Locus specific regulation of gene silencing by protein sumoylation in *S. cerevisiae*

A Thesis submitted to the University of Hyderabad for the award of Ph. D. degree in the Department of Biochemistry, School of Life Sciences

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June, 2015

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DECLARATION

I, **Abdul Hannan,** hereby declare that this thesis entitled **"Locus specific regulation of gene silencing by protein sumoylation in *S. cerevisiae*"** submitted by me under the guidance and supervision of Dr. Krishnaveni Mishra, is an original and independent piece of research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Locus specific regulation of gene silencing by protein sumoylation in *S. cerevisiae*"** is a record of bonafide work done by Abdul Hannan, a research scholar for Ph.D. programme in Department of Biochemistry, School of Life Sciences, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Signature of the Supervisor

Head of the Department

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ACKNOWLEDGEMENTS

It is my privilege to express my gratitude to my supervisor **Dr. Krishnaveni Mishra** for her constant guidance and encouragement during my tenure in the lab.

I thank **Prof. N. Siva Kumar,** Head, Department of Biochemistry, and former Head, **Prof. K. V.A. Ramaiah, and O.H. Shetty** for making my research feasible with excellent infrastructure and lab facilities.

I thank **Prof. P.Reddanna** Dean, School of Life Sciences, and former Deans, **Prof. M. Ramanadham, Prof. A.S. Raghavendra, Prof Aparna Dutta Gupta and Prof R.P. Sharma** for providing the general facilities of the school.

I would like to thank my Doctoral Committee members **Dr. Naresh Babu V. Sepuri and Dr. Shramishta Banerjee** for assessing my research work in between and for the useful discussions.

I am also grateful to the non-teaching staff members of the Department of Biochemistry, School of Life Sciences for their kind assistance and cooperation.

I cannot finish without expressing my gratitude towards all my labmates**: S. Tirupatiah, P.Nagesh, E. Sreesankar, Yashpal, Neethu, Hita, Imli, Gurranna, Sangeetha, Kathir, Pallavi,** lab attendant **Aamir and Anil** and all the **MSc project students.**

I thank all my teachers since my childhood whose guidance and encouragement at each step was instrumental in shaping my career.

I thank **D. Shore, A. Johnson and S. Gasser** for strains and plasmids and I would also like to thank **Dr. Rakesh K Mishra,** CCMB, for allowing us to use his lab facilities to do ChIP.

I thank **DST, UGC, CSIR, and DBT funding bodies** for providing necessary reagents during my work. I also thank the DST-FIST, UGC-SAP and UoH DBT CREBB for funding several research facilities in the School.

And I am deeply grateful to the **University Grant commission and Indian Council, India** for their financial support.

I thank my friends **Sapan, Barnali, Jhon, Sanjeeb, Tridib, Ranjit, Pabitra, Ananad, Ramcharan, Prasanta, Bikas, Kiran, Heerock, Rishan, Mubarak, Dipjyoti, Sulinder, Amina, Suchira, Jayanta, Purba, Bhaskar, Nikhil, Pratha, Manash, Jahnu, Rajesh, Laxmi, Roshni and Rhishi** for their help, support and affection.

I would like to express my gratitude to my parents, and my brothers for their motivation, encouragement.

Abdul Hannan

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List of Abbreviations

5-FOA	5-Fluoroorotic acid
ADE2	Adenine requiring
ADH1	Alcohol Dehydrogenase
AMP	Ampicillin
BSA	Bovine serum albumin
CEN	Centomere
ChIP	Chromatin immunoprecipitation
CTD	C-terminal domain
DAPI	4', 6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra acetic acid
EST	Ever Shorter Telomeres
ESC1	Establishes silencing complex 1
GAL	Galactose
GLU	Glucose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Homologous recombination
HRP	Horseradish peroxidise
HML	Hidden MAT left

HMR	Hidden MAT right
HIS	Histidine
LB	Luria-Bertani broth
LEU	Leucine requiring
LiAC	Lithium Acetate
MMS	Methyl methane sulfonate
mRNA	Messenger RNA
MAT	Mating type
MLP	Myosin like protein
NAD	Necotinamide adenine dinucleotide
NET1	Nucleolar silencing establishing factor and telophase regulator
NUP60	Nuclear pore 60
NSP	Nucleoskeletal like protein
NHEJ	Non-homologous end joining
NPC	Nuclear pore complex
OD	Optical density
ORC	Origin recognition complex
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIAS	Protein inhibitor of activated STAT
Pol	Polymerase
PVDF	Polyvinylidene Fluoride
RAD51	Radiation sensitive mutant 51
RAD52	Radiation sensitive mutant 52

RIF1	RAP1-interacting factor 1
RIF2	RAP1-interacting factor 2
RTT103	Regulator of Ty1 Transposition
RAP1	Repressor activator protein
SC	Synthetic complete
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SGD	Saccharomyces genome database
SIR	Silent information regulator
SMT3	Supressor of Mif-2
SUMO	Small ubiquitin related modifier
SOB	Super optimal broth
SIZ	SAP and MIZ finger domain
SP-RING	Siz/PIAS RING
ssDNA	Single-stranded DNA
TCA	Trichloroacetic acid
Tel	Telomere
TPE	Telomere position effect
TRP1	Phosphoribosylanthranilate isomerase
UBA2	Ubiquitin activating-2
UBC9	Ubiquitin conjugating 9
UV	Ultraviolet
WT	Wild type
Yku70/80	Yeast KU 70/80
YPD	Yeast extract peptone dextrose

Chapter 1

Introduction

The eukaryotic genome is organised into chromatin, an ensemble of DNA and proteins including histones and non-histone proteins. This not only helps to confine DNA in the nucleus and protect it from damage but also provides the basis for chromatin-mediated regulation of DNA transactions including recombination, replication, transcription, repair and other important biological process. The extent of chromatin compaction/organization is not uniform throughout the genome, leading to the presence of interspersed highly compact regions called heterochromatin and less compact regions called euchromatin. Heterochromatin was initially identified cytologically in insect cells as the part of chromosome that stays darkly stained/condensed in both mitosis and interphase, contrary to the rest of the chromosome that is only visible/condensed in mitosis but becomes decondensed in interphase. The other special properties of heterochromatin observed in early studies are its tendency to localize at specific sites in the nucleus including association with the nuclear envelope and nucleolus.

Studies of the heterochromatin in many other organisms revealed its different structural and functional properties and discovered molecular mechanisms underlying its establishment, maintenance and inheritance. In contrast to silent genes in euchromatin, heterochromatic gene regions are not simply regulated by upstream regulatory elements, but instead are silenced, by their inclusion into a chromatin domain with a distinct physical structure that is inhibitory to transcription (Chen & Widom, 2004). At every heterochromatic structure, there are processes to start the

silencing process at a specific sequence and spread the silencing machinery from the start site across an entire region.

Acetylation of histone regulates many cellular processes, and unique acetylation marker, either single or in multiple combinations, produces specific outcomes. As an example, the acetylation pattern of newly produced histones is necessary for their assembly by histone chaperones into nucleosomes. Moreover, the chromatin compaction and folding are regulated by acetylation of histone H4K16. Deacetylation of H4K16 is essential for heterochromatin spreading, on the other hand, acetylation of H4K16 acts as a barrier to heterochromatin spreading. Finally, histone acetylations critically regulate gene transcription, but recent studies shows that deacetylation of some specific sites plays an important role in activation as well (Shahbazian & Grunstein, 2007). Many histone acetyl transferases (HAT) and histone deacetylases (HDAC), with different specificities for different histone proteins and for unique sites on individual histones have been identified. Heterochromatin establishment is also regulated by other modifications of histones including methylation by site-specific histone methylases and phosphorylation, especially in higher eukaryotes. Understanding how these enzymes generate distinct modification patterns and maintain the function is an important challenge (Shahbazian & Grunstein, 2007).

1.1. Yeast heterochromatin

Saccharomyces cerevisiae has served as a useful model system to understand the molecular basis of heterochromatin establishment although it does not possess the classical "darkly staining" chromatin. The molecular mechanisms that establish silent chromatin in yeast are similar to the processes that establish heterochromatin

in higher eukaryotes. There are three heterochromatin loci present in *S.cerevisiae* namely, telomeric, *HM* and *rDNA* (Bi, 2014; Kueng et al, 2013).

1.1.1. Heterochromatin at HM loci

Investigation of heterochromatin in *S. cerevisiae* started from early genetic analyses of how the mating type of this single celled fungus was determined. Haploid yeast cells have either *a* or the *alpha* mating type, and *a* and *alpha* cells can mate to form an *a/alpha* diploid. It was found that three loci are there in the haploid genome, *viz.*, *MAT*, *HMR* and *HML* that encode factors responsible for mating type determination. But only the *MAT* locus is active transcriptionally, whereas *HML* and *HMR* are silent. This results in mating genes present at the *MAT* locus determining the mating type of haploid cells. Cell having the *MATa* allele (having a1 and a2 genes) is a "*a*" cell, on the other hand the cell having *MAT-α* allele (having *α 1 and α 2 genes*) is an α cell. The *α 1 and α 2* genes are also present at the *HML* locus, and *a1* and *a2* genes are present at *HMR*. As *HML* and *HMR* are silent, they never contribute to the mating type of the host under normal conditions, but instead act as donors in the gene conversion event that result in mating type switching of haploid cells. Intrigued by the observation that the mating cassettes (*α 1– α 2 and a1–a2*) are transcribed when located at *MAT* but repressed/silenced at *HML* and *HMR* loci, many early investigations have led to the understanding of this phenomenon. Figure 1 shows a schematic representation of the *HM* locus. Genetic and molecular biological studies have shown that *cis* acting elements and trans-acting factors are involved in the silencing of the *HM* loci. The *cis*-acting elements are small sequences dubbed silencers that flank the *HML* and *HMR* loci, each containing a combination of two or

three binding sites for the Abf1 (B) and Rap1 (E) proteins and origin recognition complex (A).

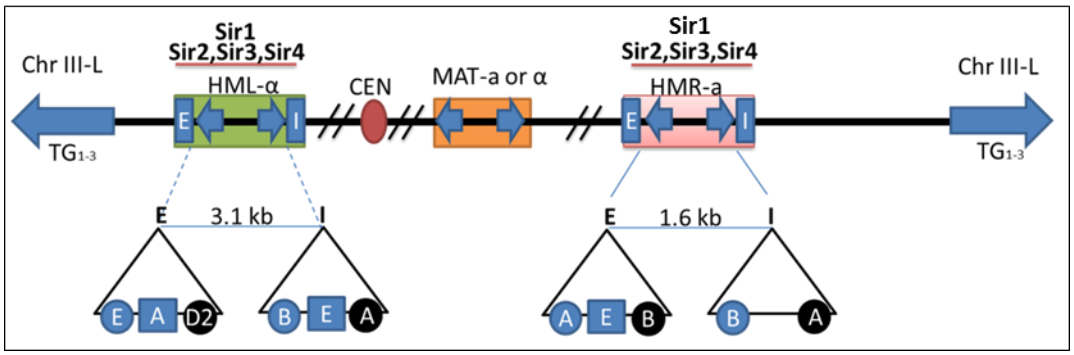

Figure 1: Heterochromatin at HM loci. *On chromosome III, the HMR and HML loci are present on the right and left of the chromosome. The MAT locus, which determines the mating factor expression is on the right arm.*

An additional SUM1 binding site is present on *HML-E* silencer. The trans-acting factors consist of the silencer binding proteins and Sir1, Sir2, Sir3 and Sir4 proteins that are components of silent chromatin of the *HM* loci. Silencing is established through protein-protein interactions between the silencer binding proteins and the Sir proteins. Rap1 interacts with Sir3 and Sir4 and recruits them; Abf1 also interacts with Sir4 and recruits Sir4; Orc1 interacts with Sir1 and Sir1 in turn interacts with Sir4 to recruit Sir proteins to the silencers. Thus Sir4 and Sir1 are recruited to the silencer through multiple protein-protein interactions. Sir4 in turn interacts with both Sir3 and Sir2 and recruits them. Sir2, a conserved NAD+ dependent histone deacetylase, deacetylaes nucleosomal histones. Deacetylated histones are bound by Sir3 and Sir4 and this recruits more Sir2 to the nucleosome-Sir4/3 complex. The nucleosome-bound SIR complex subsequently deacetylates the neighbouring nucleosome, which in turn recruits a new SIR complex. By repeating this process of nucleosomal deacetylation and SIR complex deployment, SIR complex promotes its own propagation on the nucleosome array in chromatin and

serves as an integral part of heterochromatin (Cockell et al, 1995; Moazed et al, 1997).

The early piece of proof linking histones/chromatin to transcriptional silencing in yeast was the discovery that deletion of part of the N-terminus of histone H4 led to de-repression of the *HM* loci (Kayne et al, 1988). Furthermore, studies showed that mutating H4 N-terminus, especially lysines 5, 8, 12, and 16, de-represses *HML* to various degrees (Laurenson & Rine, 1992). It was thought that the lysines were important for silencing because of the positive charge that they have. But, replacing all four lysines 5, 8, 12, and 16 by arginines abolished transcriptional silencing despite the fact that the positive charges were preserved. Studies has shown that lysine acetylation was known to be correlated with gene expression activity in general, it was proposed that lysine acetylation at H4 N-terminus, or the lack of it, played a role in transcriptional silencing. This idea was accepted widely when histones H3 and H4 at the silent *HML* and *HMR* loci were found to be hypoacetylated when compared with those at the active *MAT* locus. The path breaking finding of the first histone acetyltransferase and first histone deacetylase in 1996 dramatically impacted the studies of chromatin and chromatin-mediated gene regulation (Brownell et al, 1996). HAT Gcn5 found in yeast is a known transcriptional coactivator and HDAC Rpd3 is a transcriptional repressor again confirms the correlation between histone acetylation and gene expression but also strongly suggests that cells actively regulate the level of chromatin acetylation as a means of gene regulation. After that, many HATs and HDACs have been identified in various eukaryotes (Carmen et al, 2002).

1.1.2. Heterochromatin at telomere

In addition to the *HML* and *HMR* loci, transcriptional silencing is also prevalent at subtelomeric regions in the budding yeast. Sir2, Sir3, and Sir4 are also essential for telomeric silencing, and formation of silent telomeric heterochromatin follows a similar process as that of *HM* loci. The SIR-dependent transcriptional silencing of genes in subtelomeric domains is called Telomere Position Effect (TPE) (Kueng et al, 2013). The telomeric repeats bind multiple molecules of Rap1 that are involved in recruiting Sir proteins and initiating the formation of telomeric heterochromatin. As Rap1 is the only repressor that recognizes specific telomeric repeat DNA sequences, it might be the key protein that provides the specificity by which heterochromatin is established at the telomeres.

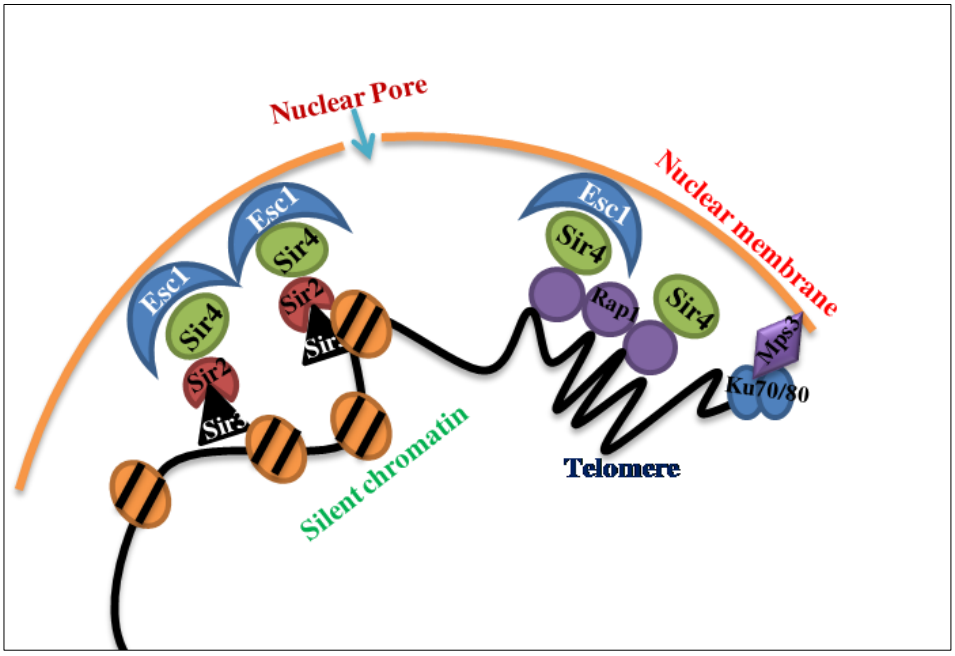

Figure 2: Heterochromatin at telomeric loci. *Genes placed close to the telomeric repeats are silenced. Telomere is anchored to the nuclear periphery by Esc1 and Mps3.*

In fact, a study shows that the Rap1 carboxy-terminal amino acids 635-827 interact with Sir3 and Sir4 carboxy-terminal fragments in the yeast two-hybrid system (Moretti & Shore, 2001). Also, when the C-terminus of Rap1 is tethered to a DNA sequence through a heterologous GAL4 DNA-binding domain it can silence adjacent genes even at internal chromosomal loci. This silencing requires Sir2, Sir3 and Sir4 (Buck & Shore, 1995). If Sir3 or Sir4 are anchored by a DNA-binding domain (of GAL4 or LexA) at the telomere in a mutant containing a defective Rap1 carboxyl terminus, it is able to silence adjacent genes. In addition, the sequence independent DNA end binding protein complex, Yku70/80 heterodimer is also present at the telomeres. Yku80 interacts with Sir4 and helps stabilize the Sir4 recruitment to telomeres. Similar to Rap1, deletion of Yku70/80 abolishes TPE and tethering Yku70/80 can establish Sir-dependent silencing (Mishra & Shore, 1999).

The model that has developed from all these studies is that the heterochromatin at telomere in *S. cerevisiae* is initiated at chromosome ends at an array of 16–20bp binding sites by DNA-binding protein Rap1 (Kimmerly et al, 1988). Then the Yku70/80 complex, which binds to the DNA ends, assists Rap1 in recruitment of the silent information regulator (Sir) proteins, which are the main physical components of heterochromatinization. At the telomeres Sir4 is most likely to be the first member of the SIR complex to be recruited as it directly interacts with both Rap1 and Yku70/80 complex. Sir4 attracts the other components of the SIR complex, Sir2 and Sir3 (Luo et al, 2002). It was also seen that Sir3-Sir3, Sir3-Sir4 and Sir4-Sir4 interactions occur in the two-hybrid system. All these interactions may occur directly as they occur in *vitro* when one of the partners is attached to glutathione S-transferase (Rudner et al, 2005). If the Sir proteins and histones form a telomeric complex, we can expect that deletion or inhibiting the role of any of these

components should loosen and disperse the telomeric foci. In fact, deletion of *SIR3* and *SIR4* and of the H3 and H4 amino termini prevent the formation of the telomeric foci *in vivo* (Grunstein, 1997). More direct proof for the presence of the heterochromatic complex *in vivo* comes from co-immunoprecipitation of its components. Rap1 and Sir4 co-immunoprecipitate from nuclear extracts and immunoprecipitation of Sir3 from a whole cell extract pulls down Sir3, Sir4, Rap1 and all four core histones (Cockell et al, 1995). Sir2 is also part of this complex and it has been shown that Sir2 interacts *in vitro* and in cell extracts with Sir4, which in turn interacts with Sir3. In addition, Sir2 binds to a matrix containing Sir4 (Hecht et al, 1996; Moazed et al, 1997) Therefore, it is predicted that a complex(es) containing Rap l, Sir2, Sir3, Sir4, histone H3 and histone H4 is likely to exist in vivo (Grunstein, 1997).

TPE was originally investigated by an engineered telomere VII-L which was truncated by its terminal ~15 kb by insertion of a *URA3* reporter cassette (Gottschling et al, 1990). Truncated telomeres have been quite helpful for checking the genetic requirements for TPE. A decade later, new yeast reporter strains were made by inserting reporter constructs in sub-telomeric domains without elimination of native telomere sequences, including the sub-telomeric repeat elements X and Y' (Mondoux & Zakian, 2007). Surprisingly, out of the 17 telomeres tested for "native" silencing, one third did not display TPE. While the basic needs for silencing at intact telomeres matches with those identified using truncated telomeres, TPE at intact telomeres varies significantly from that of telomere to telomere. Given that silencing is discontinuous wherever X and Y' elements are present, one can predict that the exact site of reporter insertion plays a key role in determining the efficiency of native TPE. Consistently, recent genome-wide shows a discontinuous binding mode

for Sir3 and Sir4 at natural telomeres. Locus specific regulation of TPE at natural telomeres may well reflect an endogenous requirement to regulate different subsets of genes found in these regions (Kueng et al, 2013). At chromosome VI-R in yeast, Sir2, Sir3, and Sir4 spread approximately 3-kb region distal from the telomere, confirming their capacity to spread from initial Rap1-binding sites (Strahl-Bolsinger et al, 1997). Simultaneously with the spreading of silencing proteins, histone deacetylation is initiated. Sir3 and Sir4 are shown to bind to the N-terminal tails of H3 and H4 in vitro, with a preference for hypo acetylated histones (Carmen et al, 2002; Hecht et al, 1995).

Sir2, the first member of NAD-dependent HDACs, deacetylates histone H4 mainly at K16 and histone H3K9 and H3K14 in vitro (Schleker et al, 2010). The important function of Sir2 in enhancing silencing, however, seems to be the deacetylation of H4K16 because removal of H4 K16 affects silencing severely, whereas removal of individual H3 residues have little effect (Bi, 2014). H4K16 was found to be important for Sir protein binding because the association among full length Sir3 and H4 peptide in vitro is stopped when acetylation of H4K16 is blocked but no other lysines. These results point to us that once the SIR complex binds to the initiation sites, Sir2 deacetylates H4K16 of nearby nucleosome, which allows binding of additional new SIR complexes and, thus, continues of the spreading process. Even though non acetylated tails of histones are important for heterochromatin formation, recent studies provide hints that it is not only the unacetylated nucleosome that is crucial for spreading of the silent complex. Deletion of acetyl transferase Sas2, which acetylates H4K16, resulting in deacetylated H4K16 in sub-telomeric region, in a *sir2* deletion background, does not lead to Sir3 spreading from the initiation (Suka et al, 2002). These observations pointed out that

Sir2 has another unique role in heterochromatin formation at telomere, in addition to histone deacetylase role.

Sir2 might be a crucial structural component responsible for the spreading of the Sir proteins. It is also important to note that the other function of Sir2 might relate to the new discovery that *O*-acetyl- ADP-ribose (AAR), a by-product that results from NAD hydrolysis that is coupled with deacetylase reaction by Sir2, affects properties of the SIR complex. AAR enhances the binding of Sir3 molecules to the Sir2-Sir4 complex and enhances a physical change in the SIR complex resulting in transition of a globular to a cylindrical form in vitro (Buchberger et al, 2008).These observations indicate that the phenomenon of deacetylation by Sir2, and not only the deacetylated form of the histone tails is crucial for heterochromatin establishment. It is not known whether production of AAR is essential in vivo and, if it is, whether this happens only during the time of the establishment of heterochromatin or also for the maintenance phase.

Sir4 mutation which disrupts its interaction with Sir3, but retains Sir2 recruitment and deacetylation, results in inadequate silencing, showing that deacetylation is not enough for the spreading of SIR complex (Rudner et al, 2005). Therefore a combination of histone modifications and physical association of Sir proteins and that act of deacetylation by Sir2, all seem important for heterochromatin assembly. All of these requirements are likely to be essential for *HM* silencing as well, given the close similarity between heterochromatin at the two loci. Studies using telomere-specific DNA probes and immune detection show that telomeric DNA sequences, Rapl, Sir3 and Sir4 all co-localize in the same foci, often near the nuclear periphery in fixed diploid cells. The clustering of silent domains into foci containing 4–6 telomeres each increases the local concentration of Sir

proteins and provides the opportunity for the formation of a repressed state. This depends on two events: the property of the SIR complex to oligomerize and the anchoring of telomeres and Sir4 to the nuclear periphery. *HM* loci are also found to co-localize with these telomeric foci creating a Sir protein rich heterochromatin compartment at the nuclear periphery.

1.1.3. Heterochromatin at nucleolus

The yeast rDNA is a repetitive sequence of that has long been studied as a model system for homologous recombination. The budding yeast genome contains a single *rDNA* cluster arranged as a linear array of 150–200 *rDNA* repeats on chromosome XII. Each repeat contains a 35S *rRNA* gene transcribed by RNA Pol I; a 5S *rRNA* gene, transcribed by RNA Pol III and two intergenic spacers containing multiple functional elements and non-coding RNAs (ncRNAs) transcribed by RNA Pol II (Fig.3). An unusual chromatin structure at the *rDNA* locus is thought to both suppress recombination between repeats and repress the transcription of ncRNA by PolII. It was demonstrated that reporter genes placed in the NTS regions are silenced and a genetic screen revealed several chromatin modifiers including Sir2, Rpd3 (an HDAC), histone chaperones, chromatin remodelers etc. Interestingly, Sir2 is required for both suppression of recombination and repression of transcription by PolII (Bischof et al, 2006; Bryk et al, 2002; Mueller & Bryk, 2007; Smith et al, 1999).

In the *rDNA* array, recruitment of Sir2 is different from telomeres and mating-type loci. Here Net1, the nucleolar protein and the phosphatase Cdc14 recruit Sir2, and form the regulator of nucleolar silencing and telophase exit (RENT) complex. Importantly, *rDNA* silencing does not require Sir3 or Sir4 and in fact, the Sir3-4p

and Net1-Cdc14p compete for limiting amounts of Sir2 present in the cell. Consequently, in vivo, two complexes of Sir2 are found: one associated with Sir3/4 and another with Net1/Cdc14. Histone deacetylation at H4K16 is, however, key for spreading of the heterochromatin at the *rDNA* locus. Sir2 plays an important role in silencing of PolII genes placed at the *rDNA* although it does not seem to influence the transcription of PolI genes (Tanny et al, 1999).

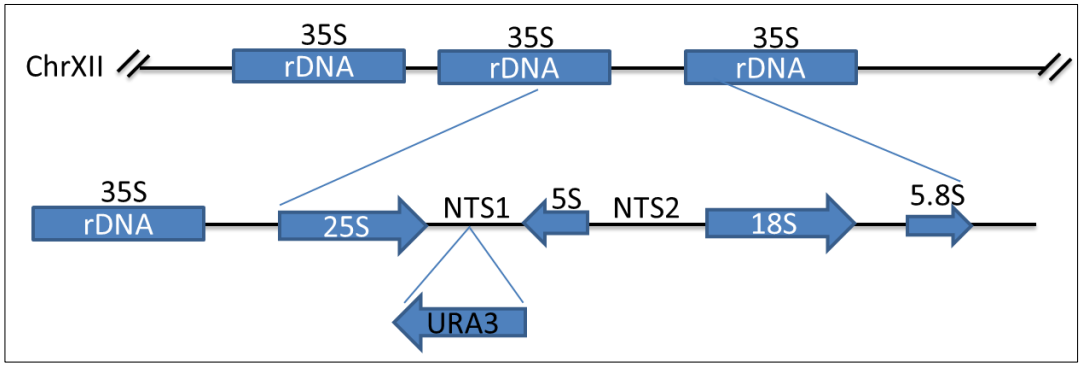

Figure 3: Heterochromatin at rDNA locus. *rDNA consist of 150-200 copies of 35S rRNA gene transcribed by Poll I and Poll II. 35S rRNA consists of 25S, 18S and 5.8s rRNA. 5S rRNA gene is transcribed by Poll III.*

Heterochromatin maintenance at *rDNA* is important for genome stability. For instance, in eukaryotes, transcription-associated recombination (TAR) is a fundamental process that is important for DNA integrity. Kobayashi and Ganley have proposed a model of TAR in *rDNA* in which RNAPII transcription of noncoding RNA (ncRNA) induces recombination, which can lead to accumulation of extra chromosomal DNA and loss of *rDNA* (Kobayashi et al, 2004). Further, this process may lead to premature cell death. Consistent with this, in mutants or cells devoid of any of the RENT subunits, multiple severe phenotypes have been observed, including premature aging, rearrangement and/or loss of genetic material, and disease (Oling et al, 2014).

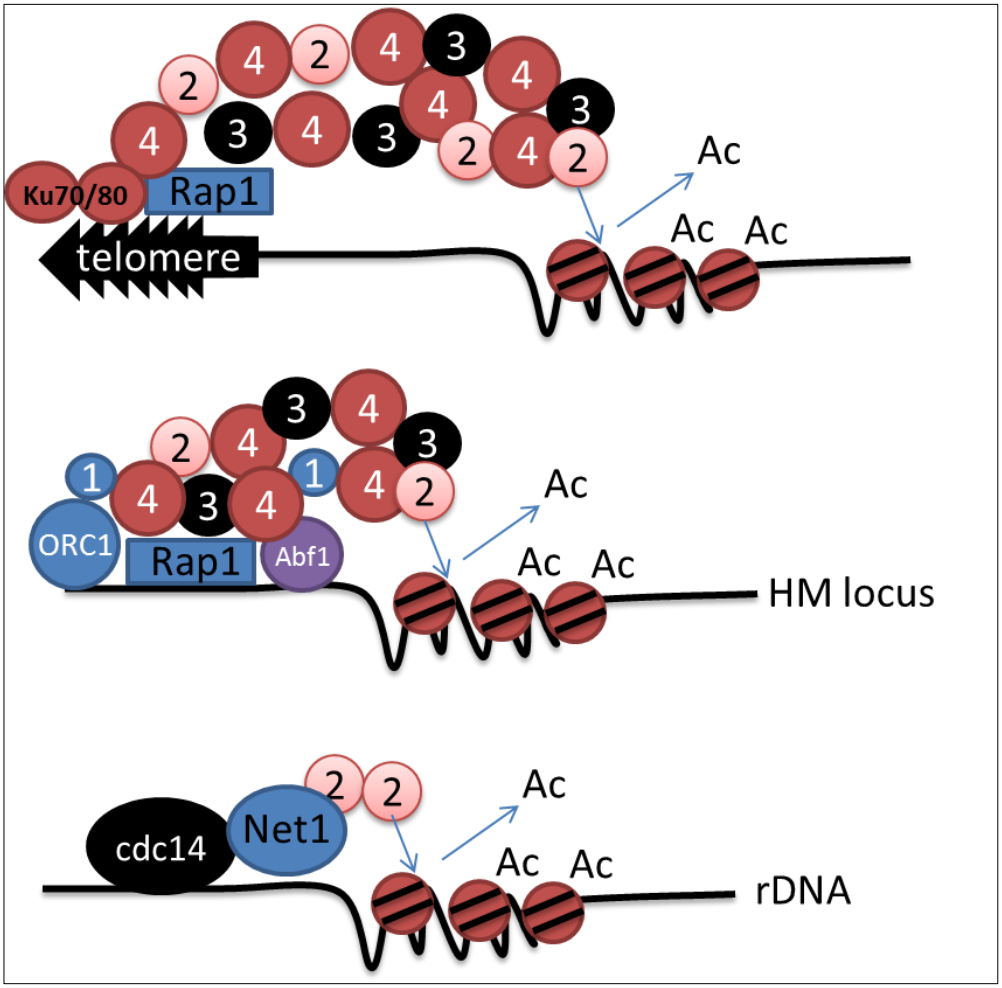

Figure 4: Heterochromatin loci found in S. cerevisiae. *Three heterochromatin loci in yeast are telomeric loci, HM loci and rDNA locus.*

1.1.4. Structure of Heterochromatin

Nucleosomes are generally more regularly distributed in heterochromatin than in euchromatin. Moreover, heterochromatin folds into higher order structures. Formation of heterochromatin also results in an increase in the negative supercoiling of DNA in it. However though heterochromatin is stable, it is not static, but is dynamic, and is subjected to regulation by cell cycle. Spreading of heterochromatin is restricted by special DNA elements called barriers that enhance the formation of active chromatin and/or exclusion of nucleosomes (Scott et al, 2006).

Heterochromatin loci tend to associate with each other and the nuclear envelope to form clusters where there is a high concentration of Sir proteins, which is thought to facilitate the making and maintenance of heterochromatin. Acetylation of a lysine residue results in the removal of its positive charge, which results in reduced interaction between histone and the negatively charged DNA, making the nucleosome assume an "open" structure (Strahl & Allis, 2000).

Establishment of heterochromatin results in the changes in the primary and higher order structures of chromatin. The primary structure of chromatin results in the distribution of nucleosomes along DNA. Formation of a nucleosome and wrapping of 147 base pairs of DNA around the histone octamer is generally a sequence-independent process. However, nucleosomes in eukaryotic genome are mostly positioned at specific sites consistently. This is likely the consequence of at least three possible causes. (i) Even though the interaction of histone–DNA is sequence nonspecific, the capability of some specific DNA sequences to form nucleosomes may be higher or lower than random sequences. (ii) An array of nucleosomes may be forced into a specific arrangement by DNA-binding proteins and chromatin remodelers. A strong DNA-binding protein may exclude histones, thereby establishing the border of a nucleosome array thereby determining the spacing of the nucleosomes. A chromatin remodeler has the ability to slide the nucleosome along the DNA. (iii) A nucleosome-binding protein may restrict the dynamics of the nucleosome and "fix" the nucleosome to one of many dynamic positions it can adopt without the protein (Bi, 2014).

Spreading of heterochromatin-specific complexes along chromatin results in the establishment of heterochromatin. But it raises the question how the nearby euchromatin locus is protected from the spreading of the protein which are

responsible for the heterochromatin establishment. Early investigation has revealed that in *Drosophila* and vertebrates chromatin boundaries coincide with "special chromatin structures" that were hypersensitive to nucleases (Vazquez & Schedl, 1994). DNA sequences that could block the propagation of transcriptional silencing were also identified in *S. cerevisiae* and named heterochromatin barriers (Scott et al, 2006). These barriers are peculiar in sequence and consist of binding sites for transcription regulators or other factors that are associated with the nuclear pore complex. This observation indicates that a barrier may function by recruiting chromatin-modifying/ remodelling complexes that promote active chromatin formation or by attaching to an "immobile" nuclear structure.

Once established, heterochromatin is stably maintained during the cell cycle, surmounting the disruptive event of DNA replication, which leads to the process of epigenetic inheritance of chromatin states. DNA replication is a semi-conservative process with each of the two single strands of DNA duplex serving as a template for the synthesis of a new complementary strand, and produce two copies of ds-DNA. Replication of DNA is intimately linked to nucleosome disassembly in front of the replication fork and nucleosome assembly on nascent DNA strands (Flotho & Melchior, 2013). The histones from earlier nucleosomes on the template chromatin are thought to be equally distributed to the two new strands of DNA, constituting half of the histones incorporated in new chromatid. The other part of the histones is newly synthesized. Hence, chromatin of newly produced DNA strands contains both old and new histones (Flotho & Werner, 2012). After a heterochromatin region is replicated, the old histones bearing heterochromatin-specific modifications may remain bound with, or recruit, heterochromatin binding complex, which would modify the new nucleosomes, therefore establishing the state of heterochromatin. In

short, heterochromatin may template its own duplication during DNA replication. Recent investigation has begun to reveal the molecular mechanism of epigenetic inheritance of chromatin states in mitosis over multiple generations and is an active area of research.

1.1.5. Proteins essential for heterochromatin establishment

Several structure and function analysis studies of Sir2, Sir3, and Sir4 have accumulated a pool of information about their roles in the formation and maintenance of heterochromatin. A brief summary of the major findings is as follows (Fig. 5). Sir2 is an NAD-dependent protein deacetylase comprising of a C-terminal catalytic domain and an N-terminal regulatory domain that interacts with Sir4. Sir2 deacetylates lysine at the N-terminal tail of histones. Sir3 contains regions that can interact with Sir4, Rap1, with the nucleosome, as well as a homodimerization domain. The N-terminal bromo adjacent homology (BAH) domain of Sir3 interacts with both the N-terminal tail of histone H4 and a surface of the nucleosome called loss of rDNA silencing (LRS) that included H3-K79 (Moazed et al, 1997). The H4 LRS domain and N-terminal tail are important for transcriptional silencing. An AAA1+ ATPase-like domain interacts with Sir4 and may also interact with the nucleosome (Ehrentraut et al, 2011). The C-terminal winged helix-turn-helix (wH) motif helps Sir3 homodimerization, which is found to be essential for heterochromatin formation. Sir4 interacts with many factors including Esc1, Sir1, Sir2, Sir3, Rap1, Yku80, nucleosome, and DNA via several domains and is thought to have a scaffold function in heterochromatin structure. Sir1 is an ORC-binding protein and the Yku70/Yku80 complex associates with chromosome ends. By binding to Sir1, Rap1, and Yku70/Yku80, Sir4, acts to initiate

the assembly of SIR complex at the *HM* silencers and telomeres. The partitioning and anchoring domain (PAD) mediates the tethering of heterochromatin to nuclear periphery by binding to Esc1, a nuclear envelope-anchoring protein. The Sir2-interacting sequence of Sir4 binds the N-terminal regulatory domain of Sir2 (Sir2N) and makes contact with the interface between Sir2N and Sir2 catalytic domain, which stimulates the deacetylase activity of Sir2 (Hsu et al, 2013).

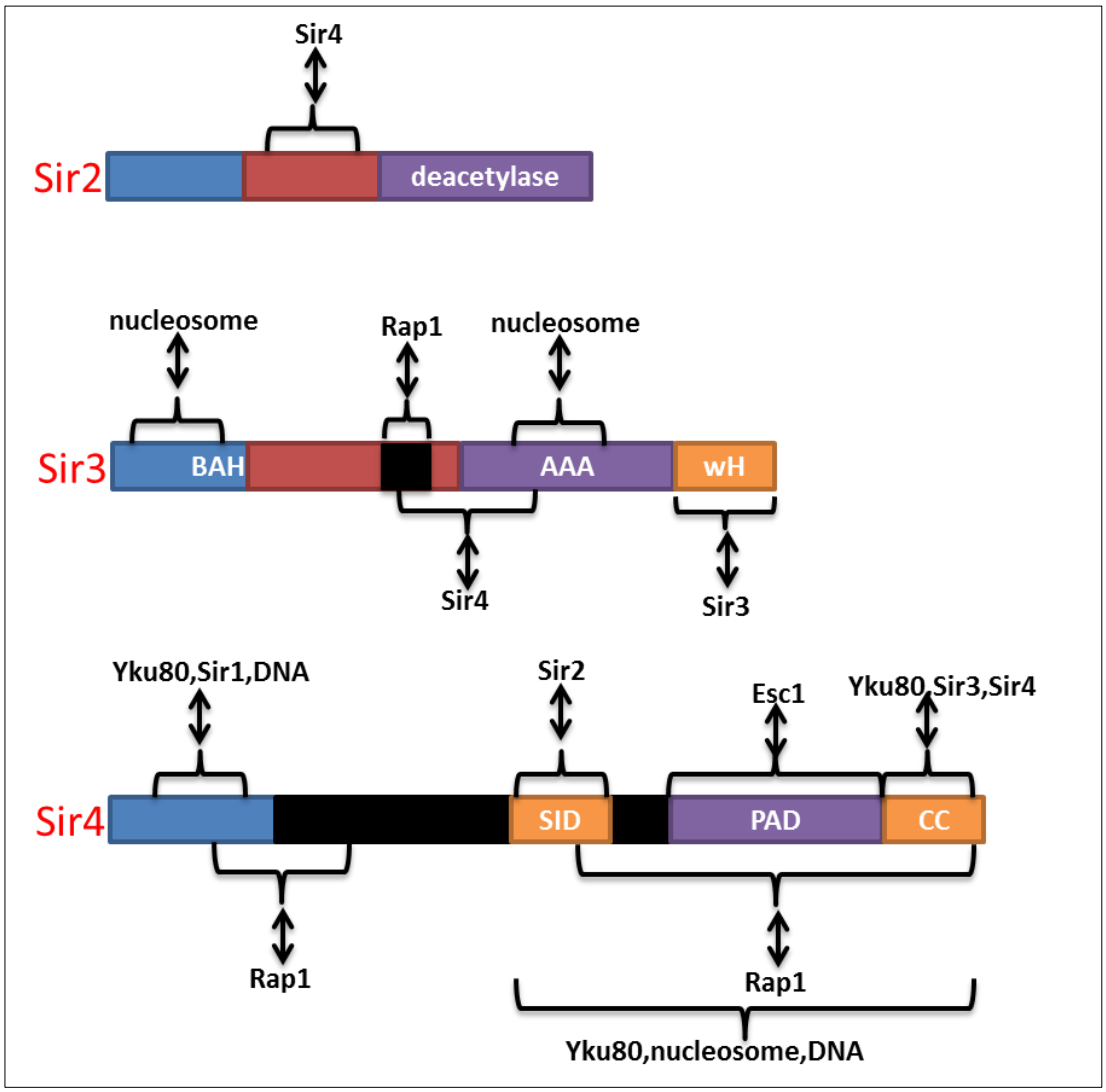

Figure 5: Cartoon representation of Sir family of proteins with the anchoring domain indicated.

1.1.6. Heterochromatin compartment in yeast

In the budding yeast *S.cerevisiae*, the 32 telomeres interact to form about 6-8 perinuclear foci of subtelomeric heterochromatin (Gotta et al, 1996). Though in yeast heterochromatin is found preferably near at the nuclear periphery or in the nucleolus, in some other eukaryotic cells heterochromatin is found in the centre of the nucleus also. In the budding yeast, it is found that the 32 telomeres interact and form six to eight perinuclear foci of subtelomeric heterochromatin and localized near envelope. Both the telomere-binding complex Yku70/Yku80 and SIR complex takes part in tethering of telomeres to nuclear envelope. Clustering of telomere increases the concentration of Sir proteins, which is thought to increase transcriptional silencing of the loci within the compartment. Since *HML* and *HMR* are situated to the left and right telomeres of chromosome III (12 and 23 kb, respectively), they are both located near a cluster of telomeres. Yku70/Yku80 was found to interact directly and efficiently with Sir4 and to play an important role together with Esc1 in positioning telomeres at the nuclear periphery. In parallel, the yeast Ku heterodimer anchors telomeres independently through the SUN-domain containing inner nuclear membrane protein, Mps3 (Schober et al, 2009). Interestingly, a recent investigation shows that upon Sir3 overexpression, the clustering of telomeres is mainly promoted by Sir3. This can be separated from the perinuclear anchoring of these foci by the use of mutants. Sir3 overexpression may result in repression without anchoring, as does the dispersion of Sir proteins away from perinuclear foci, which is provoked by mutations that release telomeres from the nuclear envelope. Under wild-type conditions, however, the local concentration of Sir3 and Sir4 molecules in perinuclear clusters of anchored telomeres clearly contributes to TPE (Kueng et al, 2013). The *rDNA* heterochromatin is maintained at

nucleolus (Rusche et al, 2003). The presence of chromosomal domain in the nucleus does not advocate the expression or repression. It also depends on the surrounding. The presence of nucleolus, Cajal bodies, splicing factor near the vicinity also influence the heterochromatin state (Dundr & Misteli, 2001). These studies underline the importance of maintaining non-random organization of protein and chromatin within the nucleus to establish a gene expression program that is finely controlled.

1.2. Sumoylation

Ubiquitin and its relatives, the ubiquitin-like proteins (Ubls), are conjugated to proteins, altering the properties of the modified proteins and hugely increasing the complexity of the proteome in eukaryotic cells. Sumoylation is a post-translational modification of protein where the glycine residue of C-terminal domain of Ubls relative SUMO (small ubiquitin-like modifier) is covalently attached to the epsilon amino group of a lysine residue of the target protein. Sumoylation is involved in cellular transcriptional regulation, transport, protein stability, apoptosis, response to stress, transcription and progression through the cell cycle (Hay, 2005). Hundreds of proteins involved in processes such as chromatin organization, DNA repair, protein homeostasis, macromolecular assembly, trafficking and signal transduction are subject to reversible sumoylation. All eukaryotes investigated to date have at least one SUMO precursor protein. Among the species which express only a single SUMO protein are yeast, *Caenorhabditis elegans* and *Drosophila*. Other eukaryotes, including higher plants and vertebrates, express several SUMO proteins. SUMO is a far relative of ubiquitin (20% identity) although in 3D it looks more similar to

ubiquitin. SUMO was first identified in mammals where it was found to be covalently linked to the GTPase activating protein RanGAP (Hay, 2005).

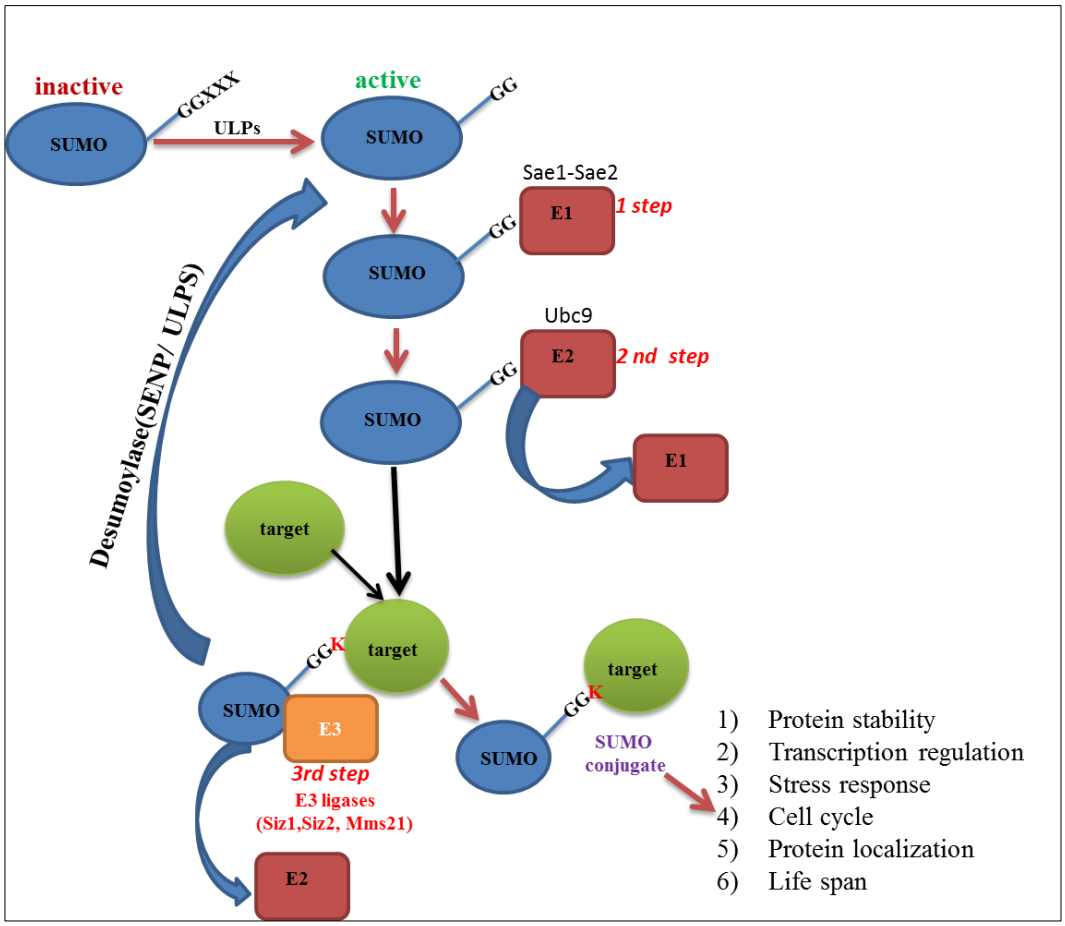

Figure 6: The sumoylation machinery. *SUMO is conjugated to the target protein via the action of E1 activating, E2 conjugating and E3 ligase enzyme. Desumoylase reverse sumoylation and release free SUMO for further conjugation.*

SUMO contains the characteristic ubiquitin fold (globular β-grasp fold) and a characteristic C-terminal Gly-Gly motif that is exposed after proteolytic maturation. Primary sequence and surface charge distributions differ significantly between Ubls, and as a consequence, each Ubl modification pathway requires distinct sets of enzymes and binding partners. A SUMO-specific characteristic is the flexible N terminus of about 20 amino acids, which seems to primarily serve as an acceptor in

SUMO chain formation. Mammals express three SUMO proteins that can be divided into two families, SUMO1 and SUMO2/3 (Hay, 2005).

SUMO is conjugated to the target by an array of three enzymes namely E1-SUMO activating, E2-SUMO conjugating and E3-SUMO ligase (Hay, 2005) (See fig. 6). The SUMO E1 enzyme is composed of two subunits, SUMO-activating enzyme subunit 1 (SAE1; also known as Aos1) and SUMO-activating enzyme subunit 2 (SAE2; also known as Uba2). Similar to other E1s, the SAE1/SAE2 E1 activates SUMO's C terminus in a two-step reaction that involves ATP hydrolysis. Formation of a SUMO adenylate is followed by dramatic E1 active-site remodelling and subsequent SAE2~SUMO thioester bond formation. Upon interaction of the thioester-charged E1 enzyme with Ubc9, which binds to SAE2's ubiquitin-fold domain, SUMO is transferred to the E2 enzyme. E3 ligase transfer SUMO from Ubc9 to the target.

The single E2 conjugating enzyme Ubc9 plays an essential role in sumoylation. In addition to providing activated SUMO, Ubc9 is directly involved in the selection of many specific SUMO targets. These targets carry a sumoylation consensus site to which Ubc9 can bind directly, albeit with low affinity. As a consequence, they can be sumoylated with E1 and high concentrations of Ubc9 in vitro. However, very few proteins are known whose sumoylation is efficient in the absence of E3 ligases; a well-known exception is RanGAP1, which binds Ubc9 with high affinity owing to an additional interaction surface (Bernier-Villamor et al, 2002). Contribution of Ubc9 to target selection opens up intriguing possibilities for regulation. Altered Ubc9 expression levels may influence steady-state sumoylation. Although mice with twofold-reduced Ubc9 levels appear normal (Nacerddine et al, 2005), elevated expression of Ubc9 may circumvent the need for E3 ligases or other

stimulatory mechanisms. Ubc9-binding partners and/or posttranslational modifications may influence Ubc9 target specificity and activity.

E3 ligases catalyze the transfer of SUMO or ubiquitin-related proteins from an E2 enzyme to a target lysine residue. SUMO E3 ligase activity has been clearly investigated for two types of proteins, Siz/PIAS (SP) E3 ligases and the nucleoporin RanBP2. Many structural investigations on SUMO and RING-type ubiquitin E3 ligases provide insights into their function. E3 ligases mediate or stabilize the interaction of the target with the charged E2, and they lock the flexible Ubl∼E2 thioester bond in an orientation that is favourable for nucleophilic attack by the target lysine. The latter activity can even be observed in the absence of target interactions, for example, with a small fragment of RanBP2 that stimulates Sp100 (Speckled 100 kDa) sumoylation in vitro. The largest family of SUMO E3 ligases is a group of SP-RING-containing proteins. Six members have been identified in humans: the protein inhibitor of activated STAT (PIAS) proteins, PIAS1 and its isoforms PIASxα and PIASxβ, PIAS3, PIASy, and Nse2/Mms21. In yeast, the SP family comprises Siz1, Siz2, Mms21 and potentially the distantly related meiosis-specific SP-RING protein Zip3. *A. thaliana* has two known SP-RING E3 ligases, At-Siz1 and At-Mms21, and encodes two related proteins with SP-RING domains, PIAL1 and PIAL2. Even though the SP-RING domain is required for Ubc9 interaction, flanking regions mediate interaction with SUMO and specific targets.

PIAS proteins are best known for their roles in transcriptional regulation. Many of these functions can be attributed to their E3 ligase activity, but others seem to depend on non-covalent interactions with sumoylated proteins. Knockout studies in mice suggest a significant redundancy between PIAS E3 ligases; however, PIAS1−/− mice show an increased protection against pathogenic infection, and

PIASxα−/− mice exhibit testis defects. Although PIASy−/− mice appear largely normal, primary fibroblasts lacking PIASy exhibit a highly reduced propensity to undergo senescence (Bischof et al, 2006). An unrelated E3 ligase is the 358-kDa nucleoporin and Ran-binding protein 2 (RanBP2; also known as Nup358). Its vertebrate specific E3 ligase region, which is natively unfolded, shows no obvious homology to other proteins. In vitro activity requires only one of two closely spaced amino acid repeat regions that can both bind to Ubc9 and SUMO. In vivo, however, RanBP2, sumoylated RanGAP1, and a catalytically inaccessible Ubc9 form a stable isopeptidase-resistant complex that represents the functional and physiologically relevant multi subunit E3 ligase. Intriguingly, this complex is built to encounter many proteins as the E3 ligase core is flanked by binding sites for nuclear transport receptors and the GTPase Ran (Flotho & Werner, 2012).

1.2.1. SUMO proteases

SUMO proteases are needed for two tasks, maturation of SUMO proteins (C-terminal hydrolase activity), and removal of SUMO from targets (isopeptidase activity). Even though SUMO maturation is essential for sumoylation, there is currently no clear evidence that this is a rate-limiting or regulated event. By contrast, desumoylation events are key determining factors for regulated sumoylation of individual target proteins. A surprisingly small number of SUMO isopeptidases has been identified to date; all of these have been categorized as cysteine proteases. The first SUMO isopeptidase, yeast Ubl-specific protease Ulp1, was identified via an activity-based screen. Many related proteases [referred to as sentrin-specific proteases (SENPs) in mammals] were subsequently identified in other organisms on the basis of sequence similarities. Two SUMO-specific Ulp/SENP proteins are

present in yeast (Ulp1 is essential; Ulp2 is not) and there are six proteins in mammals (SENP1, -2, -3, -5, -6, and -7). Ulp/SENP proteases are different in relative C-terminal hydrolase and isopeptidase activities and differ with respect to SUMO paralog preferences. Their catalytic domains do not seems to to differentiate between the individual targets to which SUMO is coupled. An important determining factor for target specificity in vivo is the N-terminal region of these proteases, which recognise interactions with specific targets and mediates distinct intracellular localizations.

The localization of various Ulp/SENP is also different. Ulp2, SENP1, SENP6, and SENP7 are localized in nucleoplasm, whereas Ulp1 and SENP2 are anchored to nuclear pore complexes. By contrast, SENP3 and SENP5 are enriched in the nucleolus. The other group of SUMO proteases currently comprises of two proteins, DeSI-1 (desumoylating isopeptidase-1) and DeSI-2. They belong to a larger family of proteins referred to as PPPDE (permuted papain fold peptidases of ds-RNA viruses and eukaryotes). Some PPPDE proteins have ubiquitin-binding domains and were previously predicted to function as ubiquitin proteases. Whether other family members also act on SUMO and/or ubiquitin is an important topic for future studies. Contrary to SENP proteases, DeSI-1 have clear target specificity in that it selectively cleaves SUMO proteins linked to BTB-ZF, but not when conjugated to other targets such as PML. Hence, depletion of DeSI-1 from cells does not lead to global changes in the SUMO pattern. One reason for this remarkable specificity may be its limited accessibility to the catalytic pocket, which is formed at the DeSI-1 homodimer interface. The most recent discovery to the list of SUMO isopeptidases is USPL1. This protein belongs to the large family of USP (ubiquitin-specific proteases) but cleaves SUMO rather than ubiquitin. It is present at low abundance

and localizes to Cajal bodies, and its zebrafish homolog C13 or f22l is essential for development. Although USPL1's catalytic domain can cleave SUMO from many targets in vitro and in vivo, its function is yet to be studied. Its knockdown in HeLa cells does not alter global sumoylation patterns, and its essential function for cell proliferation and Cajal body integrity is independent of its catalytic activity. At last it should be mentioned that the putative metalloprotease Wss-1 has been associated both genetically and biochemically with the SUMO pathway. Whether this protein works as a SUMO isopeptidase or as a SUMO-directed ubiquitin isopeptidase is currently not known.

The targets and key functions of individual SUMO proteases in mammals, how much redundancy is present in the system are questions that are only beginning to be addressed. Investigation has shown that deletion or inhibiting the function of some SENP has severe consequences. For example inactivation of the murine *SENP1* gene results in early embryonic lethality owing to a severe defect in erythropoiesis. The underlying mechanism involves failure to desumoylate the hypoxia-inducible factor 1α (Flotho & Melchior, 2013).

1.2.2. SUMO Targets

Confirmed and suspected SUMO targets contribute to virtually all aspects of cell biology. Even though SUMO enzymes are enriched in the cell nucleus, they are also found to be present in the cytoplasm. Therefore, in principle, not only are nuclear and cytoplasmic proteins accessible to sumoylation but the transmembrane proteins of the endoplasmic reticulum and nuclear envelope, the Golgi apparatus, the plasma membrane, and the outer membrane of mitochondria are sumoylated. If transient sumoylation also occurs co-translationally, as has been suggested from proteomic

studies that investigated sumoylation upon stress, the list of targets may even include proteins that are hidden from SUMO enzymes upon sorting, such as mitochondrial matrix proteins. Analysis and detection of sumoylation are difficult because of low levels of endogenous sumoylated protein at a particular time and the robust isopeptidase activity in non-denaturing lysates. This has hampered overall studies and has resulted in the characterization of only the highly abundant and/or stable SUMO targets e.g., RanGAP1, PML, Sp100. With growing work in the field, target proteins of low abundance also will become accessible. This will be leading to a rapid increase in studies that focus on the functions for sumoylation of specific targets.

1.2.3. Sumoylation Sites

Two prerequisites determine whether a protein can be sumoylated. Either the protein is able to directly interact with the Ubc9~SUMO thioester or the protein is recognized by a specific E3-ligase and thus brought into direct proximity with the charged Ubc9. In either case, an acceptor lysine has to gain access to theUbc9~SUMO thioester bond. Three distinct but not mutually exclusive mechanisms can presently be distinguished that determine lysine selection (Flotho & Melchior, 2013). One possibility is that the acceptor lysine is embedded in a short motif that is directly recognized by Ubc9 (consensus site sumoylation). Alternatively, the target contains a SUMO interaction motif (SIM) that recruits the Ubc9~SUMO thioester, which in turn allows modification of lysine residues nearby (SIM-dependent sumoylation). Finally, SUMO E3 ligases may determine site selection by binding the target and Ubc9 in an orientation that favours a specific target lysine residue (E3 ligase- dependent sumoylation). SUMO targets that require

a SIM for modification are frequently sumoylated on multiple sites, including lysine residues that are not part of consensus motifs. Mutating these lysine residues does not abolish sumoylation as long as additional lysine residues are accessible (Kuo et al, 2005). Examples of SIM dependent sumoylation include the transcriptional co-repressor Daxx, the Bloom syndrome RecQ helicase-like protein BLM, and USP25 (Chang et al, 2011). SIM-dependent sumoylation can be reconstituted in vitro with E1 and high concentrations of Ubc9 or with E1 and low Ubc9 concentrations and E3 ligases; the pattern of modification is the same under both conditions. A variation of this has been observed for some proteins that have both a consensus sumoylation site and a SIM. Sumoylation of some of these targets can be dramatically enhanced when Ubc9 is sumoylated on K14, owing to an enhanced Ubc9-target interaction (Knipscheer et al, 2008).

1.2.4. SUMO Chain

Yeast SUMO and metazoan SUMO2 family members carry consensus sumoylation sites in their N-terminal extension (one comprising K11 in human SUMO2/3, three in *S. cerevisiae* SUMO). These consensus sites are important for SUMO chain formation *in vivo* (Bylebyl et al, 2003). SUMO chains may, however, not only be restricted to consensus site linkages. SUMO chain formation can be induced, either globally or on specific targets e.g., on PML upon arsenic treatment (Lallemand-Breitenbach et al, 2008). Functional role of polysumoylation are, however, limited. They help in synaptonemal complex integrity during yeast meiosis (Cheng et al, 2006) and were found to play important roles in the recruitment of selected downstream effectors, including SUMO-dependent ubiquitin E3 ligases. Whether

SUMO chains exhibit similarly diverse signalling properties as ubiquitin chains (Komander & Rape, 2012) requires further investigation.

1.2.5. Regulation of sumoylation

It is found that a lot of proteins are sumoylated only in the presence of intrinsic or external stimuli. Even though sumoylation is regulated by a strict set of enzymes, sumoylation is frequently controlled at the level of individual target proteins. Different types of stimuli induce different types of protein modification (example phosphorylation) which in turn invite the SUMO ligases to the consensus site for sumoylation. Phosphorylation is a stimulatory or repressive mechanism for target-specific sumoylation. How does phosphorylation stimulate target specific sumoylation? One well-studied process involves a phosphorylation dependent sumoylation motif (PDSM). This is a consensus sumoylation site flanked by a phosphorylation site (KXEXXSP). Proteins bearing PDSM were heat shock transcription factor 1 (HSF1), myelin expression factor 2 (Mef2), erythroid transcription factor GATA-1 and peroxisome proliferator– activated receptor-γ (PPARγ). The negative charge introduced by phosphorylation stabilizes the interaction with Ubc9 and thereby enhances sumoylation (Mohideen et al, 2009).

Another concept in sumoylation is the requirement for simultaneous (de)modification of more than one protein that is involved in the same biological process. For example outer or inner stimuli does not induce the sumoylation or desumoylation of a single target protein; instead sumoylation of a group of proteins is altered (Flotho & Melchior, 2013). Although inhibiting any single modification may not lead to obvious consequences, inhibiting modification of several pathway components does. A case in point is yeast septins; where all three septins become

sumoylated on at least seven different lysine residues at the bud neck specifically in mitosis (Johnson & Blobel, 1999). This depends on regulated targeting of the E3 ligase Siz1 to the septin complex. Evidence first came from comparing the mammalian SUMO proteome before and after heat shock. Here, groups of proteins involved in the similar pathway has either lost or gained sumoylation collectively (Golebiowski et al, 2009). Analysis of one specific pathway, for example nucleotide excision repair had shown that DNA damage– induced sumoylation of multiple repair proteins but not of the single protein Rad16 promotes efficient nucleotide excision repair (Silver et al, 2011). Finally, Psakhye & Jentsch found that multiple proteins of various repair pathways are modified with SUMO in response to the respective DNA lesions in what they refer to as "SUMO waves." These waves are thought to be regulated at a topological level but not at the level of specific substrate recognition. Specific targeting of homologous recombination repair proteins to single-stranded DNA stretches triggers SUMO conjugation of a set of proteins by the DNA-bound SUMO ligase Siz2. Again, the sum of sumoylations rather than a single event contributes to efficient repair (Psakhye & Jentsch, 2012). These observations have many implications also for the analysis of sumoylation.

1.2.6. Functional significance of sumoylation

It is found that SUMO has role in many physiological processes. It has roles in cell cycle, DNA damage repair, equal distribution of components among mother and daughter cells, response to different kind of stress etc. In *S. cerevisiae* mutations in genes for the SUMO E1 (uba2-ts) and E2 (ubc9-ts) display cell cycle defects and arrest at the G2/M boundary (Hay, 2005). It is also found those cells devoid of SUMO cycle protein are ineffective in DNA repair. In *S. cerevisiae*, Ulp2 mutations,

a SUMO-specific protease are sensitive to some DNA damaging agents. Ulp1 mutant of *S.pombe* are hypersensitive to ultra violet radiation. Beside DNA damage response sumoylation is necessary for many proteins for their localization. It is suggested that dynamic SUMO modification of many NPC proteins is needed for their function in nuclear import (Moutty et al, 2011). It is also found that in many proteins the same lysine residue might be the target of SUMO as well ubiquitin. Hence alternate conjugation of SUMO or ubiquitin determine the fate of the protein whether it will go through ubiquitination related degradation or sumoylation related activation. For example NFkB is inactivated by sumoylated IkBα, but ubiquitination related degradation of IkBα leads to active NFkB. Hence we can say that SUMO and ubiquitin are antagonistic in nature and controlled regulation of sumoylation and ubiquitination regulates functions of many proteins for proper physiological functions (Ulrich, 2005).

1.2.6.1. Sumoylation in transcriptional regulation

In the context of regulation of transcription, sumoylation seems to promote both repression and activation. Histone sumoylation in yeast has been shown to promote gene repression by recruiting histone deacetylases. Similarly, PC2, a member of the polycomb group repressor (PRC1) complex has sumoylation activity and this activity is required to promote gene repression (Kagey et al, 2003). More recently, a regulated cycle of sumoylation/desumoylation of PC2 has been shown to be critical for PcG-mediated repression. PRC2 complex promotes tri-methylation of H3K27 (an inactivation mark) which is recognized by the PC2 containing PRC1. The sumoylated form of PC2 has high affinity for these residues and therefore promotes repression. When SENP2, a sumo protease, removes the SUMO from PC2, it loses

its affinity for these sites and therefore reduces repression. However, how promoter specificity is brought about and how the activity of the two SUMO/de SUMO activity is controlled is not clear. Similarly, Elk-1 [Ets (E twenty-six)-like kinase 1] sumoylation/desumoylation regulates genes activated by the MAPK (mitogen-activated protein kinase) signalling cascade: Sumoylated Elk1 recruits HDAC1 and deacetylates histones leading to repression of transcription in these inducible loci. Upon activation of the MAPK cascade, Elk1 is phosphorylated with simultaneous loss of SUMO, leading to reduced affinity for HDAC, in turn leading to acetylation of histones followed by gene activation (Yang & Sharrocks, 2004; Yang & Sharrocks, 2006; Yang et al, 2003).

There is also emerging evidence for sumoylation in promoting transcriptional activation. SUMO modification of Dnmt3a (de novo DNA cytosine methyltransferase), leads to gene repression by reducing its interaction with HDAC1 and HDAC2 (Ling et al, 2004). Sumoylation of p53 competes with and inhibits the MDM mediated p53 degradation, leading to activation of p53 targets (Santiago et al, 2013). A clear demonstration of sumoylation promoting transcriptional activation, with indication of possible mechanism has been done for Ikaros (Arco et al, 2005). Mutant forms of Ikaros that cannot be sumoylated are more potent repressors and using a combination of SUMO ligase and desumoylase, they also demonstrate the direct involvement of sumoylation in controlling Ikaros activity on transcription. They further demonstrate that sumoylation of Ikaros inhibits its interaction with corepressors Sin3, Mi-2b and CtBP even though it does not affect the localization of Ikaros to pericentromeric heterochromatin. In considering all the data that is available on sumoylation and transcriptional regulation, it is clear that depending on

the target protein, sumoylation can promote either activation or repression by modifying the interaction properties of the targets.

1.2.6.2. Sumoylation in gene silencing

In the context of heterochromatin establishment, recent reports implicate regulation of gene silencing by SUMO. Deletion of the SUMO isopeptidase gene *ULP2* leads to a loss of telomeric silencing, although its effect on *HMR* was not reported (Darst et al, 2008). Furthermore, deletion of *SLX5*, a component SUMO-dependent ubiquitin ligase, which leads to increased overall sumoylation also reduced gene silencing. This work also indicated that the effects of *ULP2* and *SLX5* mutation are linked to Sir2, further suggesting that SUMO homeostasis is important for gene silencing, and more specifically for the function of Sir2. Esc2, a protein that physically interacts with Sir2, and has SUMO-like motifs, influences gene silencing in a locus dependent manner (Dhillon & Kamakaka, 2000). *esc2* mutants lose TPE; additionally, Yu *et al.* (2010) see strong defects in rDNA silencing.

Taken together, these data indicate that increased sumoylation is detrimental to TPE. Work in our laboratory was one of the first to report regulation of heterochromatin through sumoylation. Through a genetic screen for genes that regulate silencing we found that elevated doses of Siz2 SUMO ligase reduces telomeric silencing and reduction of Sir2 binding at telomere. The loss of TPE is further enhanced in *esc1* deletion background. Esc1 is anchored to the nuclear periphery and tethers the silent telomeric complex at nuclear periphery. It also tethers Ulp1 SUMO protease to the nuclear periphery. Hence when *esc1* is deleted, Ulp1 cannot desumoylate the proteins at telomere, which might be the reason for enhanced loss of TPE upon *esc1* deletion background. Another report showed that

proteins responsible for telomeric silencing such as Sir3, Sir4, Yku70, Yku80 are sumoylated (Ferreira et al, 2011) and sumoylation of Yku70/80 and Sir4 regulates telomerase activity. Another report showed that the catalytic mutant of *mms21* was defective in telomeric silencing and the association of Sir proteins to many telomeres was compromised (Hsu et al, 2013). Thus recent studies indicate that gene silencing is regulated by sumoylation and possibly by directly modifying components of heterochromatin. As sumoylation is modulated by external and intrinsic signals, it is possible that dynamics of heterochromatin are linked to this process. The work presented here addresses the issue of how sumoylation regulates gene silencing.

1.3. Objective of the study

Establishment of gene silencing in yeast is a well-regulated process that involves the interplay of histones, histone associated proteins and other DNA binding proteins. The process of heterochromatin establishment and maintenance is also dynamic and responds to internal cues like cell cycle stage, metabolic state etc. and external cues like nutrients. Post-translational modifications of effector proteins could be an effective means of regulating this process. However, this possibility has not received much attention except for histone modifications and phosphorylation of a few components including Sir3 (Radman-Livaja et al, 2011). Recently, we and others have shown that deletion or over-expression of SUMO ligases like Mms21 and Siz2 have severe defect in heterochromatin establishment (Hsu et al, 2013; Pasupala et al, 2012). Several genome-wide studies have predicted that multiple components of the silencing machinery are sumoylated and a few were experimentally demonstrated to be sumoylated (Ferreira et al, 2011). With this background, the objectives of the present study were defined as follows:

- To identify the target(s) of Siz2 that regulate silencing
- To unravel the molecular basis of regulation of silencing by Siz2 target(s)

Chapter 2

Materials and Methods

2.1. Molecular biology methods

2.1.1. Polymerase chain reaction

The PCR (Polymerase Chain Reaction) was performed using Taq DNA polymerase supplied by Bioron/Jump Start Taq DNA polymerase from Sigma. Wherever the PCR products were used for cloning for protein expression, Vent DNA polymerase from NEB was used for error less amplification. A 20µl reaction volume was prepared in ddH2O with DNA (20-100ng), primers (5 pico moles), buffer (1X), dNTPs (0.5mM), MgCl2 (1.5mM) and Taq DNA polymerase (1Unit). The primer annealing temperature was identified by gradient PCR and the products were further amplified at the optimal temperature. The Eppendorff thermal cycler was used to amplify the product. The PCR products were resolved on the Agarose gel electrophoresis and confirmed. If required, the separated products were purified by using Qiagen gel extraction kit.

2.1.2. Molecular cloning

2.1.2.1. Preparation of competent cells

Ultra competent cells of *DH5α* strain of *E.coli* were prepared by Inoue method described in (Sambrook and Russell, 2001). Primary culture was made by inoculating a single colony of bacteria at 37 degree celsius for overnight. This primary culture was then inoculated (4ml, 3ml, 2ml, and 1ml) into four 250ml

conical flasks containing 100ml of SOB broth and incubated at 18-22°C with moderate until the OD reached to 0.4-0.5.Cells were harvested by centrifuging at 2500g for 10 min at 4°C. Supernatant was poured and pellet was washed with distilled cold water once. After washing cell were harvested at 4 degree Celsius and immediately kept in ice. Cell pellet was resuspended in 20ml of Inoue transformation buffer for 100ml of initial culture. Cells were harvested again by centrifuging at 2500g for 10 min at 4°C. Supernatant was discarded and centrifuge tube was stored at 4 at ice immediately. The cells were then suspended in 10ml of ice cold Inoue transformation buffer and 0.15µl of DMSO was added and mixed by swirling and stored in ice for 10 min. Bacterial suspension was aliquoted into microfuge tubes and immediately snap frozen by immersing the tightly closed tubes in liquid nitrogen. Then the tubes were stored at -70°C until needed.

2.1.2.2. DNA transformation into bacterial cells

Ultra competent *E.coli* (*DH5α* strain) cells were kept in ice for 1o minutes until it thawed. Then 100ng of plasmid was added to it and incubated in ice for 20 min. After incubation the tubes were immediately kept in 42 degree Celsius for 90second for heat shock. After heat shock tubes were immediately kept in ice for 2 min and then 1ml of SOB was added and incubated in 37 degree Celsius incubator for 20 minutes. After incubation cells were harvested by 100rpm and again resuspended in 100µl of SOB and then spread in selective plate and keep 16 hrs. for colony appearance.

2.1.2.3. Isolation of plasmid

Plasmids were isolated using different methods according to the requirement of downstream applications. Plasmids for screening clones by sequencing and restriction digestion were isolated by alkaline lysis or boiling lysis method. For transfection the plasmids were isolated using Qiagen plasmid purification kits. All three methods are explained below.

2.1.2.3.1. Isolation of plasmid by alkaline lysis method

Plasmid DNA was isolated from bacterial transformants by alkaline lysis miniprep method described in (Sambrook and Russell, 2001). Single bacterial colony was inoculated in 5ml of LB broth containing 100µg/ml of ampicilin and incubated at 37°C and 160rpm for overnight. 1.5ml of bacterial culture was transferred in a microfuge tube and centrifuged at 13k rpm for 1 min. 200µl of ice cold Solution I was added and resuspended uniformly. After solution 1 mixture was treated with Solution II and mixed properly by inverting 2-3 times. Ice cold 200µl of Solution III was added and mixed immediately by inverting several times and left on ice for 5 min and the solution turns into a white precipitate. Then the sample was centrifuged for 8 min at 13k rpm at 4°C. Supernatant was gently taken out and transferred to another clear 1.5 ml centrifuge tube. Then 0.7 volumes of 100% isopropanol was added and mixed by inverting and kept in RT for 10 minutes. DNA was precipitated by centrifuging at 13k rpm for 10 min and the pellet was washed with 500µl of 70% ethanol and once again centrifuged at 13k rpm for 2 min. Supernatant was discarded, DNA pellet was air dried and resuspended in 30-50µl of TE pH8.0 containing 30µg/ml of RNaseA.

2.1.2.3.2. Isolation of Plasmid by boiling lysis method

A 2ml of bacterial culture was centrifuged at 5000g for 2 minutes. The bacterial pellet was resuspended in 100µl of water. Equal volume of lysis buffer (0.1N NaOH, 1%SDS, 10mM EDTA) was added to the mixture. The whole mixture was boiled at 100ºC for 1-2 minutes and kept on ice immediately for 5 minutes. 50µl of Magnesium chloride was added to the mixture and kept in ice. The lysate was centrifuged at 14000g for 5 minutes and supernatant was collected. Plasmid DNA was precipitated by adding 50µl of 3M potassium acetate (pH-5.2) and kept in ice for 5 minutes. The mixture was centrifuged at 14000g for 5 minutes. To the supernatant, two volumes of isopropanol was added and centrifuged at 14000g for 5 minutes. The DNA pellet was washed with 70% ethanol, air dried and resuspended in required amount of sterile milliQ water or 10mM Tris.Cl (pH-8).

2.1.2.4. Colony PCR

An isolated colony of bacteria was resuspended in 50µl of sterile water by vortexing for 30 seconds. The resuspended bacteria were lysed by boiling for 5 minutes. The boiled mixture was centrifuged at 14000g for 1 minute and 5 µl of the supernatant was used in PCR reaction as a template to amplify the insert using either insert specific primers or standard primers present in the vector. The PCR product was confirmed on agarose gel.

2.1.2.5. Screening of the clones by restriction digestion

Plasmids were isolated using protocols as described above and presence of the insert was confirmed by restriction digestion. The restriction digestion protocol followed is described in this section. The recombinants were identified by electrophoresis on

agarose gel by comparing the size difference between vector with insert and empty vector. Restriction digestions were performed to confirm the clones, sub clone the insert into another vector and to digest the PCR product flanking with restriction sites. One or two enzymes were used with the suitable buffers supplied by the manufactures. 5U/µg endo nucleases were used to set the digestion. BSA was added if required. The reaction mixtures were incubated at 37ºC for 1-2hrs. Digests were run on agarose gel and the presence of DNA was confirmed or isolated for further use.

2.1.3. Site directed mutagenesis

Site directed mutagenesis was done to create point mutation in Sir2 where lysine was replaced by arginine. A high fidelity DNA polymerase was used for the PCR reaction. After reaction, the product is digested with DpnI. This digest is important. DpnI only cleaves at methylated sites, so it chews up the template plasmid but not the PCR product. Since the transformation efficiency of the circular template plasmid is several orders of magnitude better than the linear PCR product, without the DpnI digest a large number of colonies would be the parental. After digestion 10µl was transformed using ultra competent cells and kept 16 hrs. at 37 degree Celsius to colony to appear. The following table contains the primer used to create mutation in Sir2.

2.1.4. Vectors

pRS315 and pRS316 was used to clone Sir2. Whereas pGBD-UC1 and pGAD-C1(Xie et al, 2010) plasmid were used for yeast two hybrid assays.Sir2 was

amplified by PCR and cloned in pGBD and pGAD vector by digesting with Xma1-Sal1. Constructs made for this work are described in Table-3 and appendix.

2.1.5. RNA isolation for real time PCR

10 ml O/N culture, spin 3k, 2 min, wash with autoclaved water, freeze in liquid nitrogen. Resuspend the pellet in 400µl of TES buffer. Add 400µl of acid phenol, cap the tube, vortex for 10sec. Incubate at 65^{o}C for 60 min by overtaxing every 15 min to mix properly. Keep on ice for 5min, spin at 4 for 10 min at 13k. Collect the liquid layer. Add 400µl of chloroform and vortex for 10sec, spin at 4^{o}C degree for 10 min at 13k. Collect the liquid layer. (After spin keep at RT for 10 min before collecting the liquid layer).Take carefully, always try to avoid chloroform coming to the pipette tips, chloroform has the tendency to come towards the tip. Add 1/10 volume of 3M NaOAc (pH5.2) and 2.5 volume of ice cold ethanol. Keep at -80 for 1-2 hrs. Spin at 13k, 10min, at 4 degree. Wash the pellet with 70% ethanol, dry the pellet. Add 40-50µl of water; keep at RT for 10 min.

Note: Wear gloves, sterilize everything before use, even autoclave the pipette. Don't autoclave chloroform and SDS. Don't autoclave the NaOAc-filter sterilize it. Keep separate sets of tips, centrifuge tubes and pipettes for RNA work.

TES buffer: 10mM tris(pH7.5),10mM EDTA,0.5% SDS

2.2. Biochemical Methods

2.2.1. Protein extraction and IP

50ml overnight culture of 1.5OD was taken. Spin at 4k, 2min, freeze in liquid nitrogen immediately. Frozen sample can be kept in -80 freezer for further use. Add 1ml of lysis buffer (high salt) and add glass beads to half the volume of lysis buffer. Break the cells in bead beater, 30min pulse, 2min halt (4 times), at room temperature. Collect the lysate by pipette and spin at 13k, 15 min. Collect the supernatant. Avoid the white layer above the lysate. Add 1-2 µg of antibody, keep in ice for 2hrs (for myc Ab 0.3-0.4 µl is used). Add 40µl of protein A sepharose for rabbit antibody and protein G for mouse antibody. Incubate at 4 degree O/N with rotation. Spin at 4k, 2min, collect the beads. Wash the beads with high salt buffer for 1min. Spin at 4k, 2min collect the beads. Wash the beads with low salt buffer for 1min for two times. Spin at 4k, 2min collect the beads. Add 60-80µl 1XLaemmli buffer, boil at 65 degree for 10 min. Spin at 10k, 2min; collect the supernatant, load 10µl in gel. High salt buffer: 50mM HEPES(7.4-pH), 0.25% NP40, 500mM NaOAc, 5mM EDTA, 5% glycerol, 1mM DTT. Low salt buffer: 50mM HEPES, 150mM NaOAc, 5mM MgAc, 1mM DTT, 1mM PMSF.

2.2.2. SDS PAGE gel electrophoresis

The protein extract obtained from the total lysate of cells was resolved by denaturing Poly Acrylamide Gel Electrophoresis. This method was described by Laemmli 1970 (Laemmli, 1970). 5% Stacking gel and 10% resolving gel were cast generally using 1.5mm thick gel casting apparatus supplied by Hoefer. Protein samples were boiled in loading buffer or Laemmli buffer (50 mM Tris.HCl (pH 6.8), 100 mM DTT, 7%

SDS, 0.1% bromophenol blue, 10% glycerol) for 5 minutes and loaded onto the gel and electrophoresed at 30mA on stacking gel and 40mA when it reaches resolving gel. Pre stained molecular weight markers obtained from Biorad was loaded along with the samples.

2.2.3. Coomassie staining of the PAGE gel

After the run, gel was removed and stained with coomassie brilliant blue stain (0.025% in acetic acid: water:ethanol in 10:40:40 volume ratio) for 2-3 hours and destained in destaining solution (acetic acid:methanol:water 10:40:40) by changing it several times until protein bands were seen against a clear background. If the protein has to be transferred onto membranes coomassie staining was avoided, but proteins were stained after transferring on to membrane using Ponceau-S.

2.2.4. Transfer of proteins to membranes by wet transfer method

The proteins resolved by SDS-PAGE were transferred onto PVDF membrane for western blotting. Transfer membrane (Immobilon-P) was activated by dipping in 100% methanol. The membrane was equilibrated with transfer buffer before stacking into the wet transfer apparatus cassette. The order of assembly was "Sponge – three Whatmann papers – Gel – Membrane - three Whatmann papers-sponges". All the above mentioned materials were equilibrated in transfer buffer, (39mM Glycine, 48mM Tris.HCl, 0.037% (w/v) SDS & 20% (v/v) methanol) the cassette was assembled and kept in the transfer apparatus by keeping the gel towards cathode. Transfer was performed by applying 400mA for 2-3 hours or 225mA for

overnight under cold condition. The membranes were dipped in methanol, dried immediately and used for further studies.

2.2.5. Ponceau-S staining of the membranes

Proteins transferred on to the membrane were stained using Ponceu-S stain (0.5gm dissolved in TCA and then diluted to 100ml water) for 10 minutes. The membrane was washed in water by shaking until bands were clearly visible against the background. After recording the stain was completely removed by washing with water.

2.2.6. Immuno-detection of proteins by western blotting

Western blot was done to detect protein. PVDF membrane containing the transferred proteins was blocked in 5% non-fat dry milk in TBST (150 mM NaCl, 10mM Tris-HCl pH8.0 and 0.1%Tween 20) buffer for one hour at RT. Then the membrane was incubated with appropritae dilutions of required primary antibody for 2 hours at RT. The membrane was washed thrice in TBST buffer for 10 min and incubated with secondary anti-rabbit/mouse-HRP antibody (1:10,000 in 1% BSA in TBST buffer) 1 hour at RT. Membrane was washed thrice with TBST for 10 min. BioRad detection reagents and BioRad Versa-Doc instrument were used for detecting the protein of interest as directed by manufacturer's instructions.

2.3. Antibody used in this study

For Sir2 1:2000 dilution of primary Sir2 antibody from Santa Cruz was used (Sorolla et al, 2011). For myc protein detection we used 1:40,000 diltuion of primary

anti Myc antibody (Ab-9106) (Tavernier et al, 2012). Nop1 and Nsp1 from Abcam was used at 1:500 dilution for immunofluorescence.

2.4. Yeast methods

Yeast strain used in this study are described in Table-2.

2.4.1. High efficiency yeast transformation

Yeast transformations with plasmids (including genomic library) or PCR products were done based on high efficiency LiAc (Gietz et al, 1992). Primary overnight culture was taken and $5x10^6$ cells were added to the 25ml broth and incubated for 4-5 hrs at 30°C with constant shaking. $1x10^8$ cells from the secondary culture were used for transformation. Cell pellet was then washed in 1ml of 0.1M LiAc and resuspended in 0.240ml of 50% Poly ethylene glycol (Sigma Ultra).Then 0.036 ml of 1M LiAc and 0.040ml of 2mg/ml salmon sperm DNA and 100ng of plasmid/DNA fragment and then MilliQ water were added to make final volume upto 0.30 ml. Cells were vortexed slowly and incubated at 42°C for 40 minutes. Then cells were spun at 13k for 15 sec and cell pellet was resuspended in 200ml of sterile water and plated on selective dropout media. If the selection was on G418 plates, the cell pellet was resuspended in 1ml of YPD broth and incubated at 30°C for 10-12 hrs. to allow the expression of the gene and then plated on YPD media containing G418 drug (200 mg/ml).

2.4.2. Extraction of genomic DNA from yeast cells

Genomic DNA from yeast cells were isolated by using two methods in this study.

2.4.2.1. Zymolyase method

5ml overnight culture was taken and centrifuged at 3k rpm for 5 min. The cell pellet was resuspended in 0.5ml of 1M Sorbitol and 0.1M EDTA (pH 7.5) and transferred into 1.5ml microfuge tube. Spheroplasting was done by incubating the cell with 20 µl of Zymolyase 100,000U (2.5 mg/ml) at 37°C for 60min. Cells were centrifuged for 1 min at 13k rpm and the cell pellet was resuspended in 0.5ml of 50mM Tris-Cl (pH 7.4) and 20mM EDTA (pH 8.0). 50µl of 10% SDS was added to the cell suspension, mixed well and then incubated at 65°C for 30 min. 200µl of 5M potassium acetate was then added to the cell suspension and placed in ice for 60 min. Cells were centrifuged for 5 min at 13k rpm and supernatant was transferred to a fresh microfuge tube. One volume (0.75ml) of 100% ethanol was added to the supernatant, mixed and allowed to sit at room temperature for 5 min. Then centrifuged very briefly for 2 min at 13k rpm and supernatant was poured off. DNA pellet was air dried and resuspended in 0.3ml of TE (pH 7.4) containing 20µg/ml of RNase A. DNA was incubatded at 37°C for 30 min for degrading RNA. Then 30µl of 3M sodium acetate was added to the DNA solution and mixed. To this, 0.2ml of 100% isoproponal was added and mixed once again. DNA was recovered by centrifuging at 13k rpm for 2 min. The supernatant was poured off, DNA pellet was air dried and resuspended in 30µl of TE (pH 8.0).

2.4.2.2. Rapid isolation of genomic DNA from yeast cells

5ml of YPD overnight culture was taken and harvested by centrifuging at 3k rpm for 5 min. Pellet was washed in 0.5 ml of autoclaved distilled water and resuspended in 200µl of breaking buffer. 200µl of glass beads (0.5mm) added to the cell suspension and then 200µl of phenol/chloroform/isoamylalchohol (25:24:1) was added and

mixed. Cells were vortexed at maximum speed at RT for 2 min. 200µl of TE (pH 8.0) was added and once again vortexed briefly for 10 to 15 sec. Then the mixture was centrifuged at 13k rpm for 5 min at RT. The aqueous layer was transferred to a centrifuge tube and 1ml of 100% ethanol was added and mixed by inverting the tube 2-3 times gently. DNA was recovered by centrifuging at 13k for 5 min. The supernatant was poured off, DNA pellet was air dried and resuspended in 30µl of TE (pH 8.0).

2.4.3. Extraction of whole cell protein from yeast cells by Trichloro Acetic acid (TCA) method

Overnight culture was taken and cells harvested by centrifuging for 2 min at 4000rpm. The cell pellet was resuspended in 200µl of 20% TCA and200µl volume of glass beads were added and vortexed for 1min at high speed at RT. Cell suspension was transferred into a new 1.5 ml centrifuge tube. Glass beads were washed twice with 200l of 5% TCA and the washes were added to the previous suspension. Cell pellet was collected by centrifuging at 3k rpm for 10 min and resuspended in 200µl of 1x laemmli buffer. The laemmli buffer will turn orange coloured because of the low pH of cell pellet. Hence 20-30µl of 1M Tris base (no pH adjustment) was added till it turned blue. The sample was boiled for 3 min and centrifuged again at 13k rpm for 3min. Protein sample was transferred to a new microfuge tube and the pellet was discarded.

2.4.4. Silencing assay

Telomeric silencing was measured in strains carrying a *URA3* or *ADE2* gene near the telomere sequence. *HM* silencing was checked in strains carrying a *TRP1* gene at the *HMR* locus. For these assays, yeast cells were initially grown in nutrient rich broth or selective broth dropped out for specific amino acids (for retaining plasmids) at 30°C with appropriate rotation overnight and then the culture was subjected to 10-fold serial dilution. 5µl of each was spotted from each dilution in the respective plates: on complete medium to check the total number of cells grown and on selective medium for measuring the loss in silencing of reporter gene. In case, the reporter gene is *URA3*, then serially diluted cultures were spotted on the medium containing1mg/ml of 5-FOA (5-Fluoro orotic acid). Expression of the *URA3* gene (Orotidine-5'-phosphate (OMP) decarboxylase) leads to the conversion of 5-FOA into 5- fluorouracil, a toxic compound. Therefore strains expressing *URA3* cannot grow in this medium and those repressing *URA3* can grow. Thus, 5- FOA serves as a good indicator of the expression status of the *URA3* reporter gene. After spotting, plates are incubated at 30°C for 2-3 days and analyzed loss in silencing by observing growth of cells. In case of *ADE2* marker, the silencing was assayed by the colour of the colony. Cells with the gene in a repressed state accumulate the upstream substrate (5-aminoimidazole ribonucleotide carboxylase) which polymerizes forming a red pigment. As the colony forms through 20-25 rounds of division, the gene is stochastically repressed in some lineages and expressed in others resulting in red/white sectored colonies. Cells expressing the gene do not accumulate the substrate and are white. The cells were plated on YPD plates and incubated for 2-3 days at 30°C. Later they were kept at 4°C for about 4-5 days and analysed for the loss of silencing by observing the colour of colony.

2.4.5. Immunofluorescence

Immunofluorescence was done as described in (Gotta et al., 1996). Briefly, diploid yeast strain was grown in YPD broth. 5ml overnight culture was fixed with 0.5ml of formaldehyde and incubated at 30°C in a shaker for 20 min. Cells were then washed thrice with sterile water and resuspended in 200µl of 0.1M EDTA-KOH and 10mM DTT and incubated at 30°C for 10 min. Cell suspension was centrifuged at 3k rpm for 5 min. The cells were spheroplasted by resuspending in 200µl of YPD broth containing 1.2M sorbitol and one-tenth volume of zymolyase (2mg/ml) and incubated at 30°C for 15 min. Spheroplasts were washed thrice with 500µl of YPD sorbitol and resuspended in 100µl of YPD sorbitol. Spheroplasts were spotted on multi-well slides coated with 0.1% polylysine. They were further permeabilized with methanol and acetone by incubating for 5 min and 1 min respectively at -20°C. Spheroplasts were blocked with 1% BSA in PBS containing 0.1% tritonX100 and incubated with appropriate primary antibody for 2hrs at RT or overnight at 4°C in a moist box. Cells were then thoroughly washed thrice with PBST buffer for 5 min each and incubated with recommended dilutions of fluorescently labelled secondary antibody (Alexa Fluor 488 tagged secondary anti mouse antibody 1:500, Cy3 tagged secondary anti rabbit antibody 1:500) in dark at room temperature for 45 min in a moist box. Cells were thoroughly washed thrice with PBST buffer for 5 min each. After washes, slides were mounted in mounting medium containing DAPI and then viewed and photographed in a Zeiss AX-10microscope. Images were processed using the same software.

2.4.6. Detection of Sumoylation

Sumoylated proteins were enriched in strains carrying 8XHis tagged SMT3. 50ml yeast culture of 0.6OD was taken. Spin and immediately freeze in liquid nitrogen. Resuspend the pellet in 1ml of lysis buffer and keep in ice for 10 min. Add 1ml 50% TCA and keep in ice for 10min.Spin at13k for 10min, discard the supernatant. Dissolve the pellet with ice cold acetone, spin for 13k, 1min (repeat this process twice). Resuspend the pellet in 1ml GnCl buffer, check the pH with pH paper, add drop wise un-pHed 1M Tris to make pH to 8. Add imidazole to make 10-15mM. Keep in rocker for 20-30min at RT. Spin at 13K, RT, 10min. Collect the supernatant. Add 40 µl of Ni-NTA beads; wash the beads with urea buffer three times. Incubate the supernatant with beads for 2hrs to overnight at RT. Spin at 3K, 2 min, collect the beads. Wash the beads with urea buffer three times.(3timesX 10 min).Add 60-80 µl of Laemmli buffer (laemmli buffer should have 30mM EDTA). Boil the beads in continuously boiling water for 5-6 min. Spin ant 10k, 2 min, collect the supernatant. Load 10µl for western blot.

4M Urea buffer(20ml): Urea-4.8gram, NaPO4 (pH8)-2ml, 1M tris(pH8)-200µl,200 µl of 1M imidazole, water up to 20ml.

6M GnCl buffer (20ml): GnCl-11.4gram, NaPO4 (pH8)-2ml, 1M tris (pH8)-200µl, Water up to 20ml.

Lysis buffer (10ml):10N NaOH-1.85ml, βME-740µl, water up to 10 ml.

500mM NaPO4 (pH8) (10ml): 9.3ml of 500mM Na2HPO4 and 670 µl of 500mM NaH2 PO4 was mixed.

Precautions:

1. OD should be 0.6-1.
2. Lysis buffer should be fresh.
3. After adding GnCl buffer, all steps should be done at room temp.
4. Wash the Ni-NTA beads with urea buffer properly before adding Laemmli buffer, otherwise it will make clumps.
5. Boiling should be continuous, don’t boil in heat block, yield is not good, and it should be always in continuously boiling water.

Table 1: List of primers used in this study

Name	***SIR2 point mutation***
F-109	AGTGTCAATGTCCAATGACGTTTTG*AGA*CCAGAGACGCCC
R-109	GGGCGTCTCTGGTCTCAAAACGTCATTGGACATTGACACT
F-132	TTTTCTATGGTCCCTCCTTCACT*AGA*CGAGAGTCTCTCAA
R-132	TTGAGAGACTCTCGTCTAGTGAAGGAGGGACCATAGAAAA
F-215	TGTTGAAGACCCATTGGCAAAAAGGCAAACAGTTCGTCTA
R-215	TAGACGAACTGTTTGCCTTTTTGCCAATGGGTCTTCAACA
	SMT3 amplification
F primer:	5ATGTCTGCAGATGTCGGACTCAGAAGTCAATC3
R primer:	5ATGTGCGGCCGCAATCTGTTCTCTGTGAGCCTC3
	Sir2 amplification for FLAG tag
Fpromoter	ATATGCGGCCGCGAAATGATTATTAGCAGTCTTTCTCCC
Rpromoter	ATATTCTAGACCAGCTTTAATGTGCCGATGAGGG
FSir2-Sma1:	ATATCCCGGGACCATCCCACATATGAAATACGCCG
RSir2-Sal1:	ATATGTCGACACCACCTCCTTTCTTTGACCCAACG
Name	***Sir2 amplification for GBD construct***
FSir2 -ATG:	ATATCCCGGGATGACCATCCCACATATGAAATACG
Sir2R-sal1:	ATATGTCGACAGTGAGATGGGCGGTACATG

Table 2: List of strains used in this study

Name	Description	Source/Reference
KRY2	W303-1A (*leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*)-*MATa*	(Thomas & Rothstein, 1989)
KRY3	W303-1B (*leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535*)-*MATα*	(Thomas & Rothstein, 1989)
KRY12	KRY3 except *adh4::URA3-TelVII L-MATα*	(Pasupala et al, 2012)
KRY18	KRY2 except *sir2::KanMx*	(Cuperus et al, 2000)
KRY19	KRY3 except *sir2::KanMx*	(Cuperus et al, 2000)

KRY360	KRY3 except *RDN1::mURA3-HIS3 hmr::TRP1 adh4::ADE2 Tel VIIL sir2::KanMx-MATα*	(Cuperus et al, 2000)
KRY670	*8XHis-SMT3:TRP1-MAT α* (GA5347)	(Ferreira et al, 2011)
KRY 790	*sir2::kan 8XHis- SMT3His-MATα*	This study
KRY 717	*sir2::KanMx esc1::kanMx 8XHis-SMT3:TRP*	This study
KRY788	*sir2::kanMx esc1::kanMx*Tel *VIIL URA3-MATa*	This study
KRY793	*sir2::kanMx Tel VIIL URA3-MATa*	This study
KRY868	*sir2::kanMx SIR4-Myc-KanMX*	This study
KRY 870	*ade-,can-,his-,leu-,ura-trp-, sir2::HIS3,Net1-myc::LEU2(LPY4724)*	(Garcia & Pillus, 2002)
KRY878	KRY 670 *except siz2::HIS3*	This study
KRY883	KRY 670 *siz1::HIS-MATα*	This study
KRY872	*mms21-11::KanMx-MATα*	This study
KRY883	*siz1::HIS3 8x-His-SMT3:TRP*(353X731)-*MAT α*	This study
KRY878	*siz2::HIS3 8x-His-SMT3*(693X215)-*MAT α*	This study
KRY1507	*mms21-11::KanMx 8x-His-SMT3-MATα*	This study
KRY737	*SIR4*-13Myc::*KanMx 8X-His SMT3-MATa*	This study
KRY705	*SIR3*-13Myc::*HIS3 8X-His SMT3 MATa*	This study
KRY734	*8XHis SMT3::hygro Yku70myc::TRP-MATa*	This study
KRY697	*Yku80-13XMyc::KanMx-His SMT3:TRP1 MATα*	This study
KRY 1540	*Sir2::KanMx rDNA::ADE2*	This study

Table 3: List of plasmids used in this study

Name	Description	Source of insert/method
CKM94	Sir2 Xba - Xho in pRS415	David Shore

CKM95	Sir2 in Xba/HinDIII in pRS316	David Shore
CKM206	CKM6(YEplac181)+SIZ2	XhoI fragment of SIZ2 in SalI cut CKM6
CKM263	CKM5+*SIZ2*	SphI/EcoRI fragment from CKM206
CKM289	pGBDU-C1	Clonetech
CKM292	pGAD-C1	Clonetech
CKM294	pBluescrtipt	Clonetech
CKM433	pRS316+*His-FLAG-SIR2*	PCR product of Sir2 cloned as Xma1/Sal1
CKM434	pRS316+*His-FLAG-SIR2(K215R)*	PCR product of Sir2 cloned as Xma1/Sal1
CKM435	pGBD-UC1+*SIR2*	PCR product of Sir2 cloned as Xma1/Sal1
CKM436	pGBD-UC1+*SIR2(Sir2-SUMO)*	PCR product of Sir2-SUMO cloned as Xma1/Sal1
CKM437	pGAD+*NET1*	Cloned in pGAD-C1 with EcoR1
CKM438	pGAD+*SIR4*	David Shore
CKM439	Sir2-K106R (in CKM94)	This study
CKM440	Sir2-K132R (in CKM94)	This study
CKM441	Sir2-K215R (in CKM94)	This study
CKM442	Sir2-K106-132R (in CKM94)	This study
CKM443	Sir2-K106-215R (in CKM94)	This study
CKM444	Sir2-K132-215R (in CKM94)	This study
CKM445	Sir2-K106-132-215R (in CKM94)	This study
CKM446	Sir2-SUMO (in CKM 342)	PCR product of SUMO with Pst1/Not1 ligated.
CKM447	Sir2-K215R-SUMO (in CKM342)	This study

Chapter 3

Identification of Siz2 targets

3.1. Introduction

In work form our laboratory, we had shown that overexpression of Siz2, an E3 SUMO ligase decreases silencing at the telomeres and HM loci but not at the ribosomal locus. We also showed that Siz2 overexpression results in the reduction of Sir2 binding at telomere (Pasupala et al, 2012). Other reports from literature also implicated protein sumoylation in gene silencing. Esc2, a member of a conserved family of proteins that have SUMO-like domains, interacts with Sir2 with its SUMO-conjugating motif and is necessary for the maintenance of heterochromatin structure at *rDNA* (Miele et al, 2009). Loss of a SUMO-dependent ubiquitin ligase, Slx5, also leads to loss in silencing at telomeres and *rDNA* but not *HM* loci (Darst et al, 2008). Recently, it was shown that the catalytically inactive *mms21* also led to loss of telomeric silencing and reduction in Sir3 occupancy at the subtelomeric loci (Wan et al, 2013). In order to understand how these various SUMO ligases and Ubls influence silencing it is important to identify the targets of these activities. Yeast telomeres are bound to nuclear envelope by two partially redundant pathways involving Sir4 and Yku70/80. It was shown that PIAS-like SUMO E3 ligase Siz2 sumoylates both Yku70/80 and Sir4 in vivo and promotes telomere anchoring to the nuclear envelope (Ferreira et al, 2011). Genome-wide SUMO protein analysis has shown that multiple proteins involved in gene silencing are sumoylated (Denison et al, 2005; Hannich et al, 2005; Panse et al, 2004; Wohlschlegel et al, 2004).We were

therefore interested in testing if all of these proteins showed a *SIZ2* overexpression dependent alteration in sumoylation status.

3.2. Results

3.2.1. Identification of Siz2 targets

In our study, as mentioned in materials and methods, we tagged *SMT3* with 8X-His. The only genomic copy of *SMT3* gene, encoding the SUMO protein was now tagged with 8-His at the N-terminus. After confirming the equal expression of tagged *SMT3* with untagged *SMT3* by quantitative RT-PCR, protein extract was made and pull down was done using Ni-NTA agarose beads. Since nickel has affinity towards histidine, it will bind to *8XHis-SUMO* and therefore all proteins containing the His-tagged SUMO should be enriched. To detect whether sumoylated proteins are precipitating with *8X-His-SMT3*, western blot was done against the protein of interest. If a protein is sumoylated it will show slower migrating band in western blot because of the SUMO conjugation. By using this method we studied the sumoylation status of Sir3, Sir4, Yku70 and Yku80 and found that Sir3 and Sir4 sumoylation is Siz2 dependent. The 8-His-tagged SUMO strains containing myc-epitope-tagged Yku70, Yku80, Sir3, or Sir4 were first generated for these experiments. For detecting Sir2, we used antibodies to Sir2 (Santa Cruz). The epitope tagged strains have been described earlier (Mishra & Shore, 1999; Pasupala et al, 2012). These strains were then transformed with empty vector or 2μ *SIZ2* plasmid, and total sumoylated proteins were enriched on a nickel column, separated on SDS-PAGE, and detected by appropriate antibody. In Figure 7, panel-A was developed with anti-myc antibody and shows that one of the three sumoylated forms

of Yku70 is increased upon Siz2 overexpression; Yku80 does not show any change in sumoylation upon *SIZ2* overexpression. Sir3 and Sir4 sumoylation also clearly show elevated sumoylation upon *SIZ2* overexpression. In panel B, sumoylation of Sir2 was tested in a similar experiment.

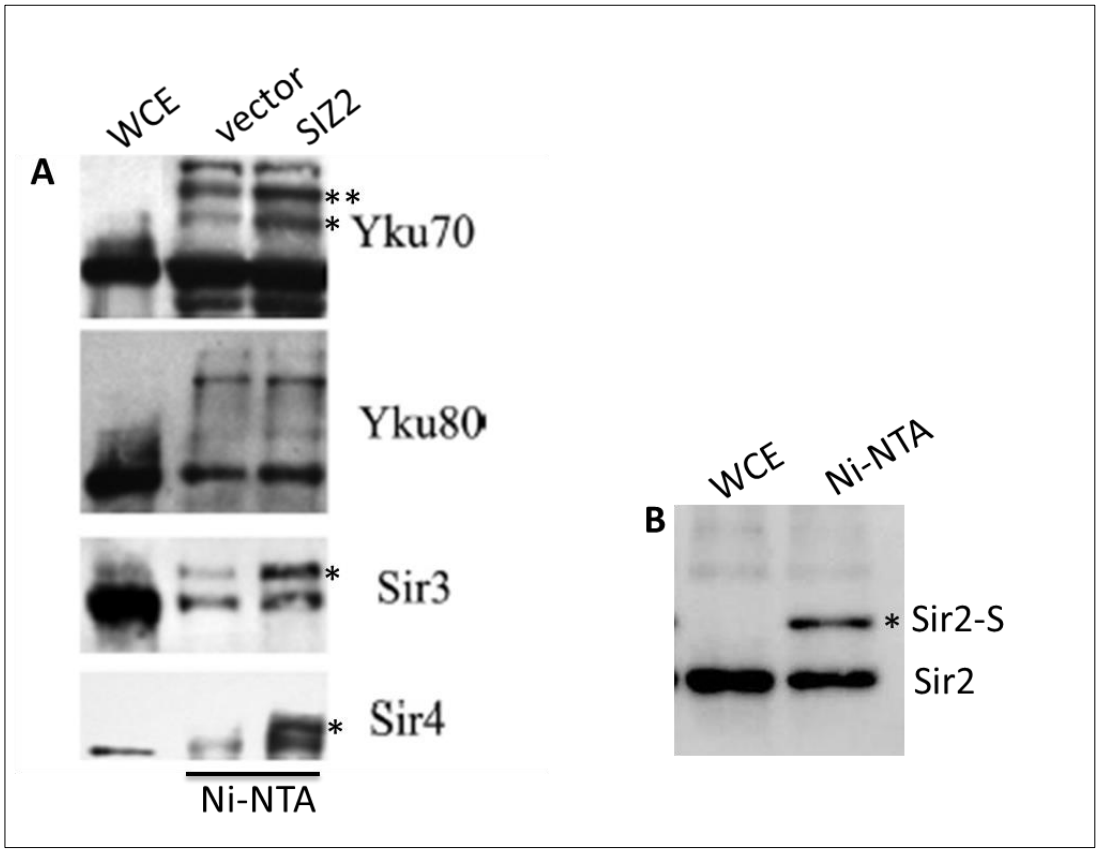

Figure 7: Siz2 expression leads to elevated sumoylation. *Panel A) lane 1 contains whole-cell extracts, lane 2 contains protein from cells transformed with vector, and lane 3 contains protein from cells transformed with 2μ SIZ2. Slower-moving bands are visible only upon enrichment on the nickel column. The right of the panel indicates the protein being detected. Panel B) Sir2 is sumoylated, lane 1 shows WCE, lane 2 shows slow migrating Sir2-SUMO band in Ni-NTA pulldown. (*) indicates the sumoylated forms.*

We find that Sir2 is also sumoylated. Therefore, these results confirm and extend previous results by showing that Sir2, Sir3, Sir4 and Yku70/80 are all

sumoylated. Sir3 and Sir2 had been shown for the first time to be sumoylated in this study.

3.2.2. Sir2 sumoylation is Siz2 dependent

Our previous work has indirectly implicated Sir2 as a possible target of Siz2: Upon Siz2 overexpression we found a reduction in Sir2 association with telomeres. Therefore, we focussed our attention on Sir2 and sumoylation of Sir2 by Siz2 for the rest of our study. We performed a pull down of sumoylated Sir2 as described above using a His-tagged *SMT3* strain and in a strain carrying untagged *SMT3*. As shown in Figure 8, neither whole cell extracts (lanes 1,2) nor pull down from untagged strain (lane 3) could detect the slow moving Sir2 band, which was clearly enriched in the pull down from the tagged strain (lane 4). This confirms that Sir2 is sumoylated. We next asked if this sumoylation was Siz2 dependent. We therefore generated *siz1, siz2* and *mms21* deleted strain with *8X-His-SMT3* and repeated the same experiment to determine which of the three SUMO ligases is responsible for Sir2 sumoylation. Interestingly we have found that Sir2 sumoylation is solely dependent upon Siz2. Whereas deletion of *siz1* or *mms21* (Fig.9 lane 4 and 6 respectively) does not affect Sir2 sumoylation, deletion of Siz2 abolishes Sir2 sumoylation completely (Fig.9 lane 5).

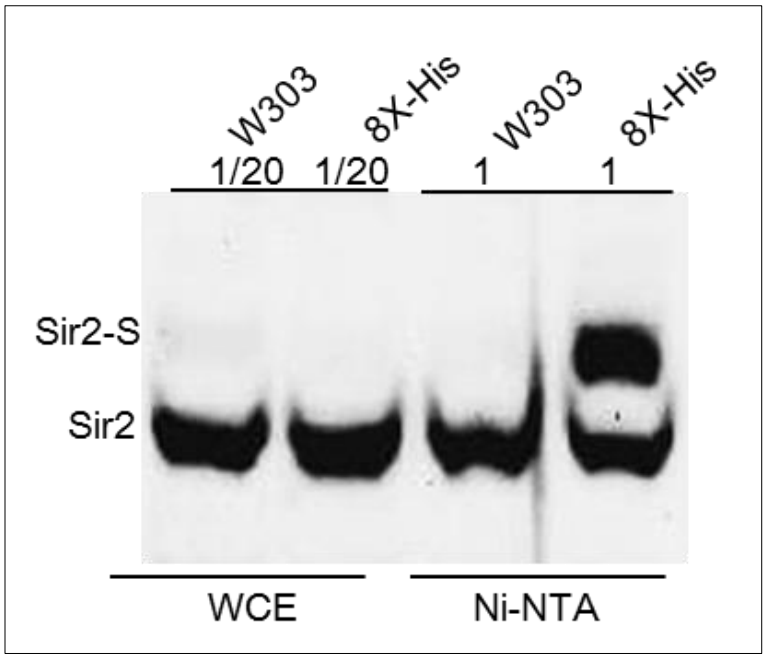

Figure 8: Sir2 is sumoylated. *Lane 1 and 2 are the whole cell extract. Lane 3 shows absence of Sir2-SUMO when cell extract without 8X-His-SMT3 was subjected to Ni-NTA pull down, lane 4 shows sumoylated Sir2 when 8X-His-SMT3 was pulled down with Ni-NTA and slower migrating band of Sir2-SUMO has appeared.*

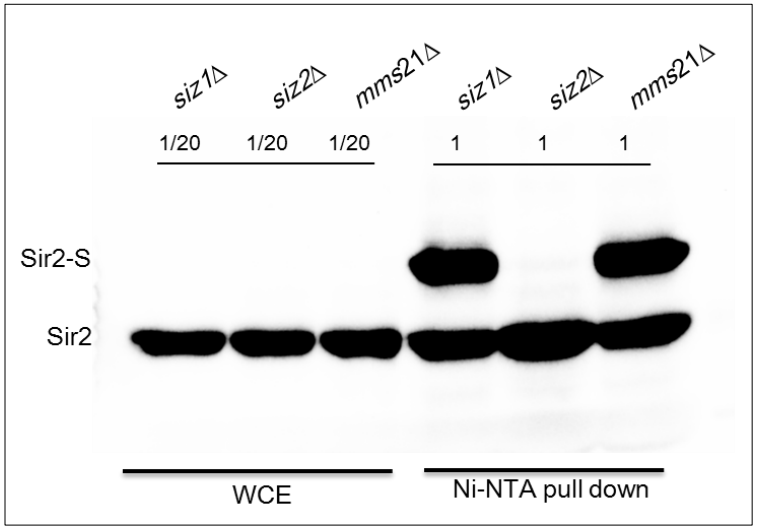

Figure 9: Sir2 sumoylation is Siz2 dependent. *Lane 1, 2 and 3 are the whole cell extracts. Lane 4 and 6, pull down from strains carrying deletion of either siz1 or mms21-11::KanMx have slower migrating sumoylated Sir2 band. Lane 5 is the siz2Δ which is not showing Sir2 sumoylation.*

3.3. Discussion

In this study we found that Sir3 and Sir4 are sumoylated by Siz2 because there are increased amounts of sumoylated fraction found in Ni-NTA pulldown experiment where Siz2 was overexpressed. We also found higher amount of Yku70 sumoylation in Siz2 overexpression background. We did not observe any change in sumoylation of Yku80 upon Siz2 overexpression. Next we asked if Sir2 is sumoylated since we had reported that binding of Sir2 at telomere reduces when Siz2 was overexpressed; moreover sumoylation of Sir2 has not been studied although one genome-wide pull down of sumoylated protein detected Sir2 (Wohlschlegel et al, 2004). Our study shows that Sir2 is sumoylated and Sir2 sumoylation is Siz2 dependent. Since Sir2 binding at telomere reduces upon Siz2 overexpression, it will be interesting to investigate if sumoylation of Sir2 by Siz2 plays a role in maintaining heterochromatin.

Chapter 4

Molecular characterization of Sir2 sumoylation

4.1. Introduction

In a protein there may be more than one site where SUMO molecule maybe conjugated. Though SUMO is conjugated to a consensus site ψKx(E/D), where "ψ" is a hydrophobic amino acid, "x" is any random amino acid and K is the target lysine residue, there are examples of many important protein sumoylation where SUMO is conjugated to non-consensus sequence. It is also notable that often SUMO is not conjugated to the consensus sequence even though a protein carries one. We wanted to identify the lysines in Sir2 that are targets for Siz2. In our study we used freely available online tool GPS-SUMO to identify the putative SUMO binding site and we identified three putative lysine namely K106, K132 and K214/5. After identifying the sites we created point mutation at the lysine residues where lysine was replaced by arginine. This will block sumoylation but is unlikely to alter the structure as a lysine to arginine change will maintain both the positive charge and bulky nature of the amino acid at this site. By site directed mutagenesis method we created single mutants K106R, K132R, K214R, K215R, double mutants K106/132R, K106/215R, K132/215R, and a triple mutant K106/132/215R. After creating the point mutants we studied the sumoylation status of the mutants by Ni-NTA pull down experiments.

4.2. Results

4.2.1. Multiple sumoylatable sites in Sir2

As mentioned earlier we identified three putative SUMO binding sites in Sir2 by using GPS-SUMO tool. All the sites are in the N-terminal region of Sir2.

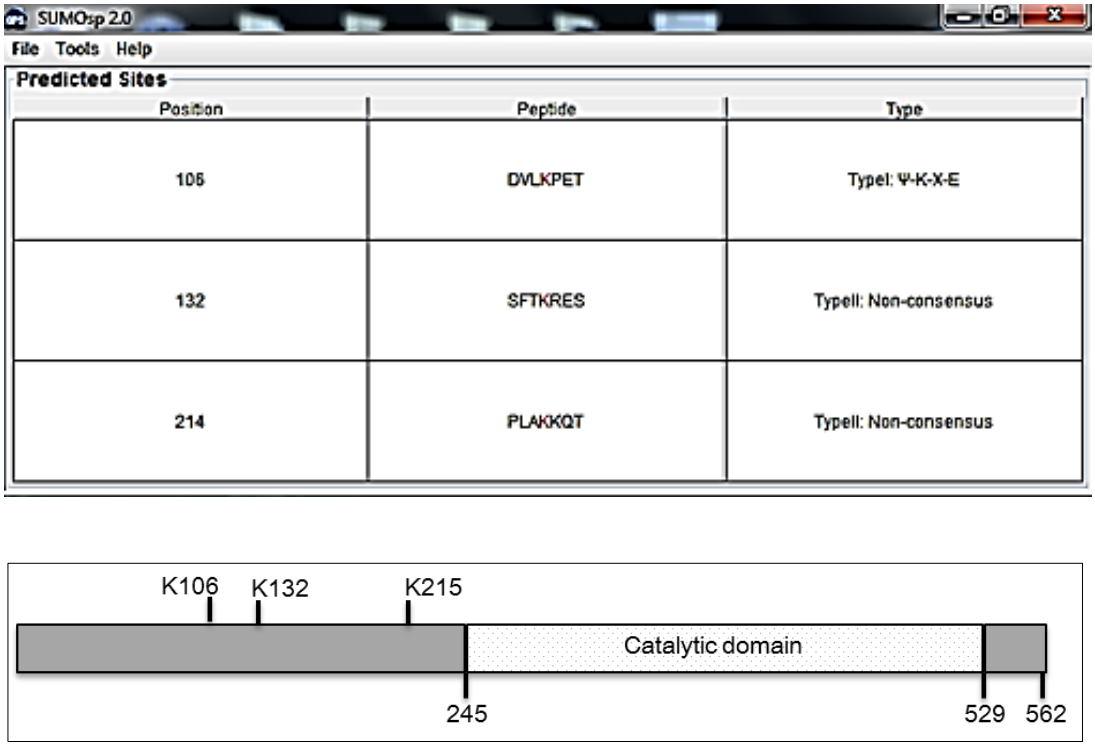

Figure 10: Prediction of SUMO binding sites in Sir2. *A) GPS-SUMO screen shot shows that there are three putative SUMO conjugation sites in Sir2. B) Schematic diagram of Sir2 with its putative SUMO conjugation sites.*

After identifying the targets we created the point mutation by site-directed mutagenesis, confirmed the mutation by sequencing and then transformed them into *sir2*Δ strains carrying His-tagged *SMT3*. We first checked the expression of all the mutants by western blot using anti-Sir2 antibody and confirmed equal expression of all the mutants.

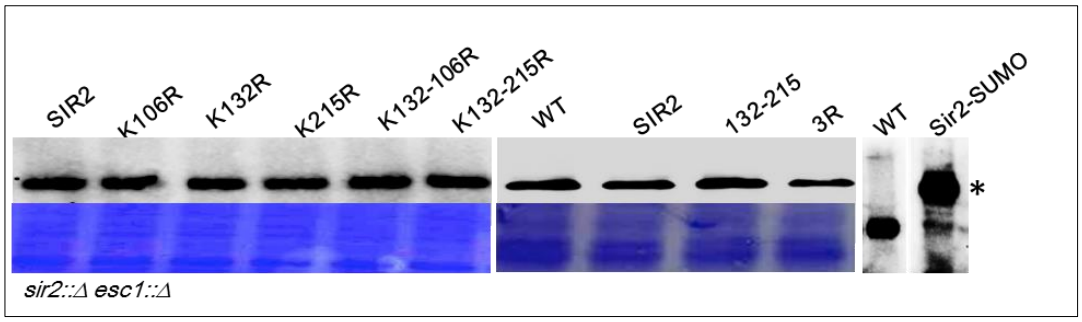

Figure 11: Point mutants are expressed equally. *Total protein extracts from sir2Δ strains carrying the indicated WT or single or double or triple point mutants on a plasmid were separated on SDS-PAGE and western blots developed with Sir2 antibody. Lane 12 showing slower migrating SUMO tagged Sir2 corresponding stained blots are shown for comparing amount of protein loaded.*

4.2.2. Three sumoylatable sites in Sir2

After confirming equal expression of all the mutants we studied the sumoylation pattern. To study the sumoylation pattern of the point mutants, Ni-NTA pull down was followed in *sir2*Δ 8 X-His-*SMT3* strain (KRY 758) containing any one of the plasmids. Our result shows that single mutant of Sir2 is not affecting Sir2 sumoylation and all the single mutants are sumoylated similar to the WT Sir2 (fig. 12, panel A). This indicates that when one site is mutated, SUMO might conjugate to the other putative site present in Sir2. Then we studied the sumoylation status of double and triple mutants and found that all the three double mutants namely K106/132R , K106/215R and K132/215R show sumoylated bands but we could not detect any sumoylated band in triple mutant (3R) (fig.12, panel B). These observations together suggest that Sir2 contains three sumoylatable sites and when all the sites are mutated, SUMO is not conjugated to Sir2.

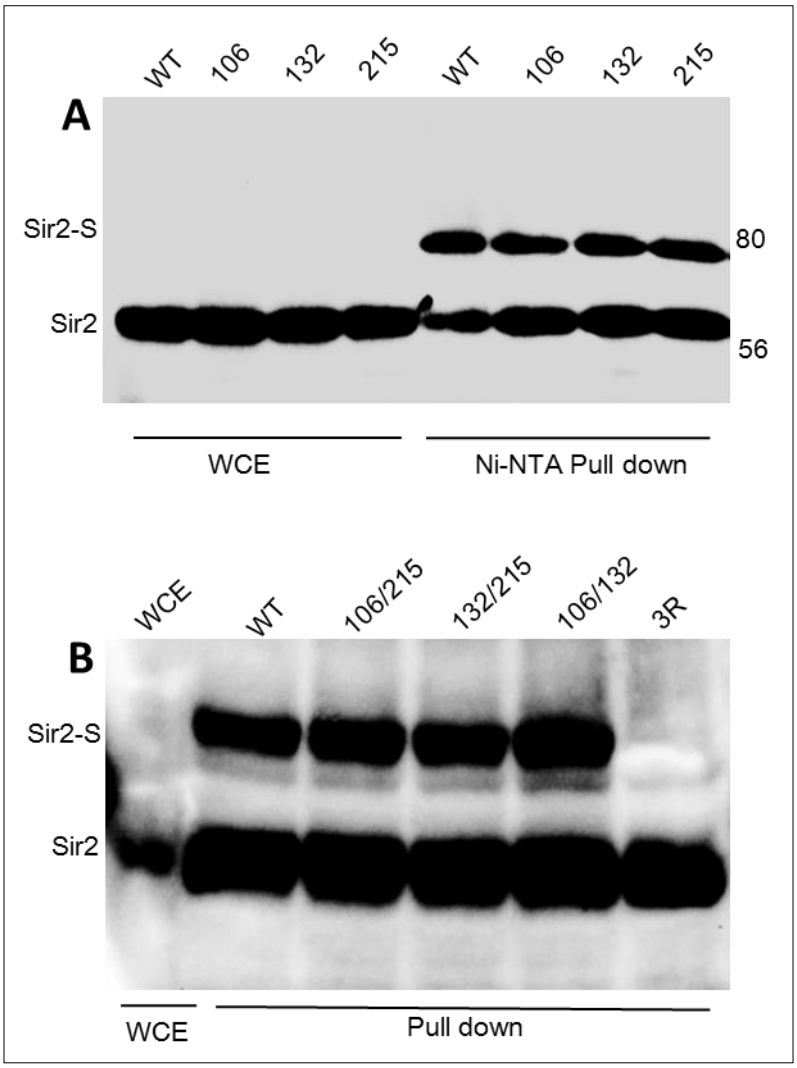

Figure 12: Sir2 contains three sumoylatable sites. *A) Single mutants are all sumoylated. Lane 1 to 4 shows the whole cell extract. Lane 5 to 8 shows pull down from the strains. Sir2 sumoylation can be detected even after replacing the lysine residue individually. In all cases, WCE is 1/20 of the IP. B) Lane 1 shows whole cell extract. Lane 2 to 5 shows the pull down from indicated double mutants and lane 6 the triple mutant. Although double mutants show sumoylation, triple mutant does not. In panel B, WCE from only one extract is shown for comparison*

4.2.3. esc1 deletion leads to the hyper sumoylation fo Sir2

We had reported that *esc1* deletion leads to enhanced loss of telomeric silencing upon Siz2 overexpression. Hence we were interested to study Sir2 sumoylation upon *esc1* deletion. For this experiment we created a strain having *8X-His-SMT3* with

*esc1*Δ*sir*Δ *(KRY 717)* and studied Sir2 sumoylation. By pull down with Ni-NTA columns we have observed that *esc1* deletion leads to the hyper sumoylation of Sir2. As shown in fig.13, panel A, we observed an additional slower moving band corresponding to molecules having two SUMO moeties conjugated (fig.13, panel A, lane 4 and 5). We have also found that *SIZ2* over expression results in the increased fraction of the mono-sumoylated Sir2 (lane 3). To further confirm the enhanced sumoylation upon Siz2 overexpression we measured the intensity of the slower migrating band by Image-J software. Intensity representation showed that SIZ2 overexpression results in increased amount of sumoylated Sir2 (Fig.13, panel B, red bar 2 and 4).

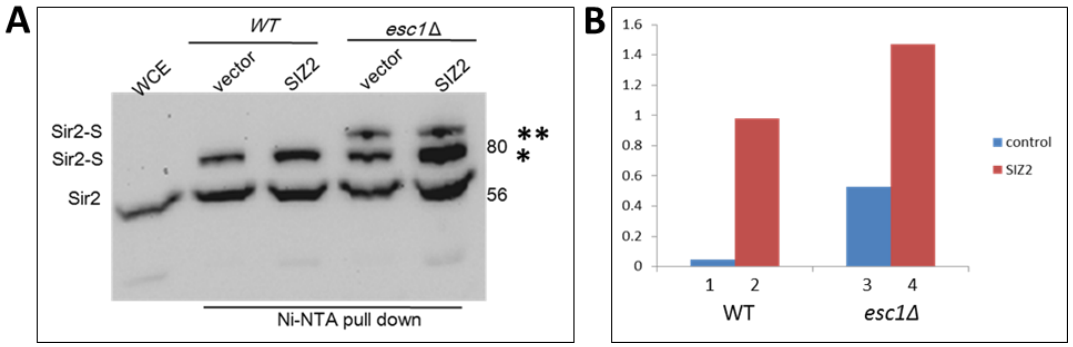

Figure 13: esc1*Δ *leads to the hyper sumoylation of Sir2. *A) Lane1 is the WCE, lane 2 and 3 shows slower migrating Sir2-S band and it is more prominent in Siz2 overexpression condition (lane 3). Lane 4 and 5 shows hyper sumoylation of Sir2 in esc1*Δ *background where three bands were detected instead of two, it also shows increased sumoylation in Siz2 overexpression background (lane5). (*) indicates Sir2-S with single SUMO, (**) indicates Sir2 with two SUMO conjugated. B) Band intensity in both WT (blue bar 1 and 2) and esc1*Δ *(red bar 3 and 4) shows that SIZ2 overexpression leads to enhanced sumoylation of Sir2 (red bar 2 and 4). Y axis shows intensity in arbitary units.*

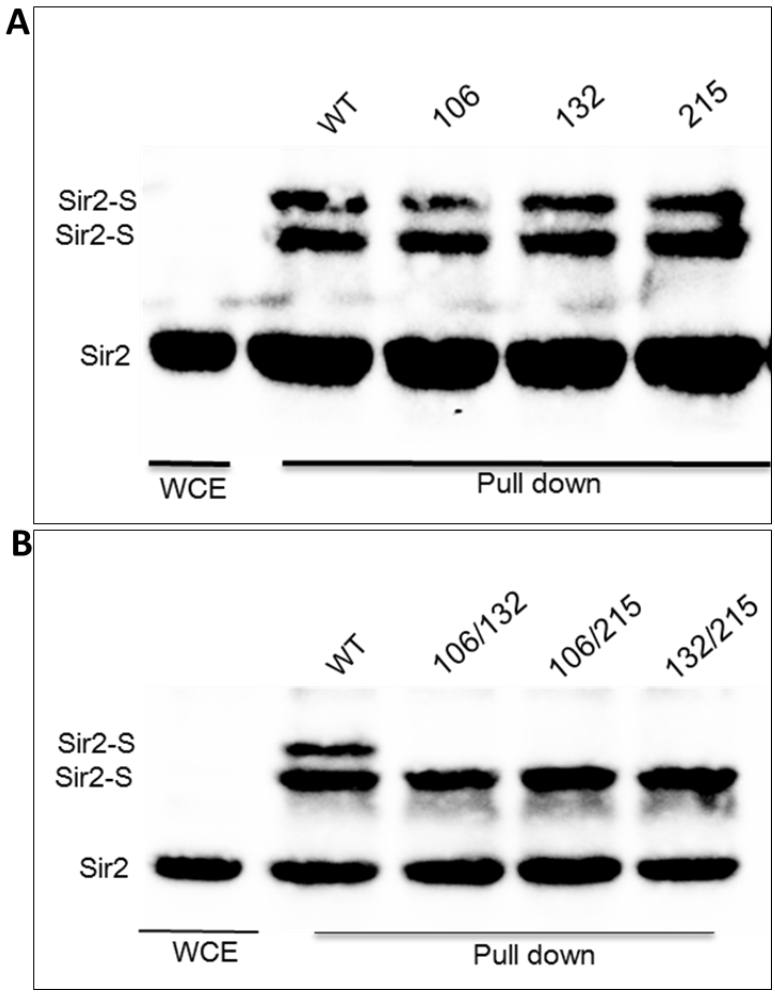

Figure 14: Two sites are possibly sumoylated in esc1Δ. *A) esc1 deletion leads to hyper sumoylation of Sir2. Lane 2 shows the sumoylation of wild type Sir2, where lane 3 to 5 show two sumoylated band in single mutants of Sir2. B) Only one sumoylated band appears in double mutant in esc1Δ. Lane 2 shows two sumoylated bands in WT Sir2, but lane 3 to 5 which are the double mutants of Sir2 shows a single sumoylated band.*

4.3. Discussion

Our study shows that there are three sumoylatable sites in Sir2 and mutating one or two sites does not affect Sir2 sumoylation but there is a complete loss of Sir2 sumoylation in the triple mutant. This shows that no additional site can be subjected to sumoylation. It is not possible to say at this juncture if all sites get sumoylated in vivo or there is one preferred site. It is possible only in the absence of the preferred site other sites get sumoylated. It has been shown earlier that when the preferred site is mutated, available nearby sites are sumoylated (Bielska et al, 2012; Figueroa-Romero et al, 2009; Maison et al, 2011). However what is clear is all three sites can get sumoylated which was clearly seen in the single mutants in *esc1*Δ cells where the two available sites get sumoylated.

Pull down with Ni-NTA shows that *esc1* deletion leads to the hyper sumoylation of Sir2 and its sumoylated fraction further increases in Siz2 overexpression background. The results of these experiments also show that upon Siz2 overexpression, Sir2 gets multi-sumoylated and not polysumoylated (i.e., additional SUMO moieties conjugated to the first SUMO which is conjugated to the target protein creating a SUMO chain). If the additional band we see in *esc1*Δ is due to polysumoylation, then in the double mutant we should continue to see the second slow moving band corresponding to two SUMO moieties on Sir2. Since we see only one sumoylated band in the double mutants, the slow moving band corresponding to two SUMO moieties is sumoylation at two different lysines.

Chapter 5

Functional characterization of Sir2 sumoylation

5.1. Introduction

As mentioned earlier Sir2 is important for heterochromatin establishment at telomere, *HM* and *rDNA* loci and deletion of Sir2 leads to complete loss of telomeric and *rDNA* silencing (Cockell et al, 2000; Gartenberg, 2000). Our results firmly establish that Sir2 is sumoylated. Therefore we investigated the functional consequence of Sir2 sumoylation. To study the role of Sir2 sumoylation in TPE, *rDNA* and *HM* silencing we used all the combination of point mutants which we created already in functional assays. TPE was originally investigated by an engineered telomere VII-L which was truncated by its terminal ~15 kb by insertion of a *URA3* reporter cassette. Truncated telomeres have been quite helpful for checking the genetic requirements for TPE. For our assay we created *sir2*Δ and *sir2*Δ*esc1*Δ with Tel VII-L::*URA3* and transformed all forms of Sir2 plasmid with and without additional copies of *SIZ2* to study the TPE. Since Sir2 is important for *HM* and *rDNA* silencing also, we followed the same procedure and created *sir2*Δ and *sir2*Δ*esc1*Δ with *HMR*Δ*E::TRP1* and *sir2*Δ with *rDNA::URA3*. In normal physiological conditions *TRP1* cassette inserted in the *HM* locus will be silent and the cells will not grow in tryptophan depleted plate. To check silencing at telomere and *rDNA* we used plates with 5-FOA. If the cells lose silencing, it will express

URA-3 gene, the gene product will convert 5-FOA to toxic substance 5' fluorouridine monophosphate, severely limiting growth of the cell (Boeke et al, 1987).

5.2. Results

5.2.1. Sir2-215 is not affected by Siz2 O/E

Having demonstrated that Sir2 is sumoylated at three sites by Siz2, we next studied the functional consequences of these sumoylations.To study the TPE with all the point mutants of Sir2 we created *sir2*Δ*Tel-VII L-URA3* and plasmids encoding WT or mutant Sir2 were transformed individually along with control vector or plasmid encoding *SIZ2*. After checking the expression by western blot it was spotted in 5-FOA plate with 10-fold serial dilution. We observed that Sir2 and all the single and double point mutants of Sir2 can efficiently establish TPE in wild type strain background in vector control i.e., without Siz2 (fig.15). This indicates that the mutation per se has no effect on Sir2 function and behaves like wild type. We used SIR2-H364A as control which has lost its deacetylase activity and can't establish TPE (Armstrong et al, 2002). Interestingly, we found that the triple mutant was not functional in TPE, suggesting that changing all three lysines to arginine in the same molecule compromised Sir2 function independent of *SIZ2* overexpression or *esc1* deletion.

After confirming that all the point mutants could establish TPE in WT background, we went ahead to study TPE in *esc1*Δ background. As mentioned in earlier report, in *esc1*Δ, upon overexpression of SIZ2 the WT Sir2 can't establish silencing at telomere (Pasupala et al, 2012). But interestingly, we find that Sir2-

K215R is not affected by SIZ2 overexpression (fig.16, panel A, row 10) and continues to establish strong silencing, even when both K106R and K132R are indistinguishable from wild type Sir2 and show exacerbated loss in silencing under SIZ2 overexpression conditions. We further confirmed our results by quantifying the effect by enumerating the colonies that grew on 5-FOA plate and could replicate the result of spotting assay (fig. 16, panel B). Thsese observations indicate that K215 sumoylation has a key role in Sir2 function.

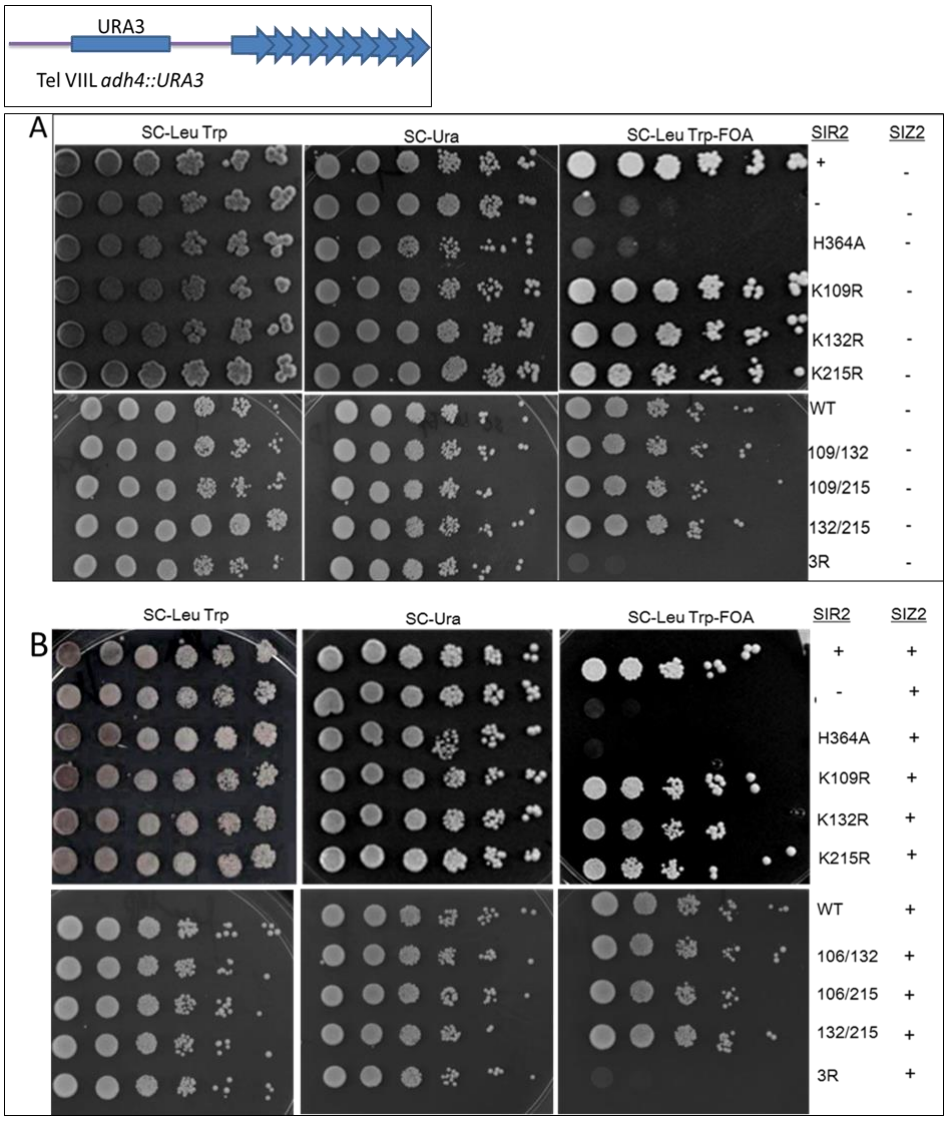

Figure 15: TPE is not affected in single or double point mutants. *A) All the indicated single and double point mutants of Sir2 were transformed into KRY 790.*

Vectors and catalytic mutant (row 2, 3) that cannot establish TPE lack growth on FOA plates; all other point mutants grow on 5-FOA. Triple mutant is unable to establish TPE in WT background (row 11). B) Point mutants of Sir2 co-transformed with plasmid encoding SIZ2 in WT. All the point mutants of Sir2 except H364A and triple mutant could establish TPE in the presence of SIZ2 in WT background.

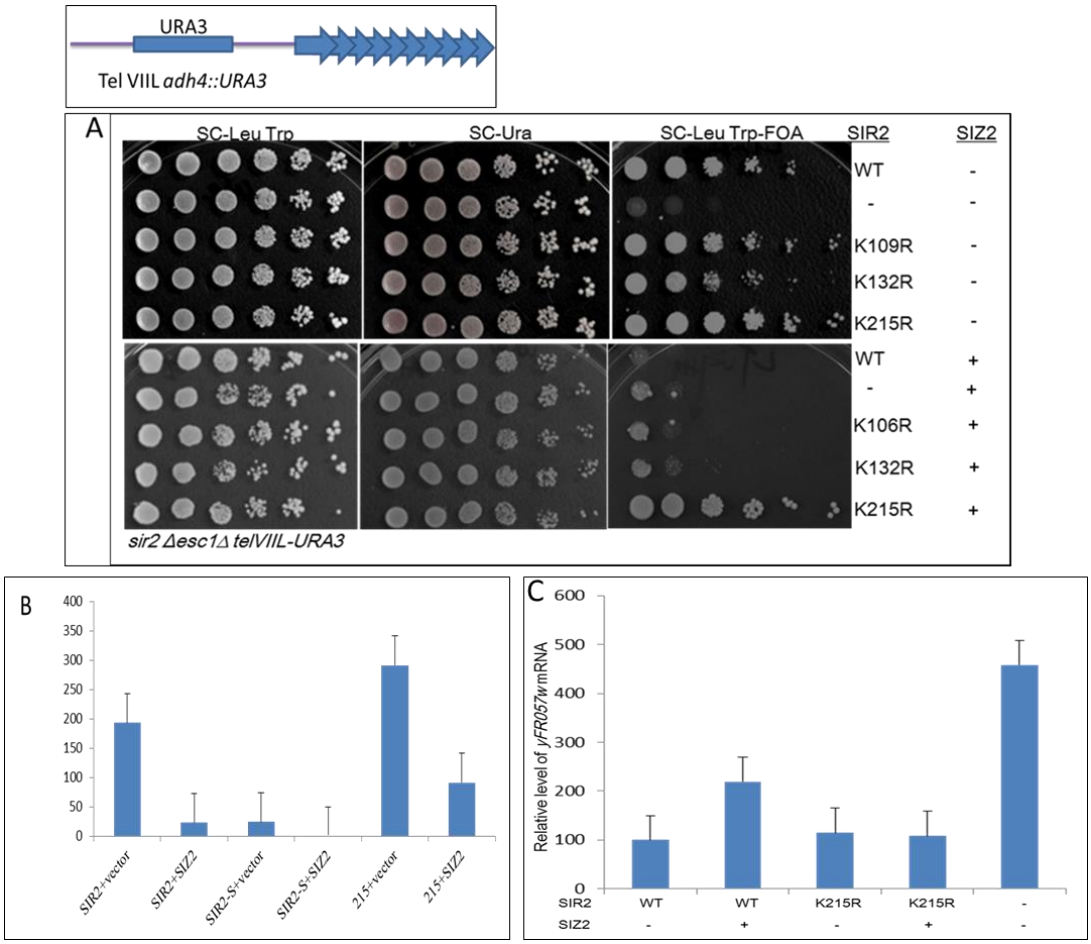

Figure 16: K215R is not affected by Siz2 O/E. *Panel A) Sir2 K215R is refractory to exacerbation of silencing defect caused by elevated Siz2 dosage in esc1Δ. KRY 788 was transformed with either wild type or indicated plasmids along with either empty vector or SIZ. Wild type and point mutants grow on 5-FOA plate in the absence of SIZ2 (row 1, 3, 4, 5). Upon elevating SIZ2 dosage, WT, K106R and K132R are unable to establish TPE but K215R is able to. B) quantification of silencing in strains shown in panel A confirms that there are more FOA resistant colonies in Sir2-215R compared to WT Sir2 in the presence of Siz2. C) Quantitative RT- PCR shows that K215R is not affected by Siz2 O/E. cDNA from esc1Δ strain transformed with indicated plasmids were made and quantitative RT-PCR was performed for*

yFR057W and normlaized against endogenous actin expression. Relative enrichment is plotted for comparison. sir2Δ shows maximum de-repression. K215R is unaffected by Siz2 O/E whereas WT is de-repressed.

To further confirm that the growth phenotypes noticed on 5-FOA plates were a real reflection of silencing, we also measured the expression of the endogenous locus, *YFR057w,* known to be silenced in a Sir protein dependent manner and was sensitive to elevated doses of *SIZ2* (Pasupala et al, 2012). Expression of the endogenous locus *YFR057w* was checked by quantitative RT-PCR. It was also refractive to loss of silencing induced by *SIZ2* when transformed with Sir2-K215R (Fig.16, panel C) as *YFR057w* expression is not increased in K215R whereas *YFR057w* expression is almost five fold higher in *sir2*Δ background. These results strongly suggest that K215R is the critical residue that influences Siz2-dependent changes in telomere position effect and in its absence; *SIZ2* overexpression has no effect on silencing.

In our earlier studies, we had observed that loss of silencing at the telomeres was accompanied by concomitant loss of Sir2 association with telomeres. We therefore tested the abundance of Sir2 and Sir2-215 under these conditions. We designed three sets of primers from the end of telomere at 0.5kb, 1.5kb and 2.5kb. Chromatin immunoprecipitation experiments were performed using Sir2 antibody in *esc1*Δ*sir2*Δ strains expressing either Sir2 or Sir2-215 alongwith vector or plasmid encoding Siz2. The enrichment of the indicated loci was quantified by ΔΔCt method using *SPS2* as internal control. We found that upon Siz2 overexpression, as expected, a 50% reduction of Sir2 occupancy at the telomere occurred. However Sir2-215 did not show any reduction from telomere (fig.17). This observation correlates with the silencing data and suggests that Sir2-215 sumoylation is

important for removal of Sir2 from telomere and if we can block the sumoylation at 215, Sir2 is not affected by Siz2 over expression.

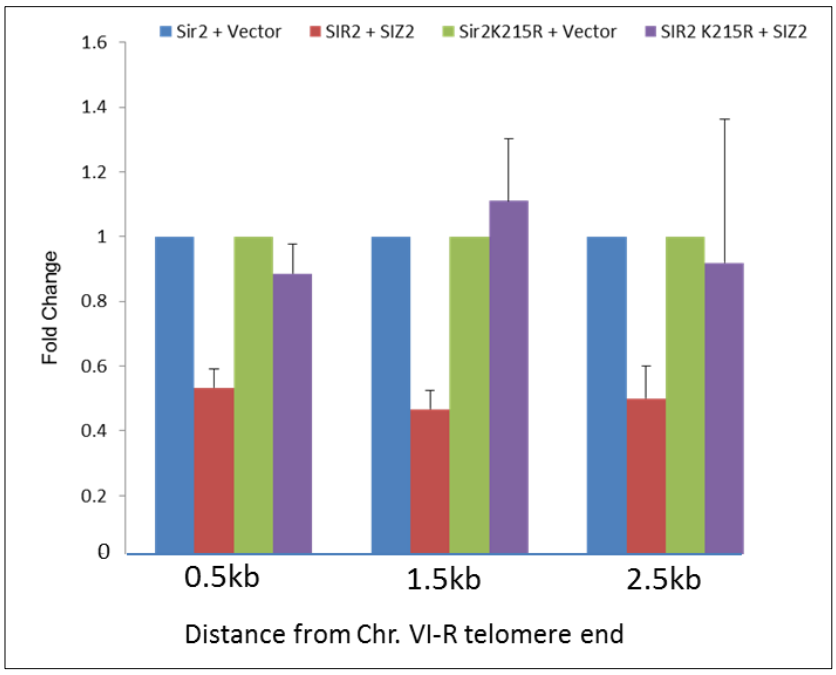

Figure 17: ChIP shows Siz2 O/E cannot reduce K215R binding at telomere. *Chromatin immunoprecipitation using antibodies to Sir2 in indicated strain combinations and quantification by real-time PCR of the telomeric loci enriched. Siz2 overexpression does not result in the reduction of Sir2-215 at telomere at 0.5kb, 1.5kb and 2.5kb region.*

We next tested the double and triple mutants for silencing at telomeres and found that, replacing lysine at 215 with arginine protected cells from loss of silencing. The double mutant K106/132R, where the only possible sumoylatable site K215 is left, was similar to WT Sir2 and lost silencing in the presence of elevated *SIZ2* doses in *esc1*Δ. But the two double mutants (K132/215R and K106/215R) containing a K215R replacement resisted the exacerbation in silencing defect induced by Siz2 (fig. 18). Together these results confirm that lysine 215 is the key residue in Sir2 that is affected by elevated *SIZ2* doses in *esc1*Δ.

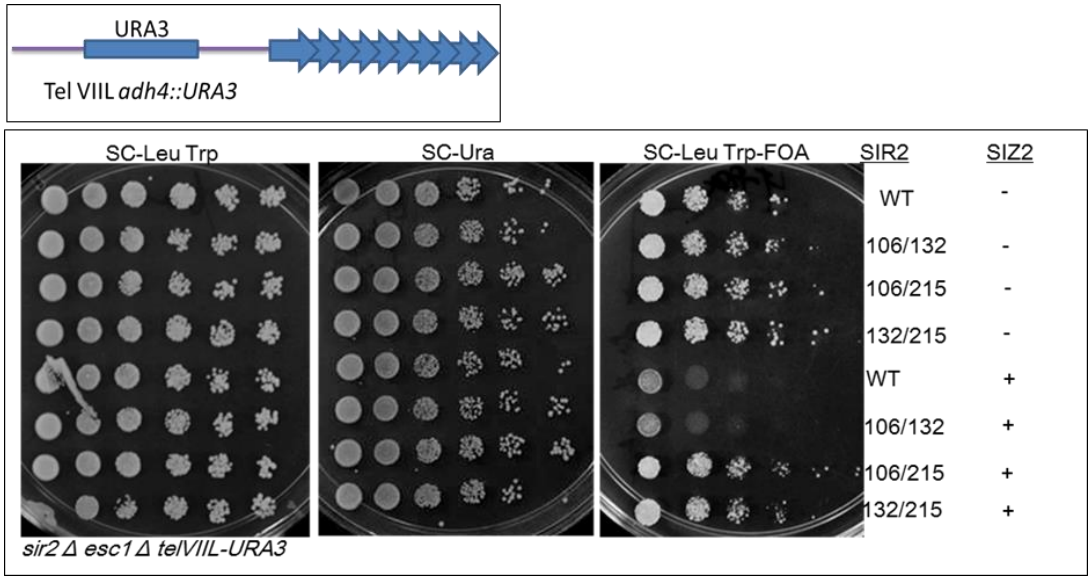

Figure 18: Sir2 K215R confers protection from loss of TPE caused by elevated Siz2 dosage in esc1Δ. *KRY788 was transformed with either wild type or indicated double mutant plasmids along with either empty vector or SIZ2. Wild type and point mutants grow on 5-FOA plate in the absence of SIZ2 (row 1-4). Upon elevating SIZ2 dosage, WT and K106/132R are unable to establish TPE but 106/215R and 132/215R are able to.*

5.2.2. Sir2-SUMO fusion is less effective at TPE

To further test the consequences of sumoylation on Sir2 function, we made a recombinant Sir2 with SUMO fused to the C-terminus similar to the Yku80-SUMO reported (Ferreira et al, 2011). We first confirmed the robust expression of this fusion protein (Fig. 19); we found that a fraction of this SUMO fusion is also further sumoylated. We tested its effect on telomere position effect. We found that this fusion protein was partially compromised for establishing silencing at telomeres under unperturbed conditions (fig. 20, panel A, row 3) even though a similar fusion with a GFP molecule was fully functional and behaves similar to WT Sir2 (fig 20, panel B). This effect was further exacerbated by increasing the dosage of *SIZ2* (fig 20, panel A, row 6), probably, by further sumoylating the consensus lysine residues.

Interestingly, in the *esc1*Δ strain, unlike wild type Sir2, Sir2-S was even less effective in silencing (Figure 20, panel A, row 9). As expected both wild type and Sir2-S were completely ineffective in establishing silencing when *SIZ2* dosage was elevated in an *esc1*Δ mutant (Figure 20, panel A, row 12). Together these observations suggest that SUMO fusion reduces the functionality of Sir2 at telomeres.

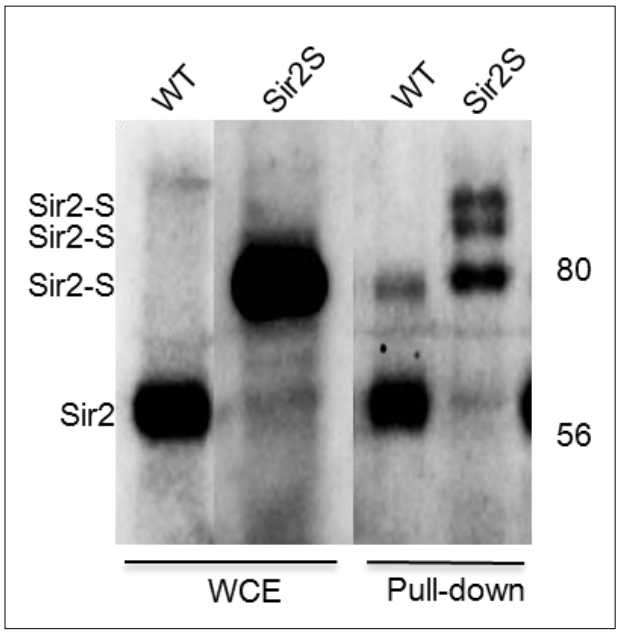

Figure 19: Sir2-S fusion is expressed stably. *Sir2-S was transformed into sir2*Δ*8X-His-SMT3 (KRY 790). Whole cell extracts (lanes 1, 2) and Ni-NTA pull down from WT and Sir2-S (lanes 3, 4) expressing cells are shown. Sir2-S is further sumoylated (lane 4). Lane 1 and 2 are whole cell extract. Lane 3 is the Ni-NTA pull down of 8X-His-SMT3 with WT Sir2, lane4 is the Ni-NTA pull down of 8X-His-SMT3 with Sir2-S.*

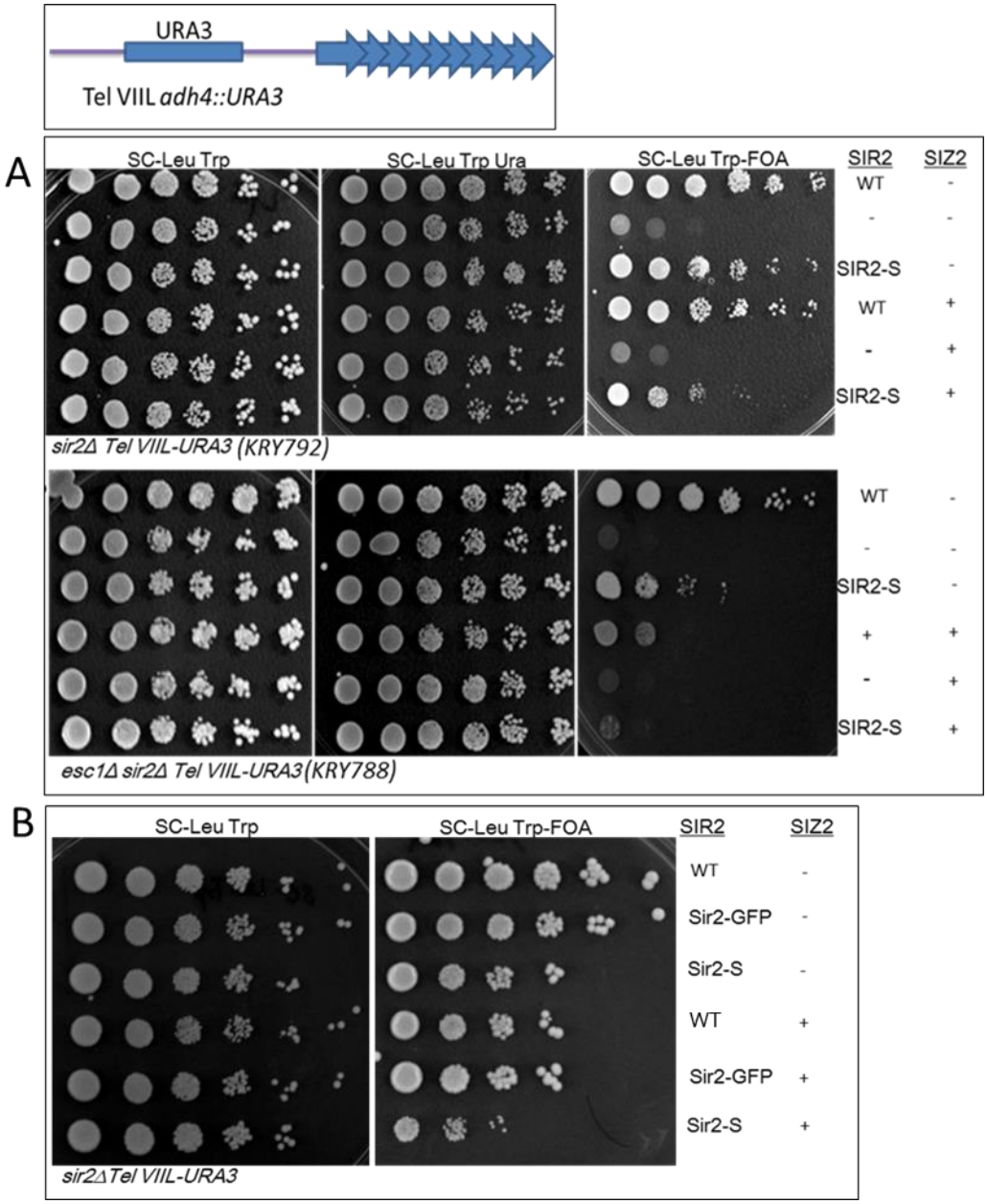

Figure 20: Sir2-S fusion is defective in TPE. *A) KRY792 and KRY788 strains were tested for TPE with Sir2-S with and without elevated doses of SIZ2. Sir2-S results in the reduction of TPE even in WT (KRY792) background (compare row 1 to row 3) and further reduction upon additional copies of SIZ2 (row 6). In esc1Δ (KRY788) Sir2-S has reduced TPE even without additional SIZ2 (row 9) and is completely defective in TPE with elevated SIZ2 dosage (row 12). B) Sir2-GFP does not affect TPE while Sir2-S shows loss in TPE. Row 1 to 3 showing TPE in vector control. Row 4-6 showing TPE at Siz2 overexpression. Sir2-GFP is capable of establishing TPE (lane 5), Sir2-S in not capable of establishing TPE upon Siz2 O/E.*

Since Sir2-S is less effective in TPE we decided to study TPE by K215R-Sir2-S. As observed when lysine 215 is replaced with arginine in Sir2-S it can establish silencing equivalent to wild type Sir2 in unperturbed cells and retains significant levels of silencing even in the overexpression of SIZ2 (fig 21, row 5). This again shows the importance of K215 in TPE.

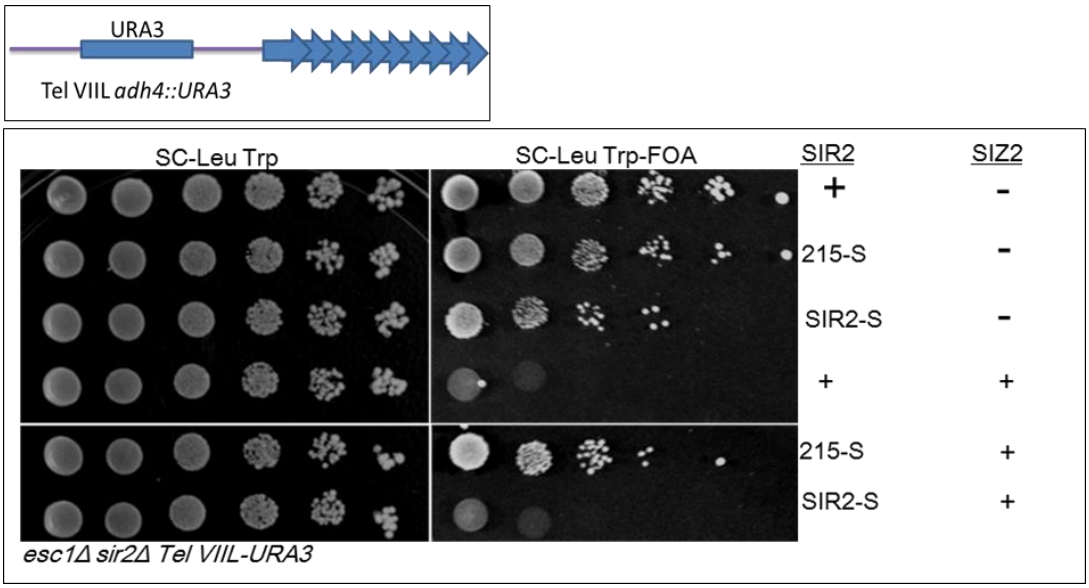

Figure 21: TPE can be established in Sir2-S by introducing K215R. *Row 1and 2 showing complete TPE by WT Sir2 and K215R, row 3 showing 50% reduction of TPE without Siz2, row 4 showing complete TPE loss when Siz2 is overexpressed. Row 5 showing TPE when K215R in introduced at Sir2-S.*

5.2.3. All the point mutants and Sir2-S could establish rDNA silencing

Sumoylated Sir2 was compromised for telomere position effect and this could be due to a reduced functionality of Sir2 induced by the fusion. To test this we performed *rDNA* silencing assays. Sir2 is required for silencing RNA polymerase II genes placed in the *rDNA* locus. This function of Sir2 is not altered when *SIZ2* dosage is elevated either in wild type or when *esc1* is deleted (Pasupala et al, 2012).

Therefore, we tested the Sir2-S in *rDNA* silencing. Simultaneously, we also tested the ability of the other point mutants generated to silence *rDNA.* These plasmids were introduced into a *sir2*Δ strain carrying *URA*3 at the *rDNA* locus (KRY 360). We then tested for growth on plates containing 5-FOA. All Sir2 constructs, including the point mutants and Sir2-S, were competent to establish silencing at the *rDNA* locus (Fig.22). This observation suggests that some specific property of Sir2, essential for telomeric silencing, was affected upon addition of SUMO, leading to reduced silencing at telomeres.

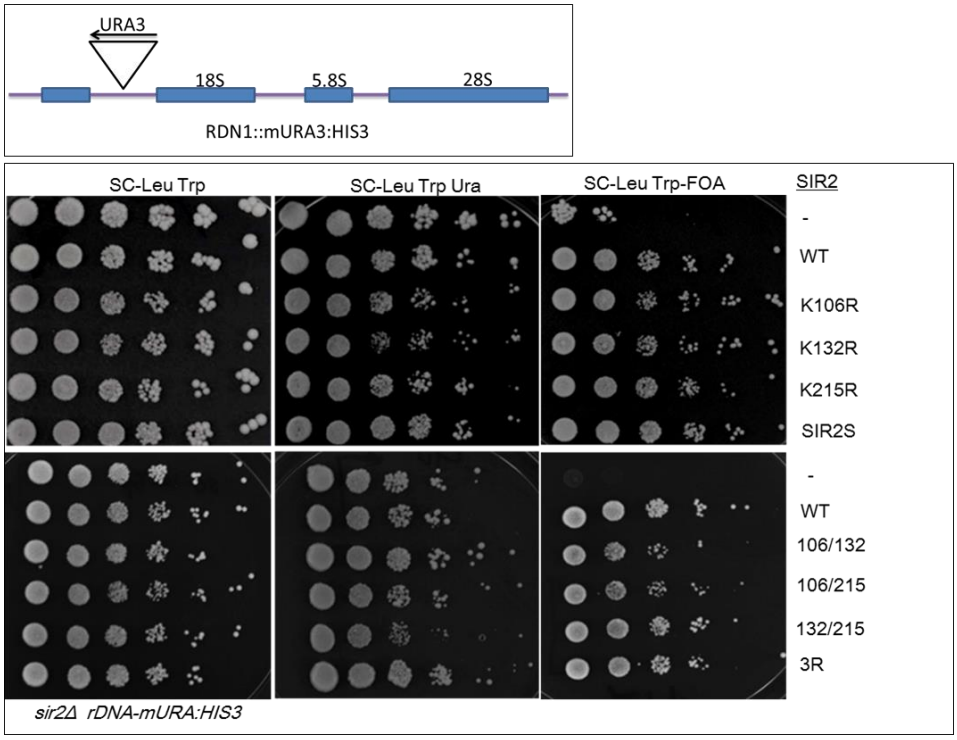

Figure 22: Sir2 mutants are functional in rDNA silencing. *KRY360 was transformed with either vector or Sir2 wild type or Sir2 mutant plasmids and tested for growth on 5-FOA to measure silencing of rDNA locus. Wild type Sir2 and all point mutants of Sir2 were able to restore silencing at the rDNA, allowing growth on 5-FOA. In the absence of Sir2 (row 1), cells could not grow on 5-FOA indicating requirement for Sir2.*

5.2.4. Effect of point mutants on HM silencing

Since Sir2 is required for *HM* silencing we went ahead to study the *HM* silencing with the point mutants of Sir2. For this study we created *sir2*Δ*hmr*Δ*aEB::TRP1* and all the modified forms of Sir2 were then transformed individually and silencing at *HMR* locus was studied. Observation shows that all the single Sir2 point mutants can establish silencing like WT Sir2 in wild type background with or without Siz2 overexpression (fig. 23). Similar to WT background, in *esc1*Δ background all the point mutants can establish silencing at *HM* locus when S*IZ2* is not overexpressed (fig. 24, Panel A, B). But unlike TPE, where only K215R could establish silencing upon *SIZ2* overexpression, we have found that all the single mutant of Sir2 and even Sir2-S could establish silencing in *HM* locus (fig 24, Panel A, B) even after *SIZ2* overexpression. Interestingly just like TPE, Sir2 triple mutant is unable to establish *HM* silencing (fig 24, Panel C) though it could establish *rDNA* silencing. The double mutants were also similar to single mutants and could establish silencing except for K106/132R, which is unable to establish *HM* silencing with or without Siz2 overexpression and further studies to be done to understand this phenotype.

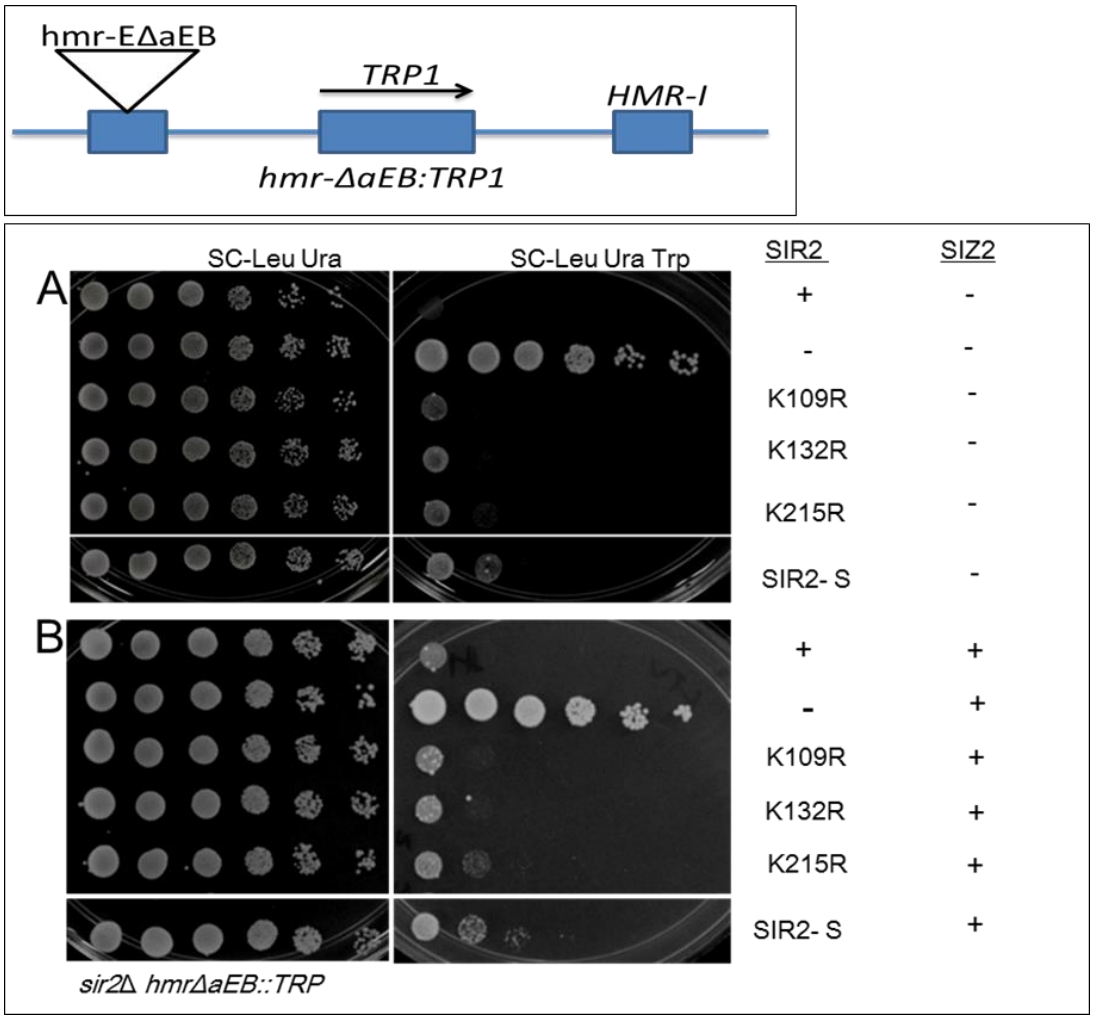

Figure 23: All the single point mutants of Sir2 could establish HM silencing in WT background. *Panel A) sir2Δ hmrΔaEB::TRP1 was transformed with the indicated plasmids and growth on Sc and Sc-Trp was tested to measure silencing at the HMR locus. All the point mutants are able to silence HMR locus (row 3-6) whereas Sir2 negative strain (row 2) was unable to establish HM silencing. Panel B) when with SIZ2 was overexpressed it could not lead to the loss of HM silencing in wild type background.*

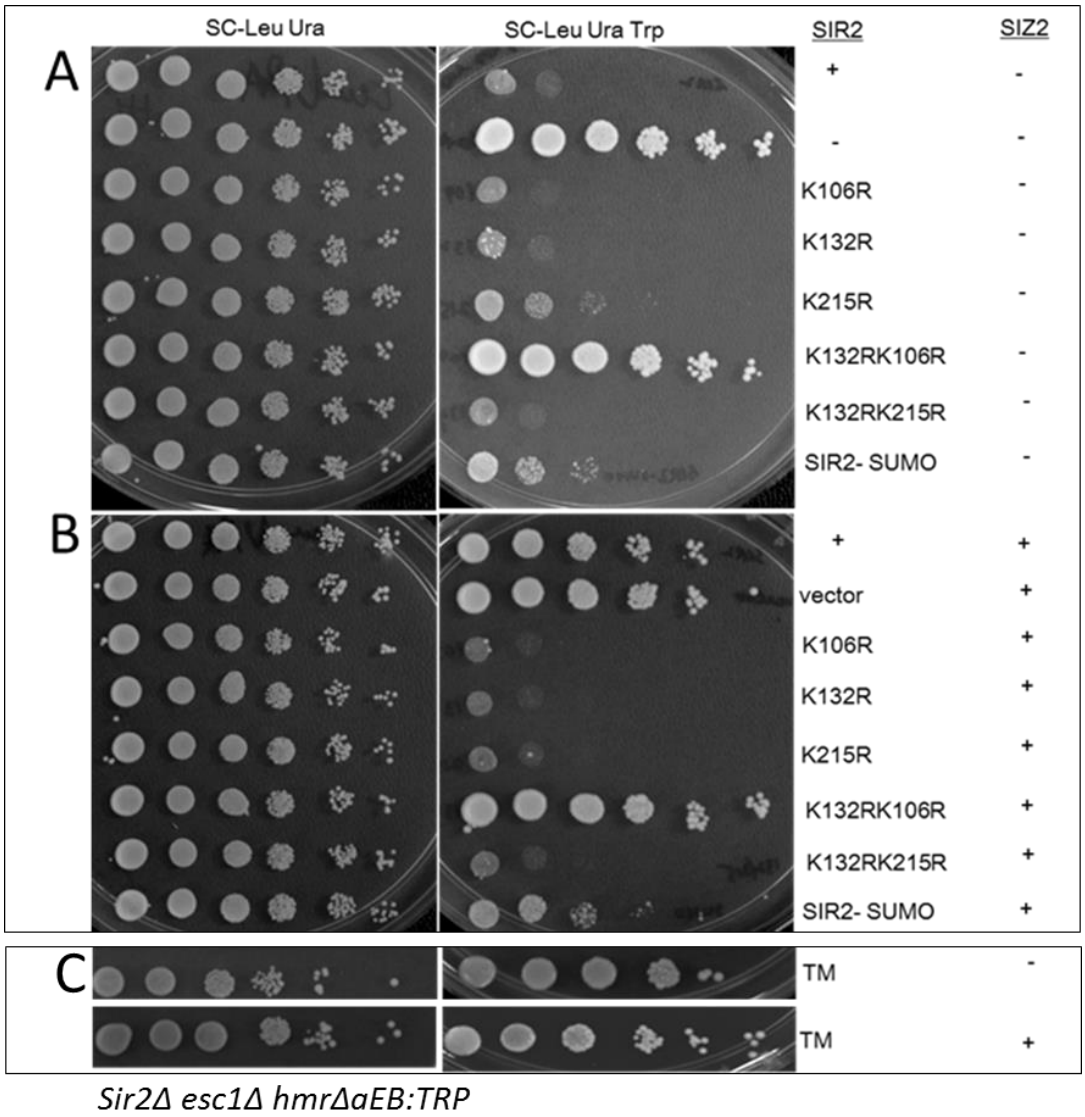

Figure 24: In esc1Δ background all the point mutants can establish HM silencing even in SIZ2 overexpression unlike WT Sir2. *Panel A) All the point mutants could establish silencing except 106/132. Panel B) Point mutants are able to establish silencing upon SIZ2 overexpression in esc1Δ (row 3, 4, 5) but WT Sir2 could not (row 1) but Sir2-S results in only very slight reduction of HM silencing. Panel C) triple mutants are not able to establish HM silencing.*

5.3. Discussion

In the work presented above we studied the effect of the non-sumoylatable mutants of Sir2 in gene silencing assays. When sumoylation of K215 is blocked by point

mutation, we observed that *SIZ2* overexpression can't affect TPE. This was observed both in the single, double mutant and SUMO fusion context, strongly suggesting that K215R was the key residue that responds to Siz2. Changing all other sumoylatable lysines had no effect on silencing defects induced by Siz2 and these mutants were indistinguishable from wild type. The triple mutant is non-functional in both telomeric and *HM* silencing although was functional in *rDNA* silencing suggesting partial loss of function. Since this loss is observed in unperturbed wild type cells, it is likely to be an innate defect in the mutant and is unlikely to be related to sumoylation.

Interestingly, the results with *HM* results are different from telomeric silencing. It appears that simply removing one sumoylatable site is sufficient to resist Siz2 effects at the *HMR*. This implies that *HM* silencing, being more robust, can resist Siz2 effects if the fraction of sumoylatable sites and hence possibly fraction of sumoylated Sir2, is reduced. In addition, the double mutant K106, 132R was, like the triple mutant, defective in silencing even in unperturbed WT cells. Although it is difficult to ascertain why this defect is observed, we propose two possible explanations. One, as the only available sumoylatable site is K215, even a small fraction of K215 sumoylated Sir2 present in unperturbed cells may render HMR silencing ineffective. This can be tested in a *siz2*Δ. Another possibility could be the unspecified defect that leads to silencing deficiency in the triple mutant maybe similar, although not so severe, in this particular double mutant. This defect may be sufficient to disrupt *HMR* silencing but not TPE. Even though HM and telomeric silencing have very similar requirements, differences also exist. For eg, the dependence of telomeric silencing on Esc1 and Yku70/80 is not observed in *HM*

silencing; *HM* silencing requires Sir1 whereas telomeric silencing does not. It is possible that the Sir2 mutations indirectly perturb one of these specific requirements.

We directly tested the effect of sumoylation of Sir2 by generating a recombinant Sir2 covalently fused to uncleavable SUMO. We infact observed that Sir2-S fusion does not efficiently establish TPE in *esc1*Δ background. There is almost 50% reduction in TPE in *esc1*Δ background even without the overexpression of *SIZ2*. This effect is further enhanced when SIZ2 was overexpressed. All these observations indicate that sumoylation of Sir2 has negative effect in TPE. But interestingly when we introdced K215R in Sir2-S it could efficiently nullify the overexpression phenotype of *SIZ2*. This again indicates that K215 sumoylation plays crucial role in TPE. This also raises the interesting possibility, that addition of SUMO reduces functionality at telomeres and this is especially severe when the K215 residue is affected. The fact that Sir2-S is defective in silencing and becomes non-functional in *esc1*Δ reinforces that SUMO itself is deterimental to Sir2 function at the telomeres but whether it is all that population or the small fraction that further gets sumoylated at K215R that is particularly potent is not clear. The property of K215R in Sir2-S would support the second possibility.

When we checked the *rDNA* silencing with the point mutants and with Sir2-S we observed that all the point mutants could establish *rDNA* silencing. This suggests that none of the changes introduced, including triple mutant and Sir-S fusion, affect the core catalytic function of Sir2, which is also required for establishing silencing at the *rDNA*. In conclusion, K215 is the critical sumoylation site in Sir2 that responds to Siz2 and this effect is specific to telomere positon effect and does not affect *rDNA* silencing.

Chapter 6

Molecular mechanistic basis of regulation of gene silencing by Sir2 sumoylation

6.1. Introduction

Sir complex assembly is required to establish telomeric heterochromatin. In yeast *S.cerevisiae* SIR complex at telomers consist of Sir1, Sir2, Sir3 and Sir4. Deletion of Sir2-Sir4 abolishes the TPE. Here, the first step is the recruitment of Sir4 to the telomere by Rap1. Then telomeric Sir4 attracts Sir2, which deacetylates H4K16 on the nucleosomes. Deacetylated H4K16 attracts Sir3 to the telomere and this cycle is repeated many times to form the heterochromatin structure. In our study we have observed sumoylated Sir2 has weak TPE. To establish TPE, recruitment of Sir2 at telomere by Sir4 is necessary. For *rDNA* silencing interaction of Sir2 with other proteins of RENT complex is important. The RENT complex consists of Net1p, Cdc14p (Moazed et al, 2004). Sir2 is recruited at *rDNA* by Net1. Our results show that all the point mutants of Sir2 and Sir2-S could efficiently establish *rDNA* silencing but some of them were defective in telomeric silencing. As one of the key difference between telomeric silencing and *rDNA* silencing is the different recruiting partners for Sir2, we asked if sumoylation influenced Sir2 interaction with Sir4 and Net1.

6.2. Results

6.2.1. Sir2-SUMO does not interact with Sir4 but interacts with Net1

To do these experiments we generated *sir2*Δ in strains carrying a Myc epitope tagged Sir4 or Net1. After introducing different Sir2 plasmids, namely wild type or K215R or Sir2-S, protein extracts were made and immunoprecipitation(Moazed et al, 1997) was done with anti Myc antibody. After the IP of Myc tagged Sir4, western blot was performed by anti-Sir2 and anti-Myc antibodies. When immunoprecipitaion was done in extracts from strains expressing Sir4-myc, we found that Sir2 was co-immunoprecipited as expected. However, in case of Sir2-S, we found only the lower molecular weight form, that is the non-sumoylated form, appearing (fig 25, panel A, lane 6). This is particularly significant because the sole Sir2 in these cells is the Sir2-S. It appears that the Sir2-S undergoes some cleavage (fig. 10B, lane 12) and the cleaved, free Sir2 is able to interact with Sir4. This cleavage is also evident in our western blots from whole cell extracts that show the presence of some cleaved Sir2 in the Sir2-S lanes. This is probably why we observe some silencing even in Sir2-S strains. But it is notable that the unbound fraction of the extract always contained the sumoylated form of Sir2 (fig 25, panel A, lane 9) ruling out the possibility that somehow all the Sir2-S was cleaved in these extracts. Similar to Sir4, to study Net1-Sir2 interaction, we used Myc tagged Net1. Net1-myc, on the other hand, was able to pull down Sir2-S efficiently from the strain where Sir2 is permanently tagged with SUMO (Fig 25, panel B). In contrast to Sir4 which could not interact with Sir2-S, Net1 was able to stably interact with Sir2-S. Taken together, these results provide

strong evidence that Sir4 interacts with the non-sumoylated Sir2 population and Net1 can interact with both free and sumoylated Sir2 (Sir2-S).

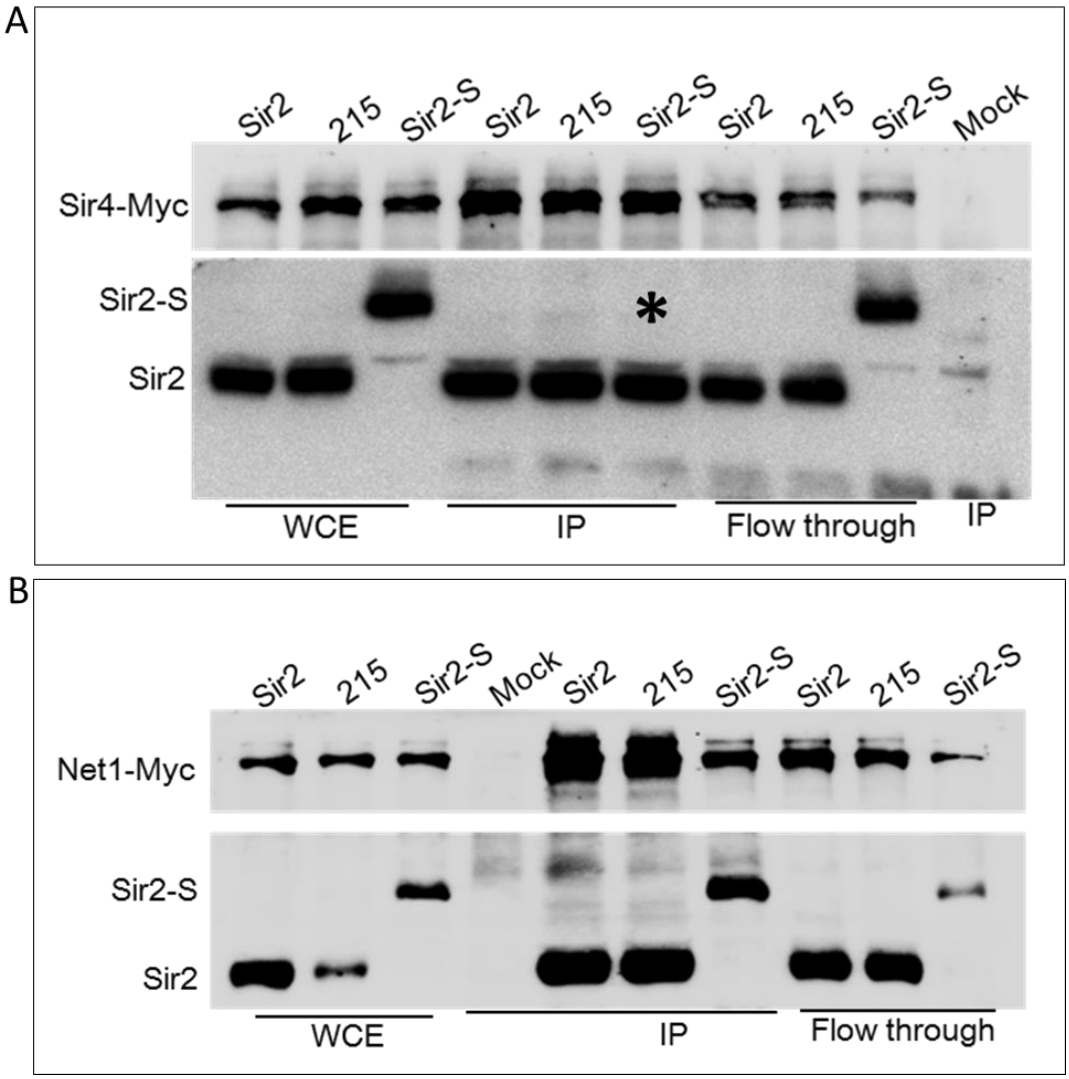

Figure 25: Interaction of Sir2 with other proteins involved in heterochromatin. *A) Sir4 does not interact with Sir2-S. KRY868 was transformed with indicated plasmids and immunoprecipitation was done using anti-myc antibodies. The immunoprecipitates were immunoblotted with Myc antibody to detect Sir4 and anti Sir2 antibody to detect Sir2. In all cases the unmodified Sir2 is co-immunoprecipitated with the Sir4-myc (lane 4, 5, 6). Even in lane 6 where only sumoylated Sir2 was present only unmodified Sir2 was co-immunoprecipitated. Lane 7, 8 and 9 shows the presence of Sir4 and Sir2 in the flowthrough. (*) shows the missing Sir2-S B) Net1 interacts with both Sir2 and Sir2-S. Total protein extracts*

from KRY875 containing indicated plasmids were immunoprecipitated with anti-myc antibodies and immmunoblots were developed with anti myc and Sir2 antibodies. Sir2 was co-precipitated in all cases and in the strain with sumoylated Sir2, sumoylated Sir2 was co-precipitated. Lanes 1 to 3 shows WCE, lane 4-7 shows IP, Lane 8-10 is showing flowthrough.

6.2.2. Yeast two hybrid shows differential interaction of Sir2-S with Net1 and Sir4

To obtain independent confirmation of the interaction data, we performed yeast 2-hybrid assays. For this assay we cloned Sir2, Sir2-215 and Sir2-S in pGBD-UC1 as in-frame fusion with the GBD, which will act as bait and Sir4 in pGAD-C1 plasmid, which will act as prey. pGAD-Sir4 was transformed in pJ694a strain with all the forms of Sir2 plasmid individually and checked for their growth in histidine and adenine dropout plates. In this strain when the prey and bait interact, transcription of *ADE2* and *HIS3 is* turned on. Correlating with our IP data, we found that in fact Sir2-S does not interact with Sir4 and hence does not grow on histidine and adenine dropout plates whereas WT-Sir2 and Sir2-K215R grew comfortably (fig. 26, panel A). To test for interaction with Net1, we cloned Net1 in pGAD which acted as prey and transformed with various Gbd-Sir2 combinations. All three Sir2 products including Sir2-S interacted with Net1, seen as robust growth on His and Ade selection plates (Figure 26, panel B). Together these observations establish that Sir2-S cannot interact with Sir4, but could interact with Net1.

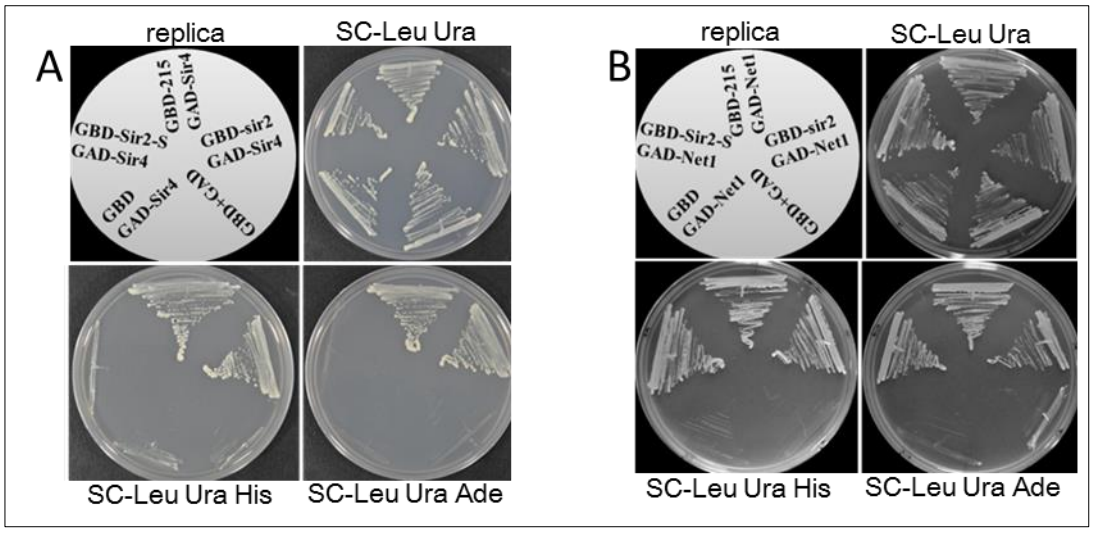

Figure 26: Sir2-S does not interact with Sir4 but interacts with Net1. *A) The first plate in the panel showing the orientation of strains streaked on the plate. Growth on the SC-Leu Ura His and SC-Leu Ura Ade shows interacting partner. B) Sir2 and Sir2-K215R and Sir2-S can interact with Net1.*

6.2.3. Sir2-S is predominantly localized to the nucleolus

In WT cells, Sir2 is found in 4-5 distinct telomeric foci and one nucleolar focus. But when Sir2 recruitment is defective, for eg., in Sir4 mutants, Sir2 shows predominat nucleolar localization (Cuperus et al, 2000). We therefore tested if localization was affected in Sir2-S. We performed immunofluorescence experiments in *sir2*Δ strains carrying either WT or Sir2-S. We used Nsp1 as a nuclear envelope marker, Sir4 as telomeric marker and Nop1 as nucleolar marker and antibodies to Sir2 to detect Sir2. All signals fall within the boundary of the nucleus suggesting that nuclear import of Sir2 is not affected upon SUMO modification (panel B). WT Sir2 co-localized with both Nop1 (fig.27,panel A) and Sir4 (fig.27, panel C) as few telomeric signals (fig.27, Panel A and B) but Sir2-S solely co-localized with Nop1 (fig.27, Panel A). This observation further strengthened our hypothesis that upon sumoylation, Sir2

migrates into the nucleolus which results in the reduction of TPE. Therefore *SIZ2* overexpression leads to the loss of TPE but *rDNA* silencing does not get affected.

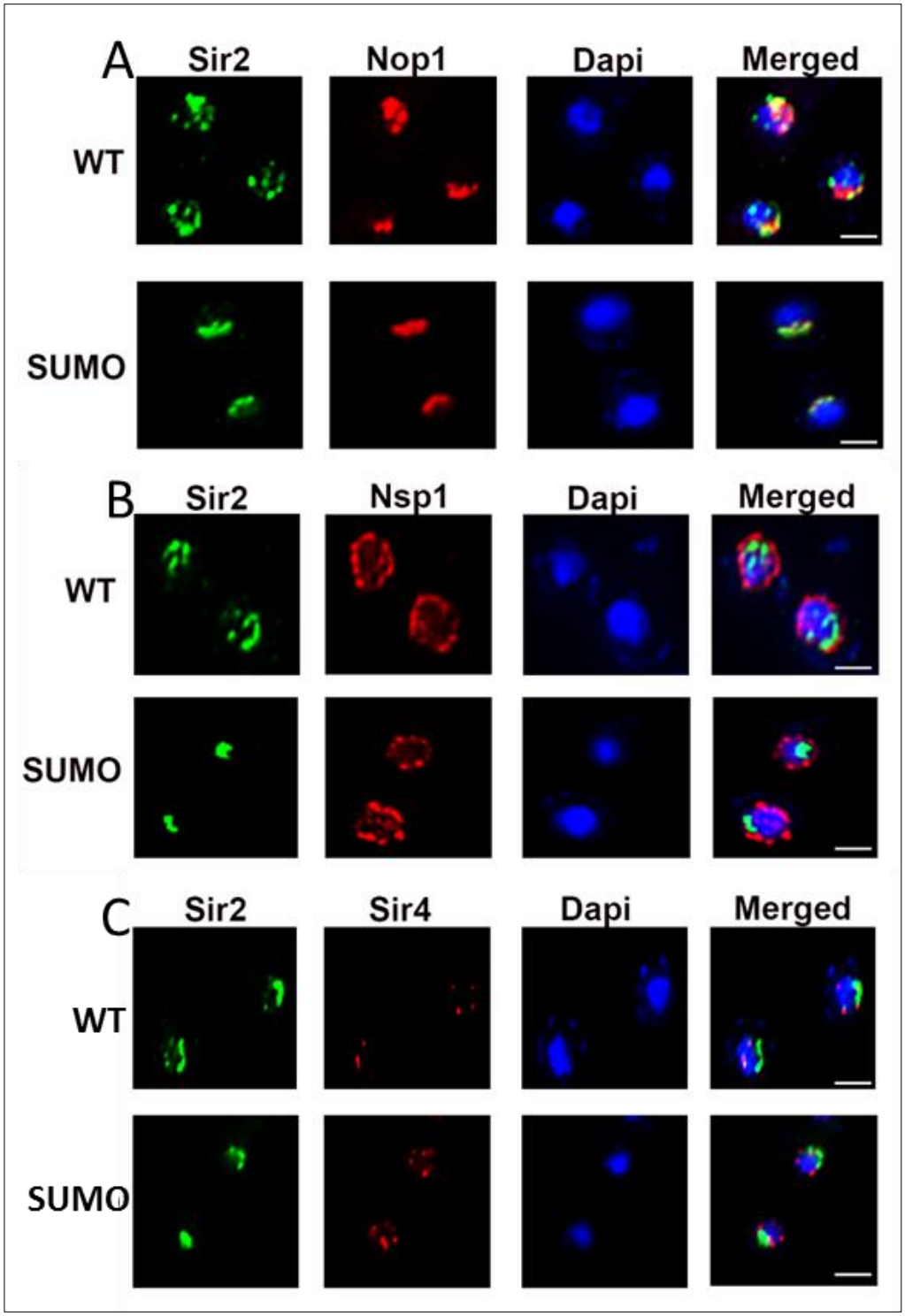

Figure 27: Sir2-S localized at nucleolus. *Immunofluorescence of with Sir2 antibodies (red) in conjunction with A) Nop1 (green) nucleolar marker. B) Nsp1*

(green) nuclear pore complex, C) Sir4 (green) telomeres. In all cases DAPI in blue indicates DNA. Sir2 (WT) appears as a prominent nucleolar spot (co-localizing with Nop1) and few telomeric spot (co-localizing with Sir4). Sir2-S shows predominantly nucleolar staining with almost no telomeric spot.

6.2.4. Sir2-S increases rDNA stability

Our immunofluorescence results indicate that Sir2-S migrates to the nucleolus which is occupied by *rDNA.* The *rDNA* locus on chromosome XII encodes 100-200copies of a 9.1kb repeat. At any given time almost half of the *rDNA* are maintained in their silent state. Sir2 localizes to the nucleolus and interacts with Net1 and Cdc14 forming the RENT complex. Absence of Sir2 can induce hyper recombination among these repeats and lead to excision of extra recombinant circles (ERC). Accumulation of these ERC's has shown to be toxic to these cells. Since our data support that Sir-S migrates to nucleolus and *rDNA* silencing is not affected, we tested *rDNA* recombination in Sir2-S background. To check *rDNA* recombination we used a strain where *ADE2* gene is placed in *rDNA* region. In normal physiological condition *ADE2* will express and it will form white colonies. But if because of *rDNA* recombination cell loses the *ADE2* gene it will form red colonies. Hence by counting the number of red colonies we can predict the recombination frequencies of Sir2 and Sir2-S. For our assay we calculated the number of half red/white sectored colonies among a total of 1000 colonies. We avoided the 100% red colonies because there might be recombination before plating the cells. Half red/white colonies are the cells which underwent recombination in the first cell division and hence half part will be red and half will be white. Our observations (fig.28) suggest that there is no *rDNA* recombination in Sir2-S strain and we could not detect a single half red/white colony among 1000 colonies. Whereas there are a

few half red/white colonies in WT-Sir2 and many in *sir2*Δ background. Interestingly, we also found a small but reproducible increase in recombination in Sir2-215 suggesting a slightly compromised recombination function. These observations again suggest that migration of Sir2-S into the nucleolus results in the *rDNA* silencing and reduction of *rDNA* recombination. The migration of sumoylated Sir2 into the nucleolus and establishing stable *rDNA* might have greater physiological role in cell survival and life span.

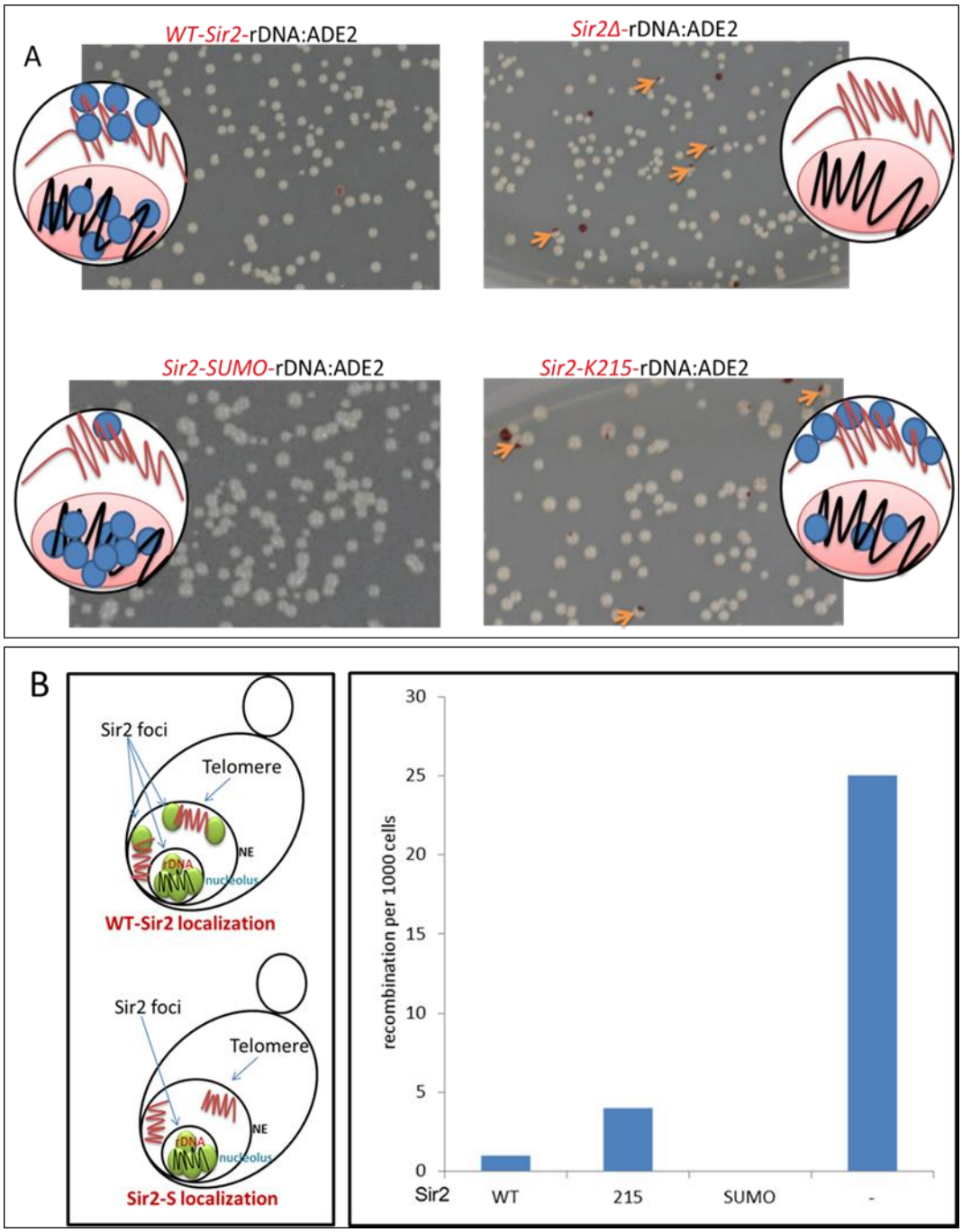

Figure 28: Sir2-S reduces rDNA recombination. *WT-Sir2, Sir2-215R or Sir2-S were introduced into KRY 1540 and plated. Red colonies indicate loss of ADE2 marker. Panel A) Sir2 deletion leads to the higher levels of rDNA recombination which results in many half red/white cells but Sir2-S leads to reduced rDNA recombination hence no half red/white cells appeared. Panel B) Schematic diagram*

*of Sir2 localization and bar diagram plotted for 1000 cells shows comparison of rDNA recombination among WT-Sir2, Sir2-215, Sir2-S and sir2*Δ.

6.3. Discussion

In normal physiological conditions Sir2 is distributed in all the three heterochromatic loci. Our results suggest that sumoylation has a key role in Sir2 distribution. When Sir2 is sumoylated it migrates into nucleolus. With changing localization of Sir2 by sumoylation the hetrochromatinization states of the three loci also changes. Sir4 which recruits Sir2 to the telomere does not interact with sumoylated Sir2 (Sir2-S) which results in reduced TPE in Sir2-S strain. On the other hand, our subcellular localization data suggest that Sir2-S migrates to the nucleolus. Hence we checked the interaction of Sir2-S with Net1 which recruit Sir2 to the rDNA. Both our IP and Y2H data suggest that though Sir2-S cannot interact with Sir4 for establishing TPE, Sir2-S could interact with Net1 to establish *rDNA* silencing.

Since Sir2-S can interact with Net1 and localize to the nucleolus, we also studied the recombination frequency of *rDNA*. Our observations suggest that *rDNA* recombination is reduced by Sir2-S in comparison to WT Sir2. Together all our observations suggest that when Sir2 is sumoylated it moves away from the telomere and migrates to *rDNA* which results in reduced TPE, strong *rDNA* silencing and reduced *rDNA* recombination. Hence we propose that sumoylation of Sir2 regulates the localization of Sir2 and this change in the localization might be helpful in many processes like DNA repair, response to different stresses, and equal distribution of components between mother and daughter cell etc. which are Sir2 functions.

Chapter 7

Sir2 sumoylation upon stress

7.1. Introduction

In the last couple of years, sumoylation has appeared as one of the main protein post-translational modification which regulates the function of proteins. It is observed that SUMO equilibrium of conjugation/deconjugation is affected by many different environmental stresses, including osmotic, hypoxic, heat, oxidative and genotoxic stresses. This regulation of sumoylation occurs either at the level of individual targets, through an exchange between stress induced phosphorylation and sumoylation, or by the equilibrium of the conjugation/deconjugation machinery (Tempe et al, 2008). Earlier observation suggested that there is an increased sumoylation upon heat shock and alcohol stress in higher eukaryotes (Saitoh & Hinchey, 2000). Many new sumoylated proteins were identified upon alcohol and oxidative stress and many of them are found to be responsible for gene expression, translation, DNA replication, chromosome separation, metabolic processes, and stress responses (Tempe et al, 2008). It is also found that the enzymes for SUMO modification cascade, such as E1, E2 and E3 are also sumoylated upon stress (Zhou et al, 2004). Taken together these studies show that sumoylation is a response observed in many cellular processes and a broad study is necessary to explore how sumoylation of proteins modulates the cellular functions. In our study we monitored how Sir2 is sumoylated by different stress as it is already shown that Sir2 is involved in many diverse cellular processes such as DNA damage-repair, cell

division, equal distribution of cell organelles among daughter and mother cell and our observations also suggest that sumoylation redistributes Sir2 within the cell.

7.2. Results

7.2.1. Alcohol treatment increases Sir2 sumoylation

To find sumoylation status of Sir2 upon different stress condition we used *8X-His-SMT3* strains. We studied sumoylation upon alcohol stress and stationary culture. For alcohol stress 0.6 OD culture were taken and treated with 5%, 10% and 20% alcohol for 1hr. After alcohol treatment cells were immediately frozen in liquid nitrogen and extract was made as mentioned earlier and Ni-NTA pull down was done. As we can see from figure 29, as alcohol concentration increases there is increase in the amount of slowly migrating Sir2 band indicating increased sumoylation.

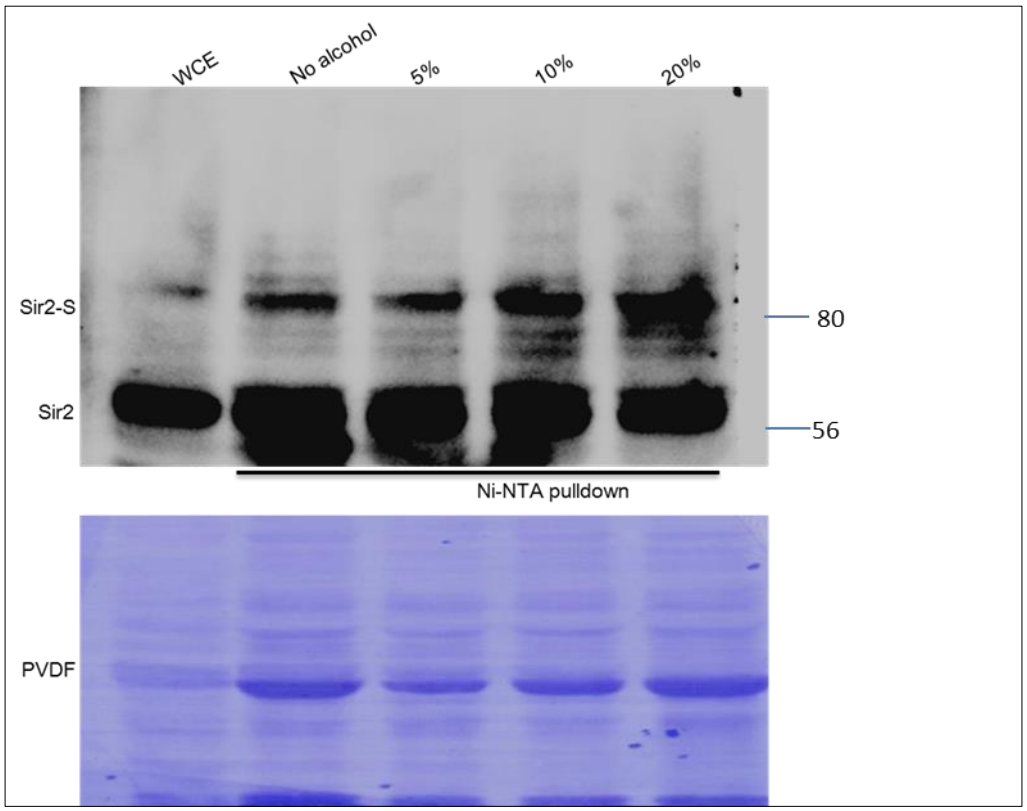

Figure 29: Alcohol treatment increases Sir2 sumoylation. *Ni-NTA pull down of untreated or treated cells were separated on SDS-PAGE and western blot was developed with anti Sir2 antibody. Compared to non-alcohol treated sample (lane 2), 10% and 20% (lane 4 and 5 respectively) has more sumoylated Sir2.*

7.2.2. Sir2 sumoyaltion decreases in stationary state

After alcohol treatment we went ahead to study Sir2 sumoylation when cells are in stationary state. To study Sir2 sumoylation in stationary state we incubated each 100 ml culture for 3, 4 and 5 days separately and did Ni-NTA pull down as mentioned earlier. As we can see there is a reduction of Sir2 sumoylation in stationary phase culture compared to overnight culture (fig. 30, lane 8). We could not detect any further difference in Sir2 sumoylation between 3, 4 and 5 day culture though (fig.30, lane 2, 3, 4). Further study is required to understand why there is a reduced Sir2 sumoylation in stationary state culture.

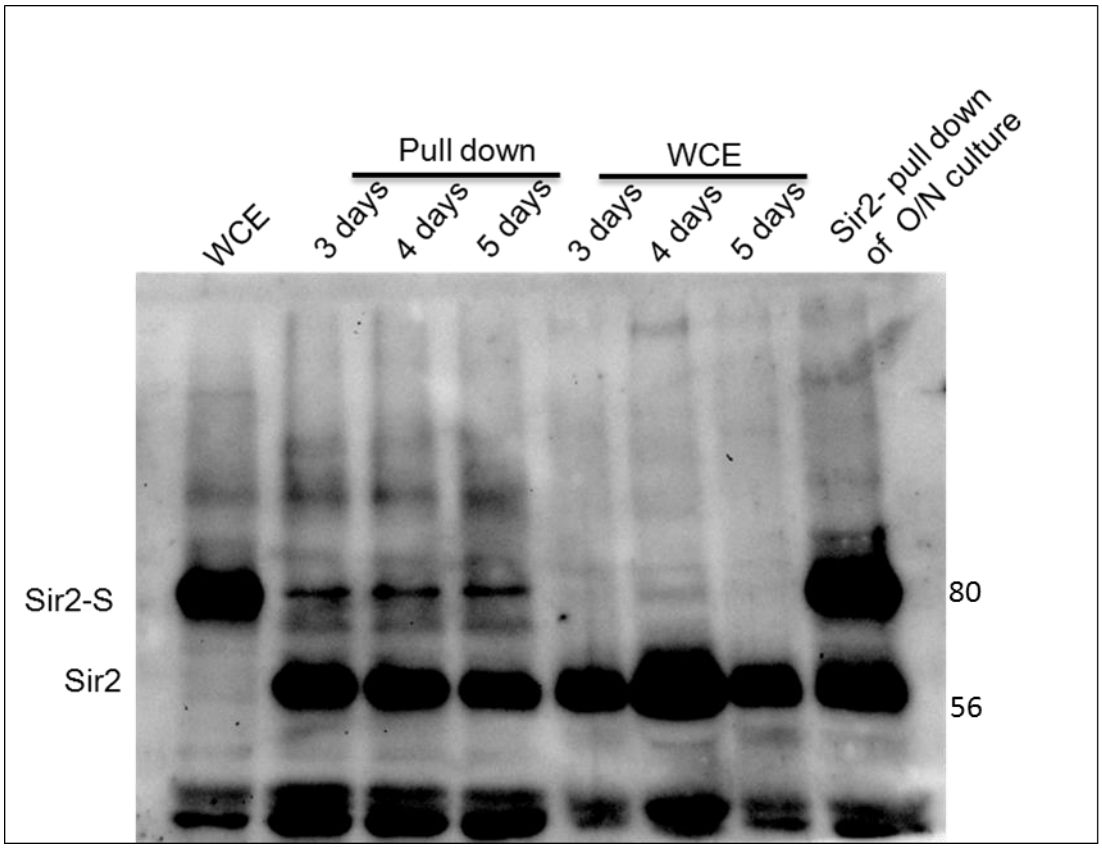

Figure 30: Sir2 sumoylation decreases in stationary phase culture. *Lane 1 is Sir2-S WCE, lane 2-4 is Ni-NTA pulldown of 3, 4 and 5th day respectively, lane 5-7 are WCE of the corresponding cultures. Lane 8 is Ni-NTA pulldown of O/N culture and has maximum amount of sumoylated Sir2.*

7.3. Discussion

Sumoylation pattern of SUMO target varies according to the stress. For example when cells are treated with UV radiation, sumoylation of Rad52 increases many folds but the underlying mechanism is not yet understood (Sacher et al, 2006). Another example is HSF1 which is highly sumoylated when cells are subjected to heat shock. There are numerous examples of specific stress and unique SUMO targets. Regarding Sir2 sumoylation we have already seen that sumoylation re-

distributes Sir2 molecule migrates into the nucleolus. Here in this study we have found that Sir2 sumoylation increases upon alcohol stress but sumoylation of Sir2 decreases when cells reach stationary phase. In natural stress condition higher percent of Sir2 sumoylation might help the Sir2 to migrate into the nucleolus stabilize and protect rDNA that reduces the cell mortality. We have not fully understood the other loci where Sir2 migrates when sumoylated and how it may regulate the cell physiology. Our preliminary results suggest that a lot of work can be done to explore the connection between Sir2 sumoylation and its function in stress.

Chapter 8

Discussions

8.1. Sir2 sumoylation and heterochromatinization

Our study has addressed how sumoylation regulates heterochromatinization in yeast. In our previous studies we had found that elevated doses of Siz2, a SUMO ligase, leads to the loss of telomeric silencing and reduction of Sir2 binding at telomere (Pasupala et al, 2012). In this work we have asked if Sir2 is a direct target of Siz2. We present several lines of evidence to show that Sir2 is sumoylated by Siz2 and in fact most of the effects of elevated dose of Siz2 are due to sumoylation at one specific site on Sir2. Firstly, we find Sir2 is sumoylated in vivo at one site (monosumoylation). Secondly, we could not detect sumoylated Sir2 in a *siz2* null mutant establishing that Siz2 is the main SUMO ligase in vivo. Thirdly, we show that elevated dose of Siz2 increases the fraction of sumoylated Sir2 and *esc1* deletion leads to the hyper sumoylation of Sir2. Fourth, we identify the sumoylation target residues on Sir2 and show that although there are three possible sumoylatable sites in Sir2, sumoylation of only one of them, K215, is sufficient to reduce TPE and also reduce the association of Sir2 with telomeres. These results suggest strongly that sumoylation of Sir2 regulates gene silencing in yeast.

In the next set of experiments we addressed the mechanistic basis of this observation. We showed that sumoylation of Sir2 has a locus-specific effect: although Sir2 sumoylation affects TPE, the nucleolar function of Sir2, both silencing

and *rDNA* recombination is not affected. Based on this observation, and the fact that Sir2K215R remains associated with the telomeres, we hypothesized that sumoylated Sir2 may not be able to interact with Sir4. We tested this hypothesis by first generating a recombinant Sir2 with a C-terminal fusion of SUMO. This construct is similar to the published Yku70/80 constructs (Ferreira et al, 2011).This approach was chosen because only a fraction of the total of a given protein is sumoylated and therefore the phenotypes observed are due to both the sumoylated and unsumoylated protein. In addition, even though we can generate loss-of-function mutants for sumoylation by mutating multiple plausible target lysines, it is not possible to generate gain-of-function mutants, like the replacement of ser/thr with aspartic acid to mimic phosphorylation, to study sumoylation. Results of our localization studies using both immunofluorescence and ChIP with this fusion protein has shown that the Sir2-S is found almost exclusively at the nucleolus. In addition, our ChIP data with K215R firmly establishes that Sir2 that cannot be sumoylated at K215 is retained at the telomeres. This was further confirmed by the inability of the fusion protein to interact with Sir4 although it retains its ability to interact with Nop1.

Based on these observations we propose the following scenario (figure 31). Sir2 is sumoylated by Siz2 and hence, in the cell, Sir2 exits in at least two forms, one that is free and one that is sumoylated at one of the three sites. We cannot distinguish between having a mixed population of monosumoylated Sir2 at anyone of the three sites versus sumoylated only at the one preferred site at this point. In either case, we propose the K215 sumoylated form of Sir2 migrates to the nucleolus. The sumoylated Sir2 can be desumoylated by Ulp1, and in such a case can be also retained at the nuclear periphery (telomeres and *HM* loci). When conditions favour additional sumoylation or when the sumoylated population of Sir2 increase, more

and more Sir2 will localize to the nucleolus and this will reflect as a loss in TPE as less Sir2 is present at the telomeres. These conditions can be produced by *esc1*Δ by displacing the Ulp1 protein from the nuclear periphery or when Siz2 activity is enhanced. We imagine that the presence of the desumoylating activity at the nuclear periphery maintains a pool of unsumoylated Sir2 that can be sumoylated and moved to other loci when conditions permit.

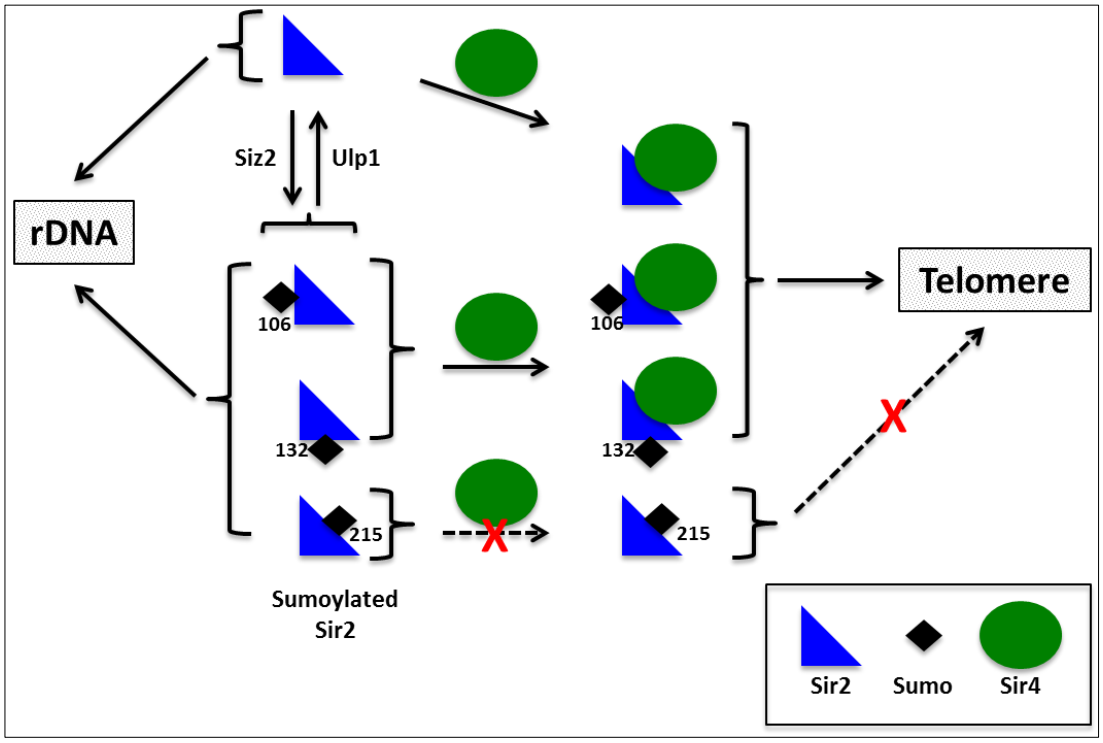

Figure 31: Summary of regulation of telomeric silencing by sumoylation. *Sir2 is sumoylated by Siz2 and both Sir2 and Sir2-S are present together. We propose that K106 and K132 sumoylation does not inhibit its interaction with Sir4 but K215 sumoylation does. Sir2, sumoylated at K215 migrates to the nucleolous.*

8.2. Other (possible) physiological consequences of Sir2 sumoylation

Our experiments show that Sir2 sumoylation alters its property of interaction with at least one of its partners and that leads to differential cellular localization. Our results also indicate that multiple sumoylation of Sir2 does not promote degradation. Therefore, sumoylation of Sir2 alters its physiology without affecting stability and provides a quick and reversible handle to regulate Sir2 function. In order to test if Sir2 sumoylation is regulated by cellular physiology, we tested two different stress conditions. We chose alcohol stress because Ulp1 migrates away from the nuclear periphery and there is a change in sumoylation status of multiple proteins when treated with alcohol (Sydorskyy et al, 2010). We chose starvation because there are several studies linking Sir2 to metabolism and chronological aging. We find differential sumoylation of Sir2 under both conditions: whereas alcohol stress increases Sir2 sumoylation, prolonging stationary phase (nutritional starvation) decreases sumoylated Sir2 population. This shows that Sir2 sumoylation is modulated by cellular physiology. Besides yeast Sir2, sumoylation of SIRT1, a homologue of Sir2 present in higher eukaryotes, was reported (Yang, 2007). It was shown that stress-inducing agents counter the anti-apoptotic function of SIRT1 by recruiting SUMO protease SENP1 to SIRT1, which in turn desumoylates and inactivates SIRT1. This results in the acetylation and activation of p53 apoptotic protein.

We therefore speculate that sumoylation of Sir2 will have consequences beyond gene silencing as Sir2 is involved in multiple other functions in yeast. In a broader model, depicted in Figure 31, we propose that under normal physiological

conditions, there is a balance of sumoylated and unsumoylated Sir2. Sumoylation is probably Siz2 mediated, and desumoylation is Ulp1 mediated. The unsumoylated form is associated with all three silent loci including telomeres, HM loci and nucleolus and sumoylated form is displaced from the telomeres and atleast part of it goes to the nucleolus. We propose that under stress conditions, Sir2 is further sumoylated. Although we have tested only under alcohol and nutritional stress, it is possible that other stress conditions like oxidative stress, DNA damging agents etc. also induce sumoylation. Again, under these conditions, we speculate that other SUMO-ligases may also target Sir2 for sumoylation. Sumoylated Sir2 displaced from the telomeres migrates, we hypothesize, possibly not only the nucleolus, but also to other loci in response to these stress conditions. As Sir2 is known to be associated with multiple other genomic sites, we think this dynamics of sequestering and release of Sir2, regulated by a simple post-translational modifcation, provides a tunable switch to regulate stress response in the cell.

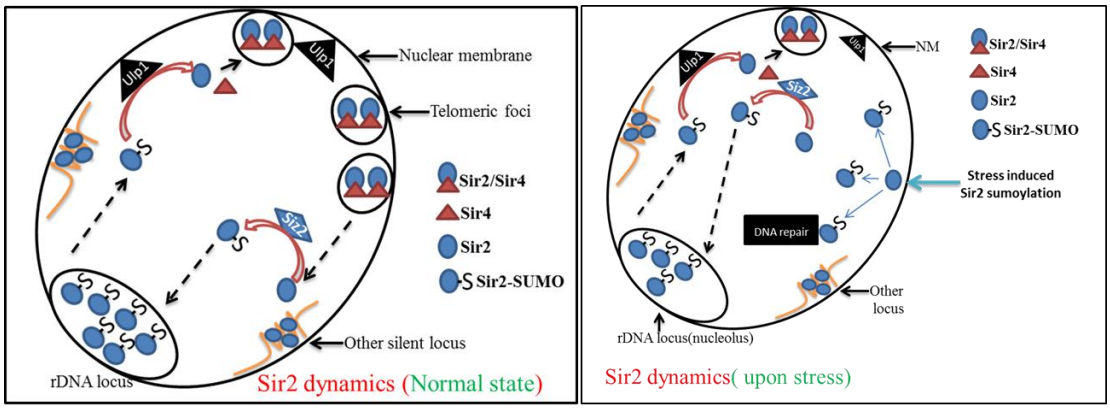

Figure 32: Proposed model for Sir2 dynamics upon stress. *Sumoylation dependent re-distribution of Sir2. Other external stimuli can regulate Sir2 sumoylation differently which might re-distribute Sir2 to other genomic and non-genomic sites. We propose that regulated activity of SUMO ligase(S) and SUMO isopeptidase(s) alters Sir2 dynamics.*

8.3. Future prospects for research on Sir2 sumoylation

Here in this work we studied how Sir2 sumoylation regulates heterochromatin state at all the three silent loci. Our observation suggests that sumoylation helps in the proper distribution of Sir2 among the loci. We also found that external stimuli such as alcohol stress, nutrition deprivation etc. regulate Sir2 sumoylation differently which might distribute Sir2 to different loci. This would be an active area of research in the future: our Sir2 localization studies only tested specific loci; in future with more sensitive assays like ChIP–SEQ we could study how Sir2 sumoylation re-distributes Sir2 among other loci. This will bring greater understanding of the physiological consequences of Sir2 sumoylation. We could also see that Sir2 sumoylation increases its migration towards nucleolus and increase *rDNA* stability by reducing *rDNA* recombination which might contribute to increase in life span. Therefore studies to investigate the regulation of life-span by Sir2 sumoylation can also be carried out. In addition, with respect to aging, apart from genome stability, distribution of components of the cytosol in an asymmetric manner also plays a role. Since Sir2 has been implicated in this process as well, that would be another active area of research. Since Sir2 is conserved throughout eukaryotes, our study has significance in the context of higher eukaryotes to understand the regulation of the physiology of aging, gene expression, cancer etc. by sumoylation of Sir2 family of proteins.

Appendix

A-1: Construction of Sir2-SUMO (Sir2-S)

To create the Sir2-SUMO fusion *SMT3* gene was amplified by following set of primers. The forward primer starts with ATG having Pst1 restriction site. The reverse primer is designed such that it devoid of last three amino acids ATY so that SUMO protease Ulp1 cannot act on the construct (Ferreira et al, 2011).

F-pst-sir2: 5-ATGTCTGCAGATGTCGGACTCAGAAGTCAATC-3
R-Not1-sir2-2G: 5-ATGTGCGGCCGCAATCTGTTCTCTGTGAGCCTC-3
Amplified *SMT3* was cloned in pBS vector (CKM 294) and named CKM311. Construct was releaed from pBS with Pst1/Not1 and cloned in CKM 342 (Sir2-GFP from David Shore).

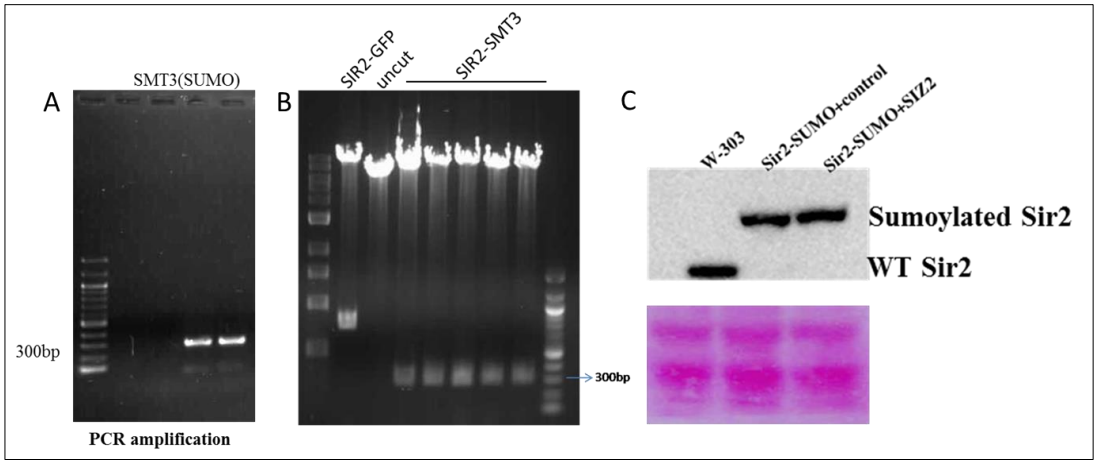

Figure A-1: *Pane1 A) SMT3 amplification by primer. Panel B) amplified fragment was cloned in CKM 342. Panel C) clones were confirmed by western blot where slower migrating Sir2-S fusion appeared.*

The CKM 342 was taken and GFP sequence was removed with Pst1/Not1 and *SMT3* amplified with Pst1/Not1 was ligated. Clone was transformed in *sir2*Δ strain and

selected on a SC–leu plate; then protein was extracted by TCA method and WB was done with anti-Sir2 antibody and slower migrating Sir2-S

fusion was confirmed. The Sir2-S fusion expressed similar to WT Sir2.

A-2: Construction of Sir2-FLAG and Sir2-215-FLAG

For His-FLAG construct Sir2 promoter, we amplified with the following primer. The forward primer contains Not1 and reverse primer contains Xba1 restriction site. After amplification, the PCR product was cloned in pRS316 having His-FLAG already cloned as Xba1/BamH1 vector (gift from Sangeetha).

Fpromoter-sir2: ATATGCGGCCGCGAAATGATTATTAGCAGTCTTTCTCCC

Rpromoter-sir2: ATATTCTAGACCAGCTTTAATGTGCCGATGAGGG

After cloning the promoter beside the His-FLAG, Sir2 gene was amplified with primer having restriction sites Sma1 and Sal1 as mentioned below which includes 150bp downstream of the stop codon.

Fsir2-Sma1: ATATCCCGGGACCATCCCACATATGAAATACGCCG

Rsir2-Sal1: ATATGTCGACACCACCTCCTTTCTTTGACCCAACG

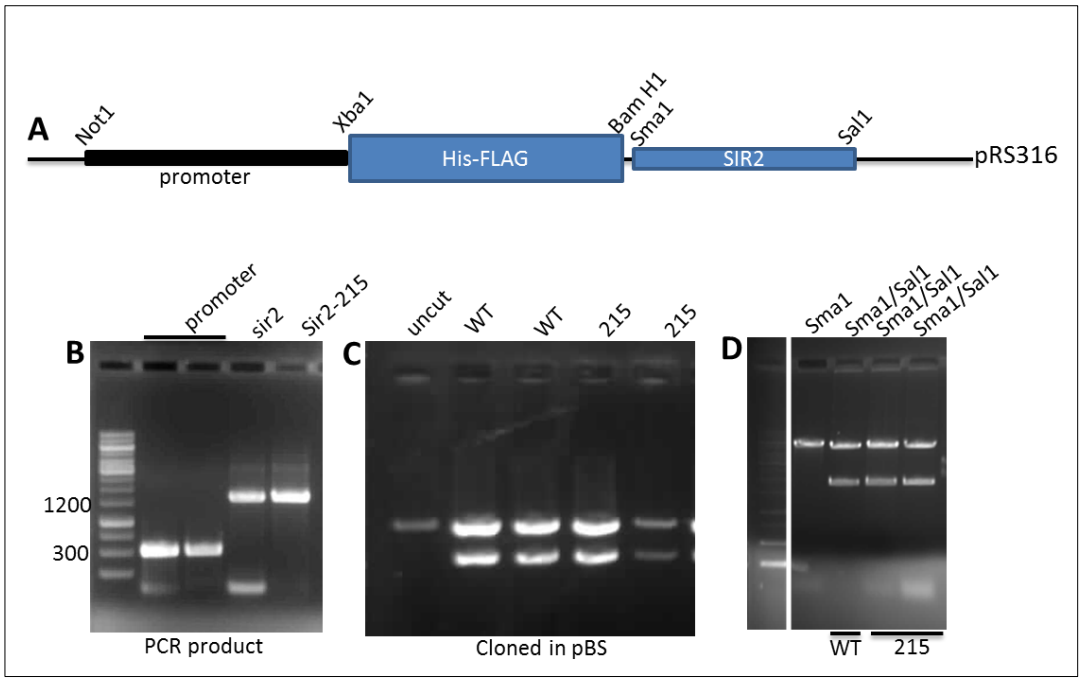

Figure A-2: *Panel A) Schematic diagram of His-FLAG SMT3, Panel B) amplification of the promoter, Panel C) Sir2 cloned in pBS, Panel D) Sir2 cloned in pRS316 with Sma1 and Sal1.*

A-3: GBD constructs of Sir2, Sir2-215 and Sir2-S

For the GBD construct Sir2 was amplified by the following primers.

F sir2 with ATG: ATATCCCGGGATGACCATCCCACATATGAAATACG

Sir2R-Sal1: ATATGTCGACAGTGAGATGGGCGGTACATG

Amplified PCR product was cloned in pBS vector. Confirmed pBS clone was selected and plasmid was isolated and digested with Sma1 and Sal1. Eluted product was the ligated in pGBD-UC1 vector digested with Sma1/ Sal1.

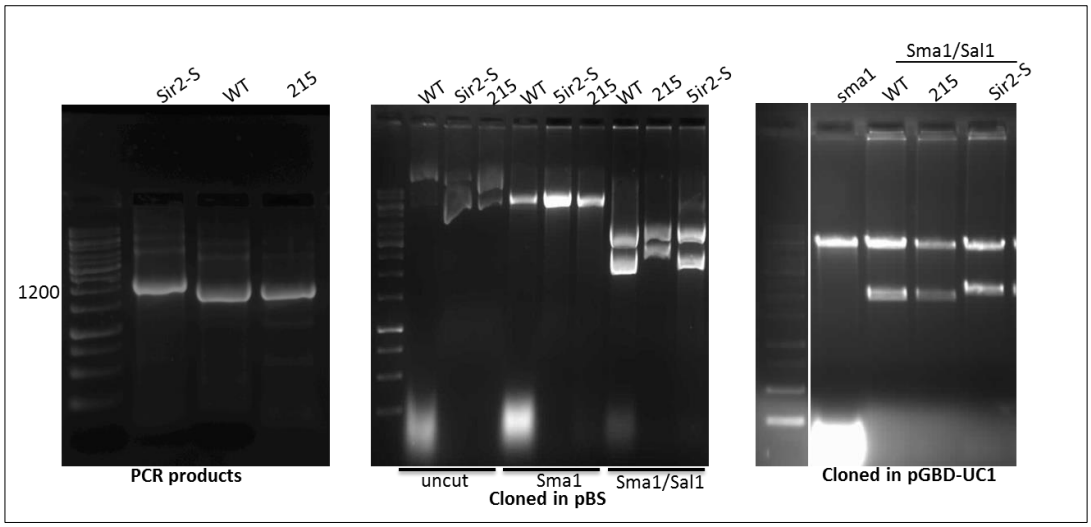

Figure A-3: *Panel 1) Sir2, Sir2-215 and Sir2-S was amplified by PCR, Panel 2) PCR products were cloned in pBS vector and confirmed by restriction digestion, Panel 3) eluted product from pBS vector was cloned in pGBD-UC1.*

A-4: SMT3 clone

SMT3 was amplified with the following set of primer and PCR product was cloned in pBS vector. Fragment was then eluted from pBS vector and cloned in pET28a vector as a BamH1/Xho1 fragment. Confirmed clone was then transformed in BL-21 and expressed with IPTG induction and western blot was done to confirm the expression.

F primer:5ATGTCTGCAGATGTCGGACTCAGAAGTCAATC3

R primer:5ATGTGCGGCCGCAATCTGTTCTCTGTGAGCCTC3

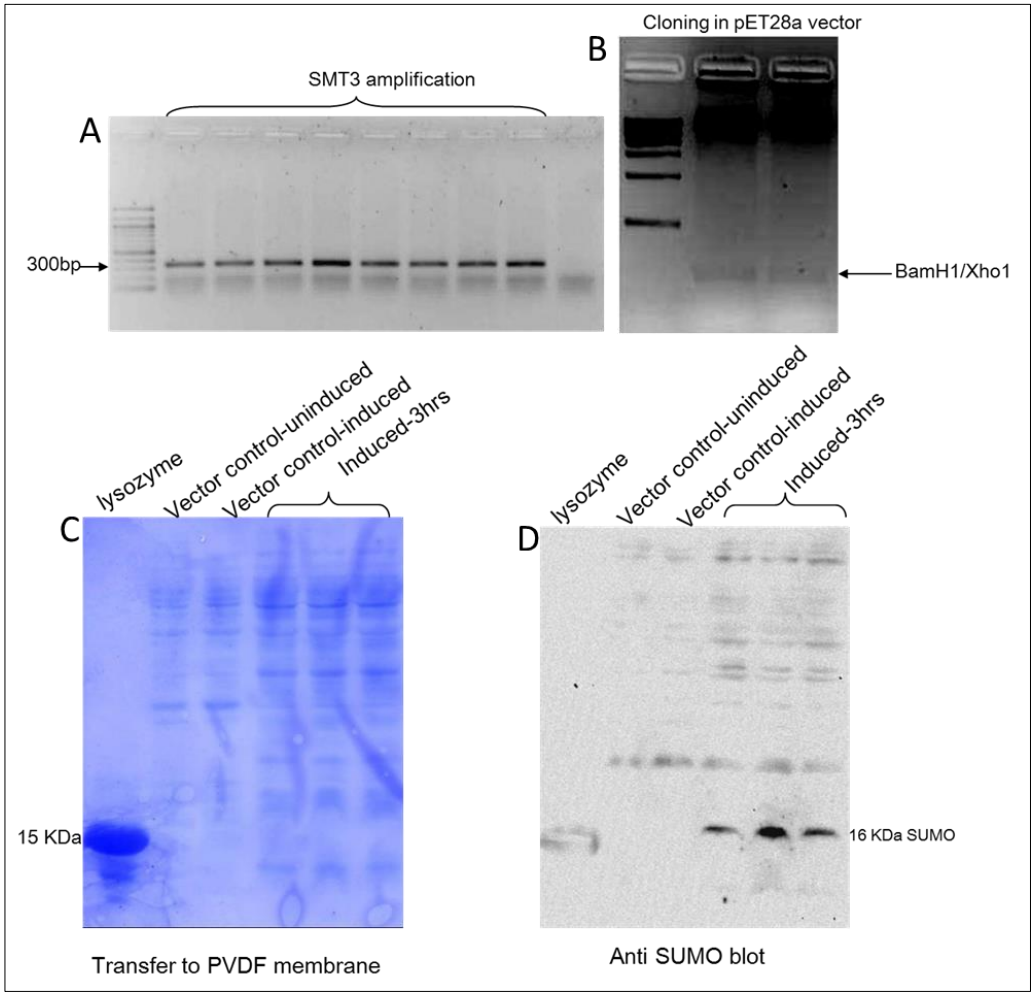

Figure A-4: *Panel A) Sir2 amplification, Panel B) cloned in pET28a, Panel C,D) western blot with anti SUMO antibody confirm the expression.*

A-5: Sir2-215-SUMO constructs

Sir2-215 was digested out from Sir2-215 plasmid with Cla1/Nco1 and ligated in Sir2-SUMO backbone as described in the picture.

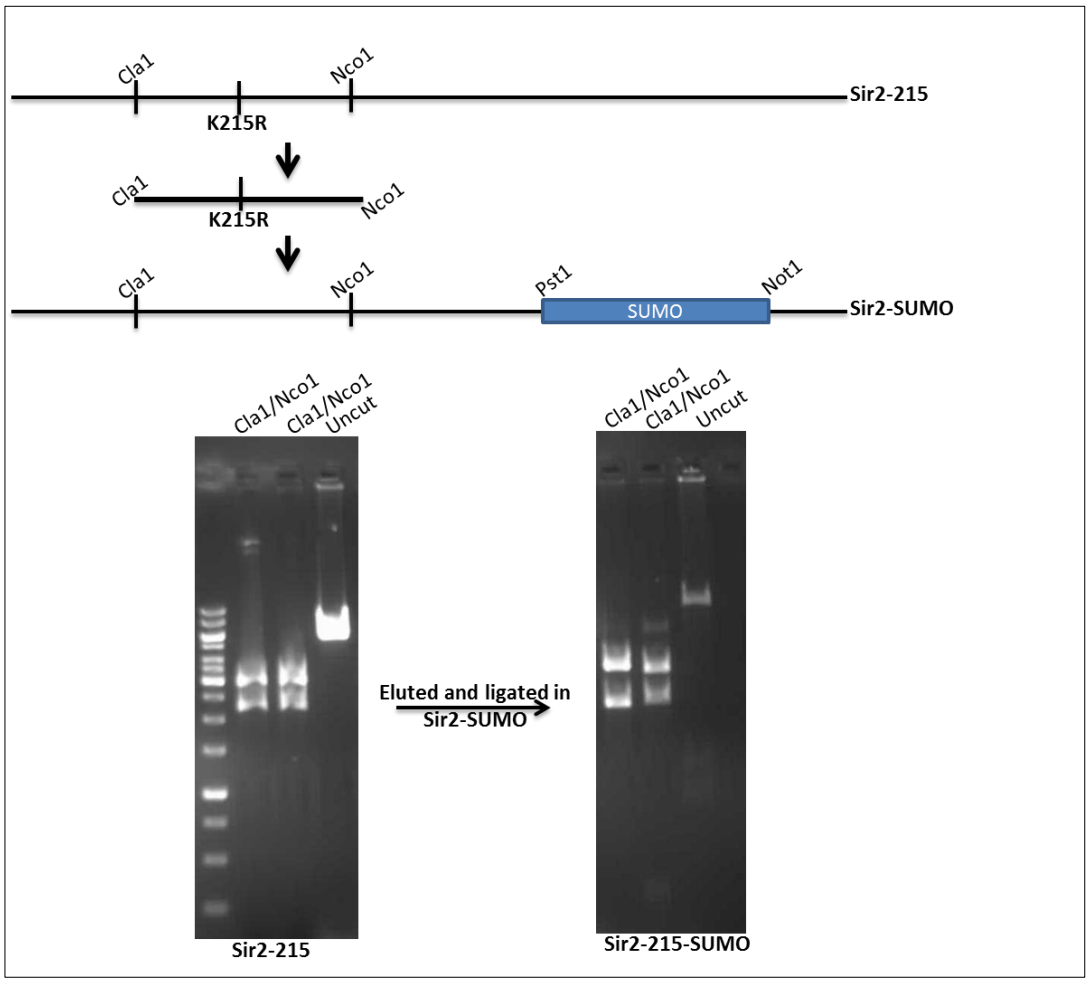

Figure A-5: *Cloning strategy of Sir2-K215R-SUMO.*

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