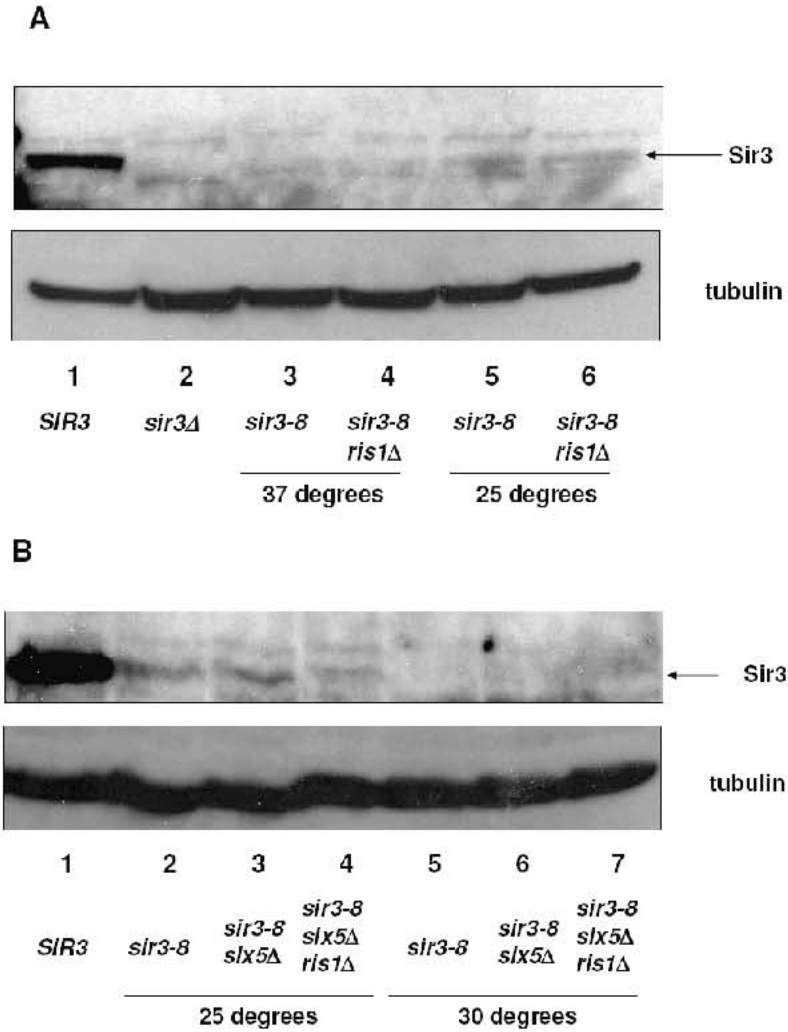


Figure 36: Neither *Ris1* nor *Slx5* contributes to the stability of the *Sir3-8* mutant protein. (A) The level of *Sir3-8* protein is not increased at 37° or 25° when *RIS1* is deleted. A *SIR3* and a *sir3Δ* strain (lane 1 and 2) were grown at 30° as controls, while a *sir3-8* (lane 3 and 5) and a *sir3-8 ris1Δ* strain (lane 4 and 6) was grown at either 37° or 25°. (B) The protein level of *Sir3-8* is not increased at 37° or 25° when *SLX5* or both *SLX5* and *RIS1* are deleted. A *SIR3*⁺ strain (lane 1) was grown at 30° as a control, while a *sir3-8* (lane 2 and 5), a *sir3-8 ris1Δ* strain (lane 3 and 6) and a *sir3-8 slx5Δ ris1Δ* (lane 4 and 7) strain were grown at either 37° or 25°. A Western blot of these samples was performed with an anti-*Sir3* antibody. Tubulin detected with an anti-tubulin was also used as a protein loading control. The arrow indicates the positions of *Sir3* and *Sir3-8*.

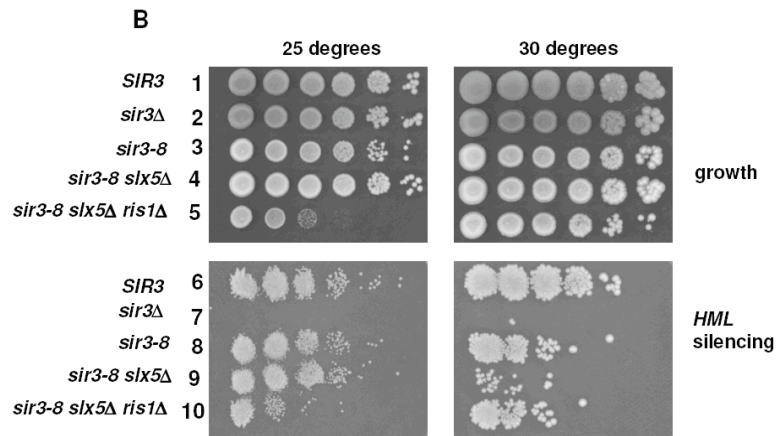
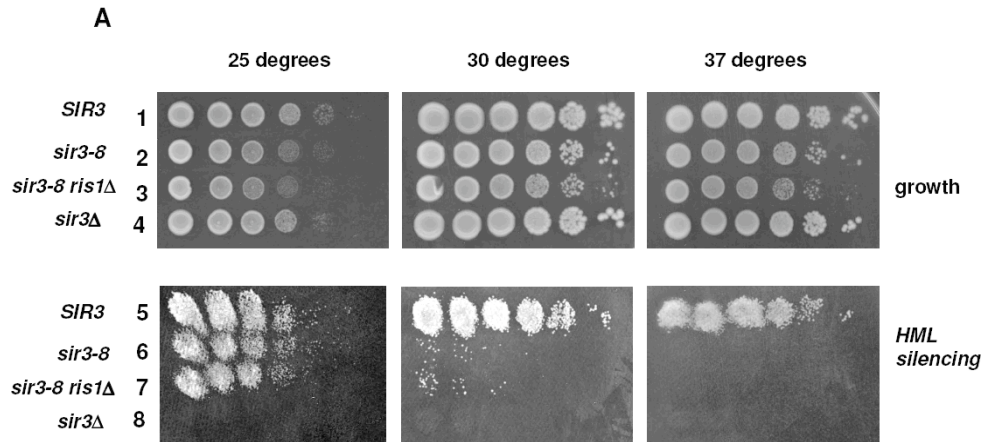


Figure 37: Deletion of *RIS1*, *SLX5* or both doesn't repress temperature-sensitive silencing defects caused by *sir3-8*. To observe growth, cells were directly spotted onto a YPD plate (growth, top). To observe *HML* silencing, cells were mixed with a *MAT α* mating tester strain and replica plating onto an SD plate (*HML* silencing, bottom). (A) Deletion of *RIS1* doesn't repress silencing defects at *HML* at 30° or 37° caused by *sir3-8*. A *SIR3*⁺ (lane 1 and 5), a *sir3-8* (lane 2 and 6), a *sir3-8 ris1Δ* (lane 3 and 7) and a *sir3Δ MAT α* strain (lane 4 and 8) were fivefold serially diluted and grown at 25°, 30° or 37° to observe growth and silencing at *HML*. (B) Deletion of *SLX5* or both *SLX5* and *RIS1* doesn't suppress silencing defects at 30° caused by *sir3-8* at *HML*. A *SIR3*⁺ (lane 1 and 6), a *sir3Δ* (lane 2 and 7), a *sir3-8* (lane 3 and 8), a *sir3-8 slx5Δ* (lane 4 and 9) and a *sir3-8 slx5Δ ris1Δ MAT α* strain (lane 5 and 10) were fivefold serially diluted and grown at 25°, 30° or 37° to observe growth and silencing at *HML*. The poor growth in lane 5 was due to the synthetic sickness caused by the *slx5Δ ris1Δ* double mutant.

Discussion

The possible regulation of Sir3 sumoylation by other post-translational modifications

As mentioned, sumoylated lysine residues are often in a short consensus sequence (I/L/V)KXE (Sampson et al, 2001). Here I showed that Sir3 is mono-sumoylated on lysine 247 within its only SUMO consensus sequence, IKIE, and I further confirmed that the whole consensus sequence is important for Sir3 sumoylation (Figure 22). Different from Sir3, even though Sir4 has 2 SUMO consensus sequences, the only reported SUMO-conjugated lysine residue, K1128, is not in the perfect consensus sequence (VKNV) (Denison et al, 2005). However, in the same study it was also shown that Sir4 is multi-sumoylated. It would be interesting to know if Sir4 can also be sumoylated within these two SUMO consensus sequences.

Previous studies reported that phosphorylation of Sir3 leads to the redistributions of silencing functions at different silent regions (Ray et al, 2003). Of note, all four of the phosphorylation sites, S275, 282, 289, 295, respectively, are fairly close to the sumoylation site, K247, which I also showed to be able to affect the distribution of Sir3 on the chromatin (Figure 27 and 28). Interestingly, some studies have shown linked regulation of SUMO conjugation to phosphorylation. It was revealed that some lysines

tend to be sumoylated when phosphorylation happens immediately downstream of the SUMO consensus sites (Anckar & Sistonen, 2007). It would be interesting to test if there is any cross-talk between these two different post-translational modifications on Sir3.

The Role of Ris1 in down-regulating the protein level of sumoylated Sir3

Recently, several proteins that contain both SUMO-interacting motifs and RING finger motifs, including the Slx5/Slx8 heterodimer and Ris1 in budding yeast, were found to target and degrade many SUMO-conjugated proteins. Slx5/Slx8 was known to have *in vitro* activity for both ubiquitylation and sumoylation (Ii et al, 2007a; Ii et al, 2007b; Xie et al, 2007). It was also shown that sumoylated Rad52 is targeted and degraded by Slx5/Slx8, which is required for the pathway of repairing DNA double strand breaks caused by DNA-damaging reagents (Burgess et al, 2007; Xie et al, 2007). Moreover, Slx5/Slx8 can also target and degrade SUMO-conjugated Mot1 that functions as a quality control of this transcriptional regulator (Wang & Prelich, 2009a).

All that we know about Ris1 in the literature is: 1) like Slx5, Ris1 binds to ubiquitin E2 (Uzunova et al, 2007), 2) like Slx5 and Slx8, Ris1 decreases the level of many sumoylated proteins (Ii et al, 2007b) and 3) Ris1 interacts with a SUMO-conjugated nucleolar protein Ebp2 through its SUMO-interacting motifs within amino acids 336 to 599, but no evidence was provided that it could control the level of Ebp2 (Shirai &

Mizuta, 2008). As a result, it is still not clear whether Ris1 has ubiquitin ligase activity and if Ris1 can specifically down-regulate any known sumoylated protein.

Here I showed that Ris1, but not Slx5, can decrease the level of SUMO-conjugated Sir3 (Figure 24), implying the different STUbL members have different preferences among sumoylated proteins. Moreover, I showed that sumoylated Sir3 also accumulated in a *ris1* RING finger motif mutant (Figure 30), indicating that the RING finger motif of Ris1 is required for decreasing the level of Sir3-SUMO. Even though the RING finger motif of Ris1 was shown to be important for decreasing the level of sumoylated Sir3, I found that the RING finger motif of Ris1 alone did not have auto-ubiquitylation activity *in vitro* (Figure 35). Of note, the level of sumoylated Sir3 was also increased in the *ris1* ATPase motif mutant (Figure 30), indicating that this unique motif within Ris1 is also required for down-regulating Sir3-SUMO. As a result, it is possible that full length Ris1 is required for the ubiquitin E3 activity *in vitro* and *in vivo* when encountering suitable SUMO-conjugated proteins. It would be important to repeat the *in vitro* ubiquitylation experiment by using a full length Ris1 with suitable ubiquitin E2. Also, a sumoylated substrate should be used.

Surprisingly, in spite of the fact that Ris1 was known to associate with Sir4, which can be multi-sumoylated, I didn't find that Ris1 could also target Sir4-SUMO (Figure 33).

Neither did I observe that deletion of *SIR4* could affect the ability of Ris1 to decrease the amount of SUMO-conjugated Sir3¹⁻³⁸⁰ (Figure 34). This indicated that the level of SUMO-Sir4 is regulated by proteins other than Ris1, and that Ris1 can still down-regulate Sir3-SUMO without association with Sir4. Further studies would be required to investigate how Sir4 sumoylation is regulated and whether sumoylated Sir4 is also required for some important functions in yeast.

The role of sumoylation and Ris1 on regulating the spreading of Sir3

As described, I showed that abolishing Sir3 SUMO conjugation or deleting *RIS1* caused more spreading of Sir3 from telomeres (Figure 27 and 28). I conclude that this led to inappropriate silencing on transcriptionally active regions since the yeast growth defect was *SIR2*- and *SIR4*-dependent (Figure 25). As a result, I think that down-regulation of sumoylated Sir3 by Ris1 is providing a protection mechanism to avoid Sir3 spreading improperly from the silent chromatin.

Of note, Ris1, originally called Dis1, was found in a genetic screen for overexpressed genes that cause dosage interference of silencing. Based on this idea, deletion of *RIS1* should improve silencing (Zhang & Buchman, 1997). In their results, Zhang and Buchman did see a slight increase in silencing of a *CYCI-lacZ* gene at an

ectopic location controlled by synthetic silencers in a *ris1*Δ mutant strain. These results support our idea that Ris1 plays a role in preventing silencing in euchromatic regions.

In spite of the fact that abolishing Sir3 sumoylation or deleting *RIS1* caused further spreading of Sir3 from telomeres, I noticed that the total amount of Sir3 was actually not changed significantly. By using the ChIP results with moderately increased Sir3 shown in Figure 28A and 28B, I did an estimation and calculated that the amount of total Sir3 was only increased about 20% when Sir3 sumoylation was abolished or when *RIS1* was deleted. This small increase of Sir3 was not observed in the whole cell extracts in my previous experiments with the Sir3 K247R mutant or the *ris1*Δ strain (Figure 22A, lane 3 versus lane 2 and Figure 24B, lane 2 versus lane 1). Based on these results, I think sumoylation or Ris1 has very little or no effects on the total amount of Sir3.

The roles of the three motifs within Ris1

As a member of STUbL family, it seemed reasonable to postulate that both the SUMO-interacting motifs (SIMs) and RING finger motif of Ris1 were important to decrease the level of Sir3-SUMO. To test this, I created *ris1* mutant strains that would abolish each motif. Interestingly, I found that the RING finger mutation caused accumulation of sumoylated Sir3¹⁻³⁸⁰ (Figure 30) and a mild synthetic sickness phenotype when *SLX5* is deleted (Figure 31), supporting the idea that the RING finger motif of Ris1

is important for lowering the amount of Sir3-SUMO and having overlapping function with Slx5.

For the *ris1* SIM mutant, I found that this mutant did not have any defect in down-regulating SUMO-conjugated Sir3¹⁻³⁸⁰ (Figure 30) nor did it cause synthetic sickness with *slx5Δ* (Figure 31 and 32). There was only one SUMO-interacting motif that had been identified when I designed this *ris1* SIM mutation (Hannich et al, 2005). Later, additional putative SIMs of Ris1 were predicted (Uzunova et al, 2007). We think our SIM mutant, as a result, could not completely abolish the binding ability to its sumoylated substrate. As a result, a better mutant that can abolish all the SIMs of *RIS1* should be made in the future.

Unique among all known STUbLs, Ris1 has a SWI/SNF-like ATPase motif in addition to its SIMs and RING finger motif. Surprisingly, we found mutating ATPase motif of Ris1 also led to the accumulation of SUMO-conjugated Sir3¹⁻³⁸⁰ (Figure 30). Strikingly, when *SLX5* was deleted, we found that a single mutation in the ATPase motif of Ris1 caused a synthetic sickness phenotype as strong as when the whole *RIS1* gene was deleted when *SLX5* is also deleted (Figure 31). Moreover, we also noticed that moderately increasing the amount of this ATPase mutant can cause a much more severe growth defect than *ris1Δ* and was even partially dominant in a *RIS*⁺ strain when *SLX5* is

deleted (Figure 32). Since both of Slx5 and Slx8 don't have an ATPase motif, it is not clear how the ATPase motif of Ris1 plays a role with the Slx5/Slx8 heterodimer.

However, we think that this ATPase motif may allow Ris1 to have more variable functions than other STUbLs. This is worth further investigation. Recently, Slx5/Slx8 was shown to target SUMO-Mot1, and this is correlated with the quality control of this yeast transcription factor (Wang & Prelich, 2009a). However, I found neither Ris1 nor Slx5 are involved in the quality control of a *sir3* temperature-sensitive mutant, *sir3-8* (Figure 36 and 37). However, it is still possible that these two STUbL members can function for quality control on different sumoylated proteins, like what Slx5/Slx8 did to SUMO-Mot1, and together regulate some important pathways in yeast.

Conclusions

In the studies on Sir3, I found that Sir3 is mono-sumoylated on K247 and the intact consensus sequence is required. Siz1 is the primary SUMO E3 ligase responsible for Sir3 sumoylation. In a *ris1*Δ mutant, the level of sumoylated Sir3 increases, while the total Sir3 level is not affected. Mutation of the RING finger or of the ATPase motif of Ris1 also caused the accumulation of Sir3-SUMO. These two mutations also have a synthetic sickness with *slx5*Δ to different extents. However, neither Ris1 nor Slx5 functions in

quality control of a Sir3 temperature-sensitive mutant protein, Sir3-8, as Slx5 does for a mot1 mutant protein (Wang & Prelich, 2009a). Although Ris1 is known to bind to one of the ubiquitin E2, Ubc4, the RING finger motif of Ris1 alone doesn't have auto-ubiquitylation activity *in vitro*.

In spite of the fact that Sir4 can be multi-sumoylated and associates with Ris1, deletion of *RIS1* doesn't cause accumulation of Sir4-SUMO, and deletion of *SIR4* doesn't affect the ability of Ris1 to down-regulate Sir3-SUMO. Overexpression of Sir3 causes it to spread from the silent chromatin into euchromatin and is somewhat toxic to yeast. This toxicity depends on Sir2 and Sir4. Abolishment of Sir3 sumoylation, deletion of *RIS1*, or both, causes further spreading of Sir3 from the silent chromatin and leads to a more severe growth defect.

As shown in Figure 38, we propose a model that reducing the level of sumoylated Sir3 by Ris1 provides a protection mechanism by sculpting the regions for Sir3 occupation from the silent chromatin. By this way, Sir3 is prevented from spreading further by an uncharacterized boundary. Once crossing the boundary region, Sir3 may have more chances to be sumoylated and in turn get down-regulated by Ris1. When abolishing sumoylation or deleting *RIS1*, Sir3 has more chances to spread across the boundary into the euchromatin that leads to inappropriate silencing.

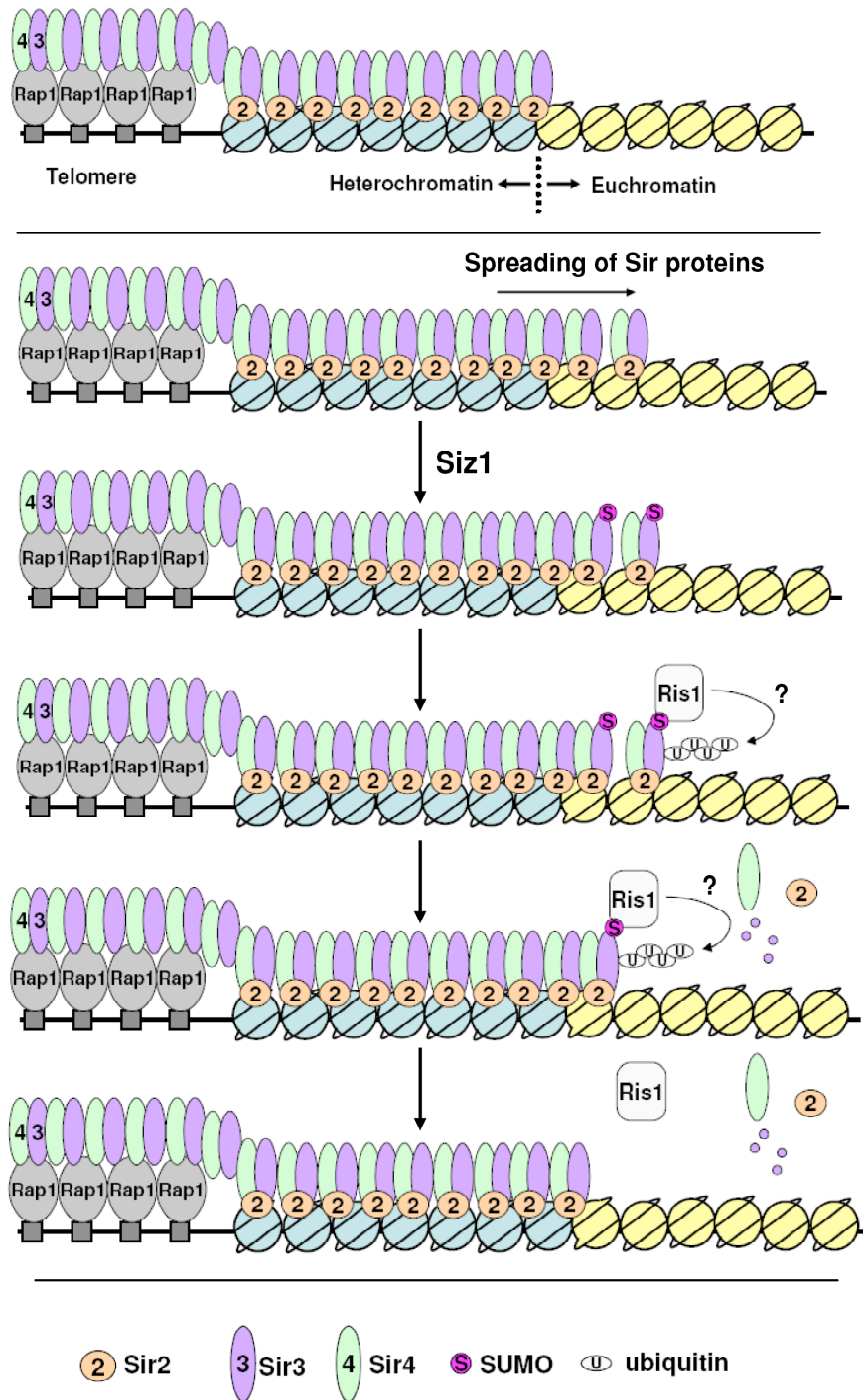


Figure 38: A model for how Sir3 spreading is affected by sumoylation and Ris1. Sir3 spreads from telomere to nearby chromatin. It is prevented from spreading further by an uncharacterized boundary or by a limiting amount of Sir proteins. When Sir3 is overexpressed and spreads across the boundary, it may have more chance to be sumoylated, probably by Siz1. Ris1 then lowers the level of Sir3-SUMO near the boundary and avoids further spreading of Sir3.

Future Plans

Better designs of *ris1* SIMs mutants

As described, I noticed that my *ris1* SUMO-interacting motif mutant didn't cause the accumulation of sumoylated Sir3¹⁻³⁸⁰ (Figure 30) and neither did it cause synthetic sickness with *slx5Δ* (Figure 31 and 32). I think this is because I didn't abolish all the SIMs within Ris1. It was shown that there are two different types of SUMO-interacting motifs, type A and type B, respectively. A type A-SIM usually contains a patch of 3–4 hydrophobic residues and is followed by a stretch of 3–4 acidic residues, while a type B-SIM contains a small sequence motif (consensus: (I/V)DL(T/D)) (Uzunova et al, 2007).

As shown in Figure 39, Ris1 has two SIMs of each type within amino acid 7 to 550. It was shown that deleting amino acids 336 to 599 of Ris1, containing three out of four SIMs, abolished the interaction with SUMO-conjugated Ebp2 (Shirai & Mizuta, 2008). Based on these findings, a new *ris1* SIM mutation in which all four conserved SUMO interacting motifs are mutagenized can be constructed by site-directed mutagenesis. Alternatively, a truncated Ris1 that the region from amino acids 7 to 550 is deleted could be made. A new *ris1* SIM mutant strain could then be created as described in the Materials and Methods section of this chapter.

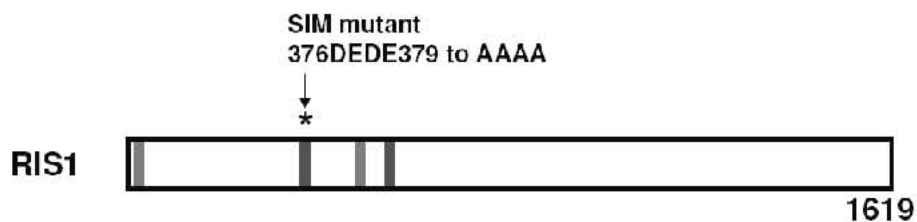


Figure 39: Schematic drawing of SUMO-interacting motifs in Ris1. The position of putative SIMs (type A in blue; type B in green) are indicated (Uzunova et al, 2007). The abolished SUMO interacting motif of the *ris1* SIM mutant used in my experiments is indicated by asterisk and arrow.

Improvement of *in vitro* ubiquitylation assays for Ris1

As shown in Figure 35, I noticed that the C-terminal RING finger motif of Ris1 alone does not have auto-ubiquitylation activity *in vitro* (Figure 35). We later noticed that the ubiquitin E2 we used for this assay, UbcH5a, may not be suitable for Ris1. One reason is that Ris1 can bind to bacterially expressed GST-Ubc4 in a pull-down assay (Uzunova et al, 2007), suggesting that Ubc4 may be the natural ubiquitin E2 for Ris1 in yeast. The other reason is that in the studies with the Slx5/Slx8 heterodimer, it was found that Slx5/Slx8, has the strongest ubiquitylation activity when using Ubc4 as the E2, while this heterodimer shows no activity when using UbcH5a as the ubiquitin E2 (Ii et al, 2007a). Based on these findings, one should repeat this *in vitro* auto-ubiquitylation assay of Ris1 by using Ubc4 as the ubiquitin E2.

Moreover, it is possible that Ris1 needs to be full length and encounter suitable SUMO-conjugated substrates to have ubiquitin E3 activity. Another study has shown that

the yield of recombinant full-length Ris1 expressed from bacteria was poor because the protein was insoluble (Uzunova et al, 2007). In order to obtain enough full-length Ris1, one would have to purify the full-length Ris1 from a yeast strain expressing GST-Ris1 by GST purification. It was also reported that a SUMO-tagged GST fusion protein, which mimics the protein-SUMO conjugation, is a suitable substrate *in vitro* for one of the STUbLs in fission yeast (Sun et al, 2007). To do this, a plasmid that expresses His-SUMO-GST fusion protein could be expressed in *E. coli*. It could be purified by a Ni⁺⁺ or GST column and the protein used as a more suitable substrate for Ris1 in the *in vitro* ubiquitylation assays.

Recently, a study has shown a method that allows chemical ubiquitylation of a protein of interest at a specific lysine residue via a technique called expressed protein ligation (EPL) (McGinty et al, 2008). One limitation of this method is the C-terminal fragment of the protein containing the lysine residue for Ub modification has to be made by peptide synthesis. Therefore, the lysine residue for chemical modification has to be close to the C-terminus of the protein due to the cost of peptide synthesis. Since Sir3 is sumoylated at K247, a perfect substrate for this method would be SUMO-conjugated Sir3¹⁻²⁵⁰. It could be used to test if the SUMO interacting motifs of Ris1 can directly bind

to this substrate and if Ris1 can act as an ubiquitin E3 ligase to poly-ubiquitylate this chemically SUMO-conjugated substrate *in vitro*.

Test if the level of Sir3-SUMO increases in an *ubc4Δ* or an *ubc5Δ* strain

As described, bacterially expressed GST-Ubc4 can pull down Ris1 from the yeast whole cell extract (Uzunova et al, 2007), although it is still not known if Ubc4 is the ubiquitin E2 of Sir3-SUMO. Of note, among all yeast ubiquitin E2s, Ubc5 shares 92% amino acid identity and has overlapping functions with Ubc4 (Seufert & Jentsch, 1990). It would be interesting to determine if Sir3-SUMO is down-regulated by Ubc4 or Ubc5. To test this, one can perform a SUMO purification assay and observe if the level of Sir3-SUMO increases in an *ubc4Δ* or an *ubc5Δ* strain.

Test if overexpressing Sir3 under different conditions does causes inappropriate silencing on euchromatin to different degrees

In Figure 25 and 26, I found that overexpressing Sir3 is toxic and this toxicity is exacerbated by loss of sumoylation or *RIS1*. Even though I performed the ChIP assays and showed in Figure 27 that the toxicity is correlated with the further spreading of Sir3 from the silent chromatin, it is still not known that whether this further spreading of Sir3 does cause the inappropriate silencing at the transcriptionally active regions.

To test this idea, one can overexpress Sir3 in a *RIS1*⁺ (*RIS1*⁺ OE *SIR3*) or a *ris1Δ*

strain (*ris1Δ* OE *SIR3*), collect the cDNAs from each sample and perform the expression microarray assays to see the decrease or increase of transcripts compared with a control in which Sir3 is normally expressed by its single genomic copy (Genomic *SIR3*). Based on our model, it is expected that the level of many transcripts should be decreased when Sir3 is overexpressed, while the extent of these reductions should be even greater when overexpressing Sir3 in a *ris1Δ* strain.

Test if Sir3 spreading is also affected by ATPase mutant or RING mutant

In Figure 27 and 28, I showed that Sir3 can spread further from telomeres when Sir3 can't be sumoylated or when *RIS1* is deleted. In Figure 30, I also showed that the level of Sir3-SUMO is accumulated in the Ris1 RING finger mutant and ATPase mutant, respectively, which is similar to the result in a *ris1Δ* strain (Figure 24B and 24C). Based on these findings, it would be interesting to know if the RING finger motif and the ATPase motif of Ris1 are required for regulating Sir3 spreading. To test this, we could overexpress or moderately increase Sir3 in these two *ris1* mutant strains and perform ChIP assays as described in Figure 27 and 28.

A high copy suppressor screen for *slx5Δ ris1* ATPase mutant

In Figure 31, I showed that a single mutation at the ATPase motif of Ris1 can cause a significant synthetic sickness, as severe as with a *RIS1* deletion, when *SLX5* is also

deleted. I further noticed that, as shown in Figure 32A, when this Ris1 ATPase mutant protein is ectopically expressed by a *CYCI* promoter in the *ris1Δ slx5Δ* double mutant, the sickness is even worse than the double mutant itself. In Figure 32B, I also showed that this ectopically expressed mutant is partially dominant in a *RIS1⁺ slx5Δ* strain.

It is important to know that only Ris1, but not Slx5 and Slx8, contains this ATPase motif. Therefore, it would be interesting to know why an *slx5Δ ris1 ATPase* double mutant can cause such a severe growth defect, especially when this Ris1 ATPase mutant protein is expressed by a plasmid. To investigate this, a suppressor screen could be done. A yeast high copy library could be transformed into a *ris1Δ slx5Δ* strain containing a *URA3* plasmid expressing Slx5 and a plasmid with a *CYCI* promoter expressing the Ris1 ATPase mutant. The transformants would be transferred to a medium with 5-FOA in order to remove the Slx5 expressing plasmid. The large colonies would then be selected and the library plasmid within the cells extracted and sequenced to find out the candidates that could suppress the severe growth defect caused by an *slx5Δ ris1 ATPase* double mutant.

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