Role of Hsp90 in maturation and regulation of Sir2 in *Saccharomyces cerevisiae*

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DECLARATION

I, Shyamasree Laskar, hereby declare that this thesis entitled, **"Role of Hsp90 in maturation and regulation of Sir2 in *Saccharomyces cerevisiae*"** submitted by me under the guidance and supervision of Dr. Sunanda Bhattacharyya, is an original and independent research work and free from plagiarism. 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. I, hereby, agree that my thesis can be deposited in Shodganga/INFLIBNET.

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CERTIFICATE

This is to certify that this thesis entitled, **"Role of Hsp90 in maturation and regulation of Sir2 in *Saccharomyces cerevisiae*"** is a record of bonafide work done by Shyamasree Laskar, a research scholar for Ph.D. programme in Department of Biotechnology and Bioinformatics, 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.

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Dr. Sunanda Bhattacharyya
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Dedicated to my beloved parents and sister

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ABBREVIATIONS:

aa : Amino acid

ADE: Adenine

bp : Base pair

BSA : Bovine serum albumin

$CaCl_2$: Calcium chloride

cDNA : complementary DNA

Da : Dalton

DEPC : Diethyl pyrocarbonate

DNA : Deoxyribonucleic acid

dsDNA : Double stranded DNA

DTT : Dithiothreitol

EDTA : Ethylene diamine tetraacetic acid

Etc : et cetera

KCl : Potassium chloride

kDa : Kilo Dalton

kV: Kilo volt

LB : Luria-bertani broth

Leu : Leucine

LiOAc: Lithium acetate

M : Molar

mg: Milli gram

$MgCl_2$: Magnesium chloride

ml: Milli liter

Min : Minute

mM : Milli molar

NaCl : Sodium chloride

NaH_2PO_4 : Sodium dihydrogen phosphate

NaOH : Sodium hydroxide

NaOAc : Sodium acetate

ng : nano gram

OD : Optical density

ORF : Open reading frame

PBS : Phosphate buffered saline

PCIA : Phenyl chloroform isoamyl alcohol

PCR : Polymerase chain reaction

PEG : Polyethylene glycol

PVDF : Polyvinylidene Fluoride

RNase : Ribonuclease

SC : Synthetic complete

SDS : Sodium dodecyl sulphate

SDS-PAGE : Sodium dodecyl sulphate poly acrylamide gel electrophoresis

ssDNA : Single stranded DNA

TBE : Tris Borate EDTA

TCA : Trichloroacetic acid

TE : Tris EDTA

Tris Cl : Tris chloride

Trp: Tryptophan

YPD : Yeast extract peptone dextrose

µg : Microgram

µM : Micromolar

µl : Microliter

CHAPTER 1

INTRODUCTION

1.1. EPIGENETICS:

The findings from the last few years are helping researchers to understand a long-standing puzzle of heritable phenomena that regulates gene expression by modifying chromatin structure. This phenomenon is defined as epigenetics [1]. Genes, packaged into chromatin, undergo a dynamic chromatin remodeling processes which are important during the initial steps of gene expression. Decrease in nucleosome density is associated with the relaxation of chromatin structure which thereby causes transcriptional activation of genomic region [2]. This includes accessibility of gene promoters and regulatory regions to various transcription factors [3]. Epigenetic factors such as DNA methylation, histone modifications, small non coding RNAs are responsible for these regulatory processes [4]. Epigenetic modification alters the chromatin structure through several chromatin-associated proteins which include linker histone 1, high mobility group proteins (HGP), histone modification enzymes and components of chromatin remodeling complex [5, 6, 7].

One of the major parts of epigenetic modifications is the histone modification. Chromatin includes nucleosomal subunit which consists of 146 base pair of DNA wrapped around an octamer of core histone. This histone octamer contains two copies of each of the histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4) proteins [8]. Lysine rich N-terminal tail of each core histone protrudes outside the nucleosome [8]. This lysine residue undergoes various modifications such as methylation, acetylation, phosphorylation, biotinylation and ribosylation which changes nucleosome arrangement causing gene regulatory regions more accessible to the transcription factors [9]. Methylation at the CpG sites within the promoter is associated with the hetero-chromatinization and repressed gene state [10, 11, 12]. On the other hand, histone acetylation at the lysine residues contributes to the transcriptionally competent euchromatin conformation [13]. Each histone modification according to its position is considered as a mark for transcriptional status of the target

gene. For example, transcriptionally active genes are associated with acetylation of H3 Lys9 (H3K9ac) and mono/di/tri-methylation of H3 Lys4 (H3K4me3/2/1) [14]. Whereas, deacetylation of H3K9, H3K9me2/3 and, H3K27me2/3 are generally associated with transcriptionally repressed genes [14].

1.1.1. EPIGENETICS AND ENVIRONMENT:

Evidences suggest that environmental factors or stresses play a critical role in chromatin remodeling and stable alterations in gene expression to the next generation. Stresses can be either intrinsic, such as spontaneous gene mutation, or extrinsic which includes biotic and abiotic stress. Pathogen attack is an example of the biotic stress whereas abiotic stress originates from unfavorable environmental conditions. Epigenetic patterns are not only influenced by environmental exposures but also altered with advancing age [15]. Environmental exposures can act across generations to influence gene expression in offspring [16]. But the mechanistic link between the environmental trigger and the observed epigenetic modifications are yet to be understood (Figure I). Histone tail modifications play an important role in response to abiotic stress in plants. For example, in tobacco and Arabidopsis, exposure to salinity, cold and abcisic acid tend to increase global enrichment level of H3S10 phosphorylation (H3S10ph), H3S10 phospho-acetylation (HeS10ph-ac), H4 Lys4 acetylation and these histone modifications lead to up-regulation of many stress specific genes [17]. Nutritional factors have been shown to modulate gene expression in animal and human models.

It has been seen that nutritional conditions and folic acid supplementation during gestation contribute to the alterations in DNA methylation at the imprinted genes [18, 19]. Experiment in mice shows that methyl supplement causes diet induced phenotypic alterations at A^{vy} locus *via* increased C_pG methylation ensuing silencing of *agouti* gene expression [20]. In mammals trans-generational inheritance of methylated DNA is seen during gametogenesis [21].

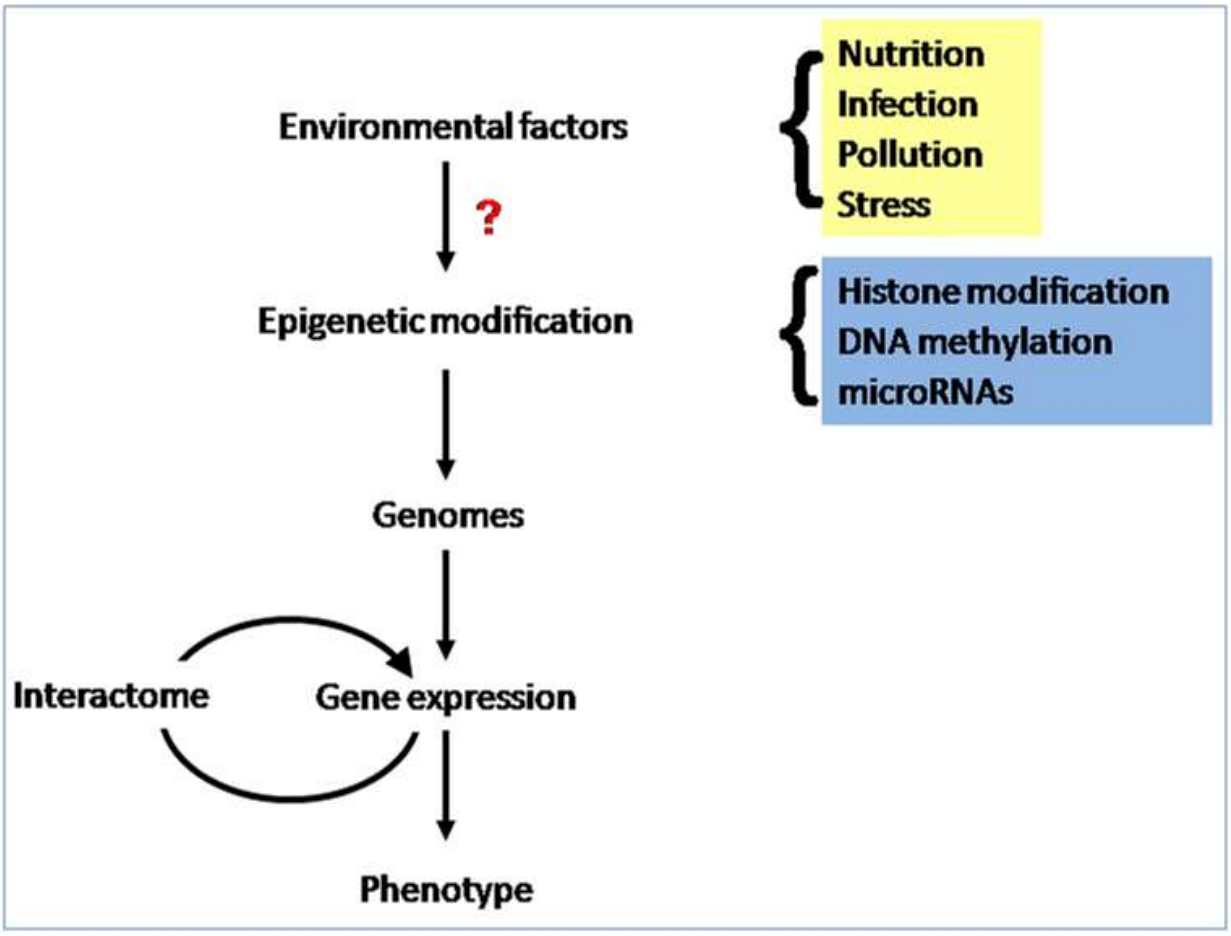

Figure I: Link between environmental factors and gene expression.

Transgenerational effects include both physiological and behavioral changes across generations. Adaptive epigenetic inheritance of mothering style and stress in rats is associated with multilevel process that involves behavioral, physiological, cellular and molecular events. High licking/grooming and arched-back nursing results in high level of serotonin in the hippocampus of the pups. This causes the up-regulation of nerve growth factor inducible protein A (NGFI-A) which in turn binds to the promoter of glucocorticoid receptor (GR) gene and increases expression of GR by DNA hypomethylation and histone acetylation [22, 23].

1.1.2. ENVIRONMENTAL TEMPERATURE AND EPIGENETICS:

Studies have explored that changes in the environmental temperature trigger epigenetic alterations in individuals. Exposure to heat shock and osmotic stress causes chromatin modification by phosphorylation of ATF2 resulting red eye color phenotype in drosophila that is stably inherited over successive generations [24]. Vernalization in plants is another striking example of epigenetic regulation of gene expression through which plants are instructed to flower early after having exposed to cold temperature [25]. It is an adaptive trait which is required to prevent flowering before winter and permits flowering in spring. In Arabidopsis and cereals, vernalization causes suppression of genes which repress flowering. Change in the environmental temperature leads to recruitment of chromatin modifying complexes to a group of flowering repressors that are silenced epigenetically by histone modifications. Also, in the fission yeast *Schizosaccharomyces pombe*, stress activated protein kinases modulate Atf1 activity and cause defect in heterochromatin assembly [26]. Epigenetic temperature adaptation processes at the central nervous system is seen in birds [27]. Environmental temperature influences the melanogenesis in various animals [28, 29]. During development environment can accomplish its specific instructions through neuro-endocrine system [30]. Polyphenism in butterfly *Araschnia levana* is a phenotypic plasticity due to different

environmental temperatures and photoperiods [30]. Environmentally controlled pigmentation in *Araschnia* is mediated by a hormone called ecdysone. In summer, while longer exposure to light and temperature induces ecdysone pulse causing black pigmentation, early pupation does not induce ecdysone during spring [30, 31]. Some vertebrates also show neuroendocrine control of phenotype such as temperature sensitive sex determination of turtles and crocodilians. Aromatase which is elevated at high temperature converts testosterone into estrogen and gives rise to female offspring [32]. Diamondback terrapins also show temperature dependent aromatase activity [33]. In some cases environmental factors control gene activity by regulating the gene product. Temperature sensitive tyrosinase is one of the best documented examples of temperature affecting protein activity in Himalayan cavies, Siamese cats, mice and guinea pigs [34]. This enzyme, folded in such a manner that under normal body temperature it is unstable and non functional. However at the extreme parts of the body where body temperature is slightly lower, this enzyme undergoes proper folding and becomes functional [35]. In Himalayan mouse, His 420-Arg mutation in tyrosinase is the reason behind different coat colors [36]. Another study shows that Himalayan mouse skin contains a tyrosinase inhibitor. At normal body temperature tyrosinase has higher affinity for the inhibitor than at lower body temperature and hence tyrosinase controls melanin synthesis in Himalayan mouse skin [37].

1.1.3. ENVIRONMENTAL POLLUTANTS AND EPIGENETICS:

Environmental pollutants play crucial role in epigenetic modifications and gene expression. Nickel (Ni), an epigenetic carcinogen, alters gene expression by DNA methylation or histone acetylation in heritable fashion [38, 39]. In spite of low mutagenic activity, Arsenic is known to cause chromosomal alterations such as aberrations, aneuploidy, sister chromatid exchange by inducing both DNA hypomethylation and hypermethylation [40]. Epidemiological findings suggest chromium

(Cr) is another potent epigenetic chromatin modifier in cells exposed to environmental chromium mainly through drinking water [41]. There are formations of DNA-protein complexes in lymphocytes upon chromium exposure.

1.1.4. EPIGENETIC STUDY PLAYS SIGNIFICANT ROLE IN BETTER UNDERSTANDING OF DIFFERENT HUMAN DISEASES:

Considerable evidences uncover the role of the measurable environmental factors which contribute to variations in normal trait that alter the risk of acquiring different diseases. It has been pointed out that partial stability and plasticity of epigenetic regulation opens the door to understand complex mechanisms behind different diseases which could not be explained by traditional DNA sequence based genetics. Food habit, drinking habit of humans, as well as rest of their physiological and biological environmental stresses are responsible for many severe illness experienced by human, for example smoking and lung disease, sunlight and skin cancer. One of the best known epigenetic modification is DNA methylation and alterations in it is associated with many different pathogenesis, the best studied of which is cancer [42]. Since the first report of association of cancer and alterations in DNA methylation was published [43], epigenetics has become a crucial component in the study of cancer biology. In cancer cells, CpG island promoter hyper-methylation of tumor-suppressor genes leads to its transcriptional silencing which is a typical hallmark of cancer cell. By contrast, there is loss of substantial proportion of methylation of genes which are normally heavily methylated [43]. Hypo-methylation causes aberrant expression of oncogenes such as *HRAS* and CT genes in testis [44] and genome wide demethylation of human gene *MAGE* occurs in tumor cells [45]. Hypo-methylation of *CAGE* is associated with stomach and liver cancer [46]. The study of relation between epigenetics and aging has been a quite interesting subject from long back. A

pioneering study in spawning humpbacked salmon showed that global DNA methylation decreases with age [47].

1.2.1. EPIGENETIC MODIFIERS:

This gene-environment interaction involves protein-DNA association to regulate gene expression. Chromatin modifying enzymes that catalyze chemical conversion of cytosine residues in DNA, or lysine, arginine, tyrosine and serine residues in histone proteins are the master regulators of gene expression and play an important role in chromatin modification. Systematic sequencing of human malignancies shows several mutations in epigenetic modifiers. Several of these mutated epigenetic regulatory genes are associated with specific disease phenotype and these mutations cause gain-of-function of genes which is potential target for drug development against diseases. Epigenetic modifying genes which causes direct cytosine methylation of DNA include DNA (cytosine-5)-methyltransferase-3A (DNMT3), Ten-eleven translocation methylcytosine dioxygenase (TET), Isocitrate dehydrogenase 1/2 (IDH 1/2) [48]. Genes encoding enzymes for histone modifications are Histone-lysine N methyl transferase (EZH2), BRCA associated protein 1 (BAP1), Mixed lineage leukemia 1-3 (MLL1-3), CREB binding protein (CREBBP), E1A binding protein p300 (EP300). Epigenetic modifiers also include genes which are required for histone enzyme complexes to function such as embryonic ectoderm development (EED), Suppressor of zeste 12 (SUZ12), additional sex combs-like 1 (ASXL1) [48]. Post-translational modifications (PTMs) of histones also play an important role in modification of chromatin compaction and hence affect the binding of effector proteins including chromatin modifying enzymes or remodeling complexes. In addition, ATP dependent chromatin remodeling complexes also contribute to epigenetic modification of chromatin compaction. ATPases are divided into four main families and these are SWI/SNF, ISWI, CHD, and INO 80 complexes [49].

In mammals, there are three active DNA methylatransferases, DNMT1, DNMT3A and DNMT3B that add a methyl group to cytosine in CpG dinucleotides of DNA. During DNA replication, one of the maintenance methyltransferases DNMT1 add methyl group to newly synthesized CpG dinucleotides on the hemi-methylated DNA. DNMT3A and DNMT3B show high expression level during embryogenesis and are primarily responsible for *de novo* DNA methylation. The TET family proteins have been shown to be involved in the conversion of 5-methyl cytosine (5-mc) to 5-hydroxymethyl cytosine (5-hmc) and thus contribute to the regulation of DNA methylation [50]. This modification inhibits the binding of silencer proteins to the methylated DNA. The regulatory regions of actively transcribed genes are associated with this enzyme [51]. (8-23)% of the patients having myeloid hematopoietic malignancies shows mutations in TET2 [52]. Knockdown of TET1/2 in mouse embryonic stem cells causes hyper-methylation of several gene promoters [53].

1.2.2. HISTONE MODIFIERS:

1.2.2.1. HISTONE METHYLASES AND DEMETHYLASES:

Maintenance of the silent and active state of gene expression during developmental process depends on methylation and demethylation of specific residues and these histone modifications are largely altered by environmental cues. Methylation at both lysine and arginine play an important role in histone remodeling. Most of the histone methyltransferases comprise SET domain for their function and methylates lysine and arginine residues. There are eight histone lysine methyltransferases (HKMTs) [54, 55]. HKMT1 is responsible for H3K9 methylation while HKMT2 targets methylation in H3K4. H3K36 and H4K20 are methylated by HKMT3. HKMT4 comprises DOT1L, a non-SET domain protein and is involved in H3K79 methylation. Mono-methylation of H4K20 occurs by HKMT5 family proteins, while HKMT6 acts as a subunit of polycomb repressive complex 2 (PRC2) and contribute to H3K27 methylation. Set 7/9 protein containing HKMT7 mono-methylates H3K4

and HKMT8 is responsible for H3K9 [55]. Four classes of histone arginine methylatransferases (HRMT) have been designated as HRMT I-IV [55]. Type I HRMT di-methylates only one guanidine nitrogen and modifies arginine of histone tail. HRMT-II is responsible for symmetrical modification by targeting both the guanidine nitrogens. While HRMT-III can act similarly as type II, HRMT-IV can methylate an internal nitrogen atom of arginine [55].

Another histone methylase is polycomb repressive complex 2 (PRC2) which belongs to the class of polycomb group proteins (PcG) which are transcriptional repressors and plays crucial role in maintenance of cell identity and regulation of cellular differentiation.

1.2.2.2. HISTONE ACETYLASES:

Histone acetylation, the most prominent post translational modification is a reversible phenomena leading to gene expression, DNA replication and DNA repair. Histone acetylases (HATs) with the help of acetyl coA co-factor attach a acetyl group to the ε-amino group of lysine residues of histone protein. HATs have been classified into five major families: HAT1, Gcn5/PCAF, MYST, p300/CBP and Rtt109. Five major families of HATs share the common structural motif of a three stranded anti-parallel sheet with a helix spanning through the length of the sheet that contributes to co-factor binding.

HAT1, found in almost all eukaryotes, has a very broad range of functions in many DNA regulatory processes [56]. HAT1 is a member of HAT-B complex and in yeast HAT1 functions in association with HAT2 protein (RbAp46 or RBBP7 in human) forming a trimeric complex [57]. In yeast, after binding to H4 and H3, HAT1-HAT2 complex is imported from cytoplasm to nucleus and becomes associated with histone chaperone/chromatin assembly factor HIF1 [58].

Extensively studied HATs include Gcn5/PCAF and MYST. GCN5, a member of GCN5-related N-acetyltransferase super family, was first found as an enzyme involved in amino acid biosynthesis in

yeast [59]. GCN5/ PCAF family members have higher affinity for H2B, Lys14 of H3 and Lys8 and 16 of H4 [60]. The MYST family of HATs is named after its members MOZ, ybf2/sas3, sas2 and tip60. MYST has high specificity for Lys16 on H4, Tip60, MORF, MOZ and HBO1 [61]. p300/CBP family of HATs is named after its members p300 (KAT3B) and CREB binding protein (CBP or KAT3A) in mammals. CBP and p300 share identical domain structures and bind to similar set of transcription factors including ETS1, c-MYB, STAT2, CREB and tend to stimulate transcription of their target genes [61]. Though Rtt109 by itself has a very low catalytic activity, but in association with its chaperones Vps75 and Asf1 it exhibits high level of acetylation. Rtt109 contributes mainly to the acetylation of H3K56 in fungi. Fungi cells which are incapable of acetylating H3K56 are extremely sensitive to DNA damage [62].

1.2.2.3. HISTONE DEACETYLASES (HDACS):

Epigenetic regulation plays a crucial role in diverse physiological and pathological cellular processes through post translational modifications of chromatin. In particular, acetylation plays a crucial role in epigenetic regulation of gene expression by opposing actions of two large families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs result in open chromatin and gene expression while HDACs are involved in closed or compact chromatin structure leading to gene repression. Mechanism of action of HDACs comprises of removal of acetyl group from its substrate leading to decrease in space between the nucleosome and the DNA wrapped around it. Functional and phylogenetic studies have shown that there four different classes of HDACs which include class I-IV. These classes differ in their structure, enzymatic function, sub cellular localization and expression pattern [63, 64]. HDACs have a conserved catalytic domain of about 390 amino acids. Class I, II and IV HDACs depend on zinc ion for their catalytic activity while class III Sir2-like HDACs deliver their catalytic activity in a nicotinamide-adenine-dinucleotide

(NAD^+) dependent manner [55]. The imbalance between these two antagonistic functions between HAT and HDAC may result in disturbance in developmental processes.

Ubiquitously expressed class I HDACs which are most closely to yeast Rpd3 include HDAC 1, 2, 3 and 4. These are mostly located in nucleus and show strongest enzymatic activity among all the classes of HDACs. There is 82 % sequence identity in human isoforms of HDAC1 and HDAC2 which share substantial amount of functional redundancy. Co-existence of these two proteins has been shown in different multi protein repressor complexes such as Sin3A, NcoR/SMRT, Co-REST, Mi2/NuRD and EST1B [63]. HDAC1 and 2 display their function only when they are present in specific multi-protein complex and modulate histone deacetylase and DNA binding activity. HDAC3 is mostly localized in nucleus and it is recruited to DNA by other HDACs (HDAC 4, 5 and 7) and co-repressors [65]. Class II HDACs are of two sub-classes having similarity to yeast Hda1. These are class IIa (HDAC 4, 5, 7 and 9) and class IIb (HDAC 6 and 10). Class IIa HDACs play important role as epigenetic regulator of gene expression as their ability to change their localization in response to specific signal. The most recently discovered class II HDAC is HDAC10. There are also two putative Rb-binding domains in HDAC10 suggesting its role in cell cycle regulation [63]. The only member of class IV HDACs is HDAC11 which shows tissue specific expression in mammals [66].

1.2.2.4. CLASS III HISTONE DEACETYLASES OR SIRTUINS:

Rising evidences unfold that reversible acetylation of proteins by acetyltransferases and deacetylases is one of the major regulatory mechanisms of gene expression. NAD^+ dependent Class III histone deacetylases or sirtuins (SIRTs) are emerging as key regulators of diverse biological processes from regulation of cell division and survival to senescence. SIRTs ranging from yeast to humans are unique in a way that they translate different metabolic states into global cellular changes. Sir2, the

founding member of sirtuin family was originally identified for its role in silencing cryptic mating type loci, *HML* and *HMR* in *Saccharomyces cerevisae* [67, 68]. There are four additional Sir2 homologs in yeast: Hst1, Hst2, Hst3 and Hst4. Hst1 forms complex with DNA-binding protein Sum1 and causes transcriptional repression of specific genes involved in the middle sporulation [69], de novo NAD^+ biosynthesis [70] and thiamine biosynthesis [71]. Though Hst2 is primarily localized in the cytoplasm, it shuttles into the nucleus and represses sub-telomeric genes [72]. Hst2 also negatively regulates rDNA silencing [73]. Hst3 and Hst4 deacetylate H3K56 and result in transcriptional silencing. These are involved in cell cycle progression and promote genome stability [74].

Sir2 homologs have been identified in other species as well. The *Caenorhabditis elegans* genome codes for four sirtuin genes: SIR2.1, SIR2.2, SIR2.3 and SIR2.4. Over-expression of SIR2.1 causes modest increase in mean life span and shows stress resistance of worms in a SIR2.1 dependent manner [75]. Over expression of dSir2 in fat body also increases longevity of *Drosophila melanogaster* in a diet dependent manner [76]. According to the amino acid sequence based phylogenetic analysis mammalian sirtuins are divided into four classes: class I, II, III and IV [77]. SIRT1, SIRT2 and SIRT3 belong to class I while SIRT4 and SIRT5 belong to class II and class III respectively, and class IV includes SIRT6 and SIRT7. Mammalian sirtuins have diverse cellular localizations. SIRT1, SIRT6 and SIRT7 are mainly found in the nucleus while SIRT2 is predominantly localized in the cytoplasm and SIRT3, SIRT4 and SIRT5 are mitochondrial proteins.

The best studied sirtuins is SIRT1 which is an ortholog of yeast Sir2. It is a NAD^+ dependent protein deacetylase. The non-histone substrates of SIRT1 include p53, fork-head box class O (FOXOs), p73, BCL6, E2F1, retinoblastoma protein (pRb), Ku70, nuclear factor κB (NFκB), P300/CBP associated factor (PCAF), peroxisome proliferator-activated protein (PPAR)γ, PPARγ coactivator 1α (PGC-1α) [78]. SIRT1 is associated with glucose metabolism, cellular differentiation,

neuro-degeneration, tumorogenesis and aging [78]. Starvation or calorie restriction positively regulates SIRT1 activity and increases cellular stress resistance ability. Some evidences show the presence of SIRT1 in mitochondria as well [79]. Cytosolic SIRT2 is involved in deacetylation of alpha tubulin (Lys40), H4 (Lys16), H3 (Lys56), NFκB, FOXO1, FOXO3a etc. [78]. It has significant role in cell cycle regulation, autophagy, DNA damage response, neurodegeneration and cancer [78]. Several studies suggest SIRT2 as a tumor suppressor protein. This sirtuin has been shown to be down-regulated in human gliomas [78]. It is also involved in the maintenance of genomic integrity by releasing severely damaged cells from mitotic arrest and forcing them into apoptosis [80]. SIRT3 is associated with mitochondrial ATP production, regulation of mitochondrial protein and fatty acid oxidation. Increased expression and activation of SIRT3 during calorie restriction reduces oxidative stress and it does that by de-acetylating and activating mitochondrial antioxidant enzyme Sod2 [81]. SIRT3 has an important role in different human malignancies. It also regulates mitochondrial structure dynamics by targeting and deacetylating OPA1, a mitochondrial fusion protein [82]. Its substrates include metabolic enzymes, components of mitochondrial respiratory chain complexes and Ku70. SIRT4 negatively regulates glutamine metabolism and hence functions as tumor suppressor [83]. SIRT5 is another sirtuin localized in mitochondrial matrix. SIRT5 mediated deacetylation of cytochrome C suggests a role of this sirtuin in stress induced, mitochondria-dependent apoptosis [84]. In nucleus SIRT6 acts as ADP-ribosyltransferase and deacetylase [85]. SIRT6 significantly contribute to telomeric functions, DNA repair, metabolic homeostasis and genome stability [78]. SIRT7, is predominantly localized in nucleus, interacts with RNA polymerase I and regulates the transcription of ribosomal genes [78].

1.2.2.5. YEAST SIR2:

All eukaryotes including *Saccharomyces cerevisiae* regulate chromatin structure both locally and globally to control transcriptional activation. Sir2 is a chromatin modifier which alters the expression of target genes through transcriptional repression. Transcriptional repression can occur locally and transiently, such as promoters of specific genes, or it can be spread across large regions of the genome that is generally repressed for long periods, sometimes for multiple generations. These transcriptionally repressed regions are called heterochromatic. In budding yeast, there are three heterochromatin regions which include *HMR*, *HML* and telomeres which are characterized by hypo-acetylation of the N-terminal tails of histones H3 and H4 [86, 87]. The repetitive ribosomal DNA (rDNA) in yeast is also included in heterochromatin and is associated with silencing of Pol II transcription within the tandem array and suppression of homologous recombination between the repeats [88, 89, 90].

In yeast Sir2 functions as a multi-protein silencing complex called Sir complex, which consists of Sir2, Sir3 and Sir4. Sir2 is the deacetylase enzyme in this multi-protein silencing complex. This Sir complex is involved in the establishment, spreading and maintenance of silent chromatin across this heterochromatin [91]. Sir2 deacetylates histones *via* cleavage of a molecule of NAD^+ into nicotinamide (NAM) and O-acetyl-ADP-ribose. NAM inhibits deacetylation activity of Sir2 and other sirtuins *in vitro* and it also negatively regulates silencing at rDNA, telomeres and mating type loci [92, 93]. Sir2 is found both in the telomere and nucleolus [94].

Silencing at homothallic loci (*HMR* and *HML*) is associated with haploid cell identity in yeast. SIR complex is recruited to the cis-acting sequences flanking the silent domains called E and I silencers of *HMR* and *HML* (Figure II). Repressor activator protein 1 (Rap1), origin recognition complex (ORC), ARS binding factor 1 (Abf1) bind to the E and I silencer region and together make a binding

platform for SIR complex. The next step of heterochromatin formation occurs by the recruitment of Sir1 which that in turn recruits Sir2-Sir3-Sir4 complex [95]. NAD^+ dependent histone deacetylase Sir2 deacetylates lysine residues on the N-terminal tails of H3 and H4. This deacetylation causes binding of Sir3-Sir4 and stabilizing the position of the nucleosome. Sir2 then deacetylates the next nucleosome spreading the silencing further. In budding yeast, there is reversible transcriptional silencing of genes near telomere called telomere silencing or telomere position effect. Telomere silencing is initiated by recruiting Sir2-Sir4 complex at the sub-telomeric regions by telomere bound proteins which include Rap1, Ku70, and Ku80 [96]. The recruitment of Sir2-Sir4 complex triggers Sir2 mediated deacetylation of lysine residues of H3 and H4 (Figure III). Sir2 mediated histone deacetylation helps Sir3 binding and spreading of the Sir complex across the sub-telomeric regions typically upto 1 or 2 kb.

Mechanism of TPE has been well documented in *S. cerevisiae*, although it was discovered in *Drosophila melanogaster* [97]. *Drosophila* species has unique and distinct composition and maintenance process but they share many features of telomeres with other species in regard to the heterochromatization of the telomeric and sub-telomeric regions. They maintain their telomeres by the transposition of the retrotransposons *HeTA* and *TART* to chromosome ends and carry several kilobases of conserved complex satellites termed Telomere Associated Sequences (TAS) [98].

HP1, a stable component of all telomeres in *Drosophila*, caps the telomere by direct binding while it contributes to telomeric silencing by interacting with tri-methylated Lysine 9 at the histone H3 tail [99, 100]. It has been demonstrated that mutation in *HP1* increases the abundance of *HeT-A* and *TART* RNA as well as their frequency of transposition to broken telomeric ends [101]. Various pathogens use TPE mechanism to acquire antigenic variation which is a highly efficient survival strategy to bypass the eradication by the host immune response [102].

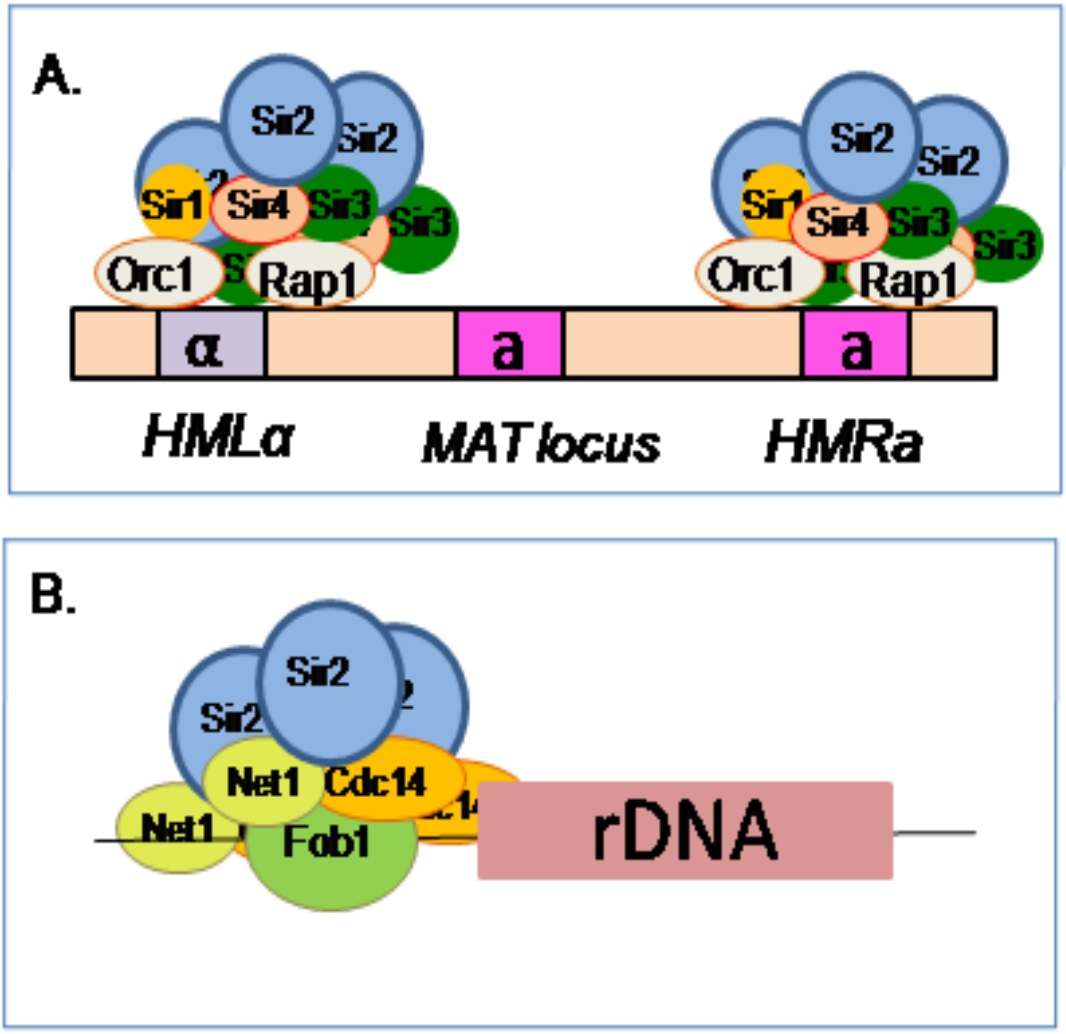

Figure II: Sir2 mediated transcriptional silencing. (A) Mating type silencing: Rap1, ORC, Abf1 bind to the silencer region and together make a binding platform for SIR complex. Then Sir1 recruits Sir2-Sir3-Sir4 complex and Sir2 deacetylates histones and recruits more Sir3-Sir4. (B) rDNA silencing: In association with Net1 and Cdc14 Sir2 forms RENT complex which is essential for the formation of silent chromatin at rDNA. RENT complex is recruited to the silencer through the interaction with Fob1 and Pol I.

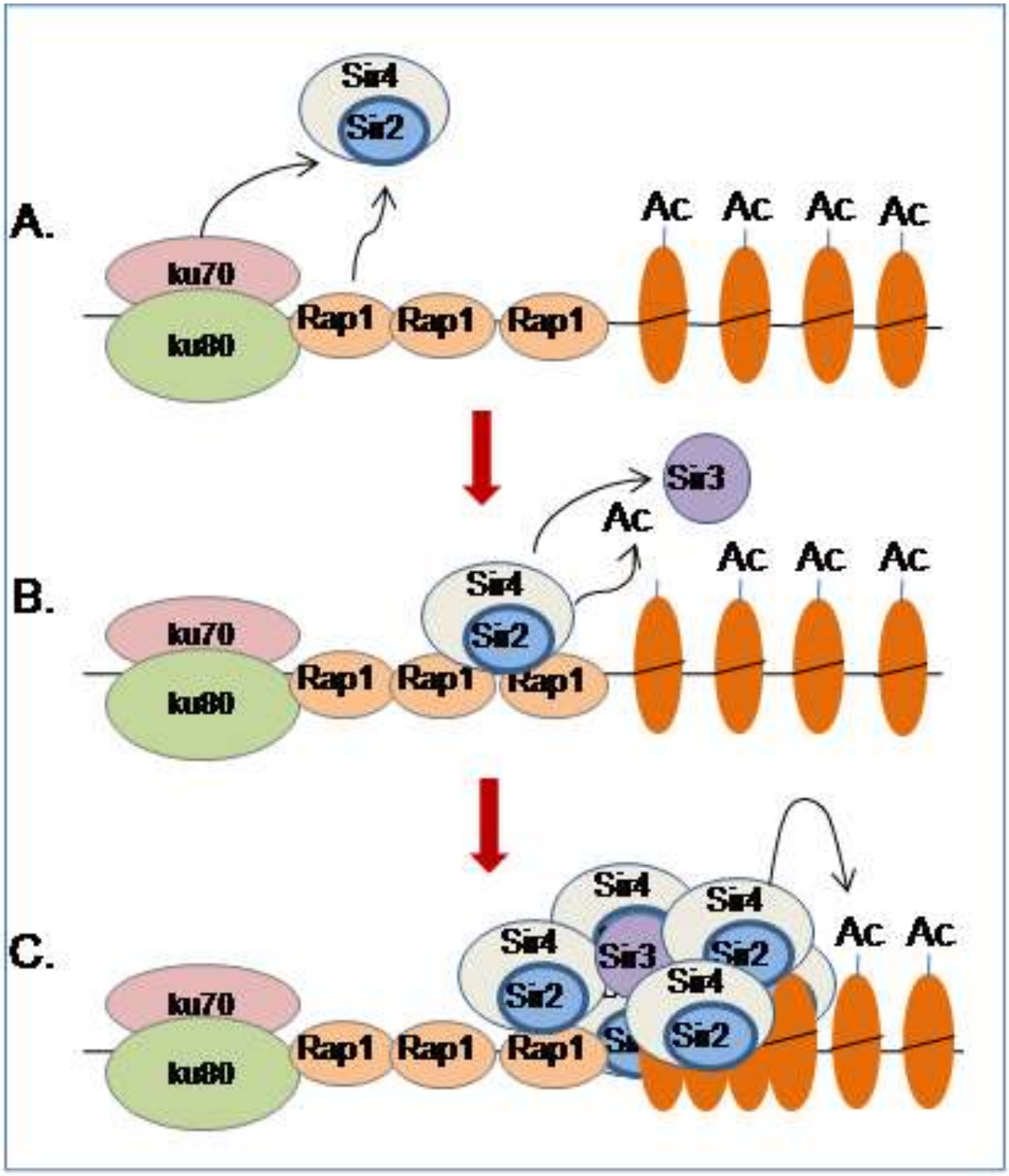

Figure III: Telomere position effect. (A) Recruitment of Sir2-Sir4 complex at the sub-telomeric region by telomere bound proteins, Rap1, Ku70, and Ku80. (B) Deacetylation of histone by Sir2 and recruitment of Sir3. (C) Spreading of Sir complex.

Such regulation has been demonstrated in *Leishmania major*, *Trypanosoma brucei*, *Plasmodium falciparum*, *Candida glabrata* and *Pneumocystis carinii*. While there is exclusive expression of a sub-telomeric vsg or MSG gene from a unique expression site in *T. brucei* and *P. carinii* respectively [103], they have approximately 20 and 100 sub-telomeric genes respectively, encoding surface glycoproteins [104, 105]. The *P. falciparum* genome encodes a SIR2 homolog (PfSir2) that is involved in silencing var gene family which encodes surface protein pfEMP1 [106]. Silent telomeric chromatin involves deacetylation of several lysine residues on histone tails particularly H4K16 [107]. Sir2 mediated deacetylation of H4K16 actively promotes high-affinity binding of Sir-complex. Spreading of the Sir-complex is controlled by opposing histone acetylation activity of other HATs, most importantly Sas2 [107]. In other organisms, most of the factors involved in TPE are the functional homologs of *S. cerevisiae* proteins. In *Schizosaccharomyces pombe*, spRap1 (homologous to Rap1 protein in *S. cerevisiae*) is recruited to the telomere by telomeric repeat-binding protein Taz1. A highly substrate specific Sir2 family member SIRT6 is involved in the silencing of sub-telomeric genes maintaining the heterochromatic feature of telomeric chromatin in human cells [108].

Sir2 is also involved in the silencing at the rDNA repeats. In this case in association with Net1 and Cdc14, Sir2 forms RENT complex (regulator of nucleolar silencing and telophase exit) which is essential for the formation of silent chromatin at rDNA. RENT complex is recruited to the silencer through the interaction with Fob1 and Pol I (Figure II) [107]. Sir2 dependent silencing extends along with Pol I transcription [107].

1.2.2.6. FUNCTIONS OF TPE:

In *S. cerevisiae* genome-wide studies of transcription levels determined that about 267 genes, located less than 20 kb from the telomeres which display a mean expression level that is roughly 20% of non-telomeric gene expression [109]. Among these genes, less than 10% are silenced in Sir-

dependent manner whereas one half is derepressed upon H4 depletion, which is about three times more than for non-telomeric genes [109]. Genome-wide study identified a lysine deacetylase specific for H2B and H3 that is involved in the Sir-independent silencing of genes clustered in domains 10-25 kb from telomere, termed *HAST* (Hda1-Affected Sub-telomeric) [110]. According to a recent study in closely related *Hemiascomycetes* species, sub-telomeric gene families are in general specific for the different species and possess different copy number and sub-telomeric distribution [111]. It has been found in several yeast species that the expressions of these genes are influenced by TPE [112, 113]. In many yeast species, during non-optimal growth conditions sub-telomeric enrichment of genes related to stress response and metabolism appears to be a conserved feature whereas, most of these genes are silenced under optimal growth conditions [110]. In budding yeast, various stress conditions like nutrient starvation, heat shock or chemical treatment can cause hyperphosphorylation of Sir3p and a consequent decrease in TPE at the truncated VII-L [114]. This also induces the expression of natural sub-telomeric genes such as *PAU* genes which are involved in cell wall constitution and drug resistance [115]. In *Candida glabrata* expression of cell wall proteins is also Sir-dependent [116]. Sub-telomeric *HAST* domains in *S. cerevisiae* contain clusters of normally silenced genes that are involved in neoglucogenesis or stress response [110]. For example, the FLO genes, involved in cellular adherence, mostly belong to these domains and are repressed in a Sir-independent way which is however dependent upon Sir2 homologs, Hst1 and Hst2 [117]. In *S. pombe*, many genes related to nitrogen starvation are also clustered in sub-telomeric regions andsilenced by Hda1 ortholog, Clr3 [118]. There may be other functions of silencing proteins at telomeres in addition to transcriptional silencing. Upon DNA double strand breaks Rap1p, Sir and the Ku complex lose their punctate, peripheral localization and become dispersed throughout the nucleus [96]. Dispersal of silencing proteins leads to recruitment of the Ku complex to chromosome

breaks, followed by movement of the Sir complex to the same sites. These events are induced even by a single double strand break. Ku complex plays important role in DNA repair [95, 119]. Moreover, in at least some genetic backgrounds, strains that are deficient of Sir proteins show hypersensitivity to DNA damage [95]. These findings suggest that telomeres provide a reservoir of repair proteins that can be readily mobilized in response to DNA damage.

Yeast aging process, a genetically programmed step, includes movement of silencing proteins away from telomeres. During aging the Sir complex re-localizes from telomeres to the nucleolus, which contains the genes that encode ribosomal RNA (rRNA) [120]. Moreover, the NAD dependent deacetylase activity of Sir2 in association with its partner proteins Sir3 and Sir4, establishes a chromatin structure that causes transcriptional silencing of RNA polymerase II (Pol II) in budding yeast [121].

1.2.2.7. TELOMERIC LENGTH AND TELOMERE SILENCING:

Increase in the length of telomeres strengthens TPE [122, 123, 124]. As an explanation, longer telomeres may allow binding of more Rap1p that subsequently recruits more Sir-complexes and thereby facilitates the formation of a heterochromatin complex allowing spreading within the sub-telomeric regions. It has been shown that inactivation of the Rif proteins improves TPE and increases telomere length by alleviating the competition with Sir proteins for access to telomere bound Rap1p [125, 126]. Silencing can be induced by insertion of a stretch of telomeric repeats at an internal chromosomal site through Rap1p binding. This effect is not as strong as telomeric silencing, which can account for Ku participation in the recruitment of silencing factors [127] and and proper sub-nuclear localization within heterochromatin compartments [128, 129]. These data suggest that the influence of telomere length on TPE is not merely the length by itself but concomitant changes in the recruitment of different factors for silencing.

According to a study by Martin Kupiec et al [130], beverages may affect human telomere length homeostasis. Using series of control experiments the authors have shown the possible effect of environment on yeast telomere length and pinpointed the underlying molecular mechanism by which environment modifies genome. In this study the authors have shown the effect of different stresses on telomere length. Alcohol and acetic acid gives rise to elongated telomere while caffeine and high temperature is associated with telomere shortening. However, there is no effect of oxidative stress on telomere length alteration. This study reveals that environmental factors regulate telomere length through Rap1/Rif1 pathway. Another study in yeast has found that over-expression of Hsc82 reverses the shortening of telomere defect due to mutations in telomere maintenance proteins Stn1 and Cdc13 [131]. Loss of telomere length has also been observed in wild type cells upon over-expression of Hsc82, or of its temperature-inducible homolog, Hsp82 [131].

1.2.2.8. REGULATION OF SIRTUINS:

Sirtuins, NAD^+ dependent protein deacetylases, are sensitive to changes in the intracellular concentration of NAD^+. In yeast, NAD^+ is synthesized from tryptophan and there are three vitamin precursors of NAD^+ which are nicotinic acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR). Biosynthesis, salvage pathways and balanced secretion or import of vitamin precursors contribute to the NAD^+ homeostasis in yeast. There is de novo synthesis of NAD^+ from tryptophan in the absence of in growth medium. Nicotinic acid phosphoribosyl transferase (Npt1) is the rate limiting enzyme in NAD^+ biosynthesis and deletion of Npt1 results in inhibition of Sir2 mediated transcriptional silencing [132]. NR gives rise to NAM by several nucleoside hydrolyses and phosphorylases in the cell. Nicotinamidase 1 (Pnc1) converts NAM into NA by deamidating and hence increases Sir2 activity. Presence of NAM in the growth medium inhibits Sir2 activity leading to *⊿sir2* phenotype [93]. Over-expression of PNC1 suppresses this phenotype by scavenging NAM

by deamidation. Pnc1 also helps in extending replicative life-span even in the absence of NAM in the growth medium.

In human cell line, it has been demonstrated that SIRT1 protein associates with HIC1 (hypermethylated in cancer 1) and the complex binds to the *SIRT1* promoter to negatively regulate its own transcription [133]. Hypermethylation of *HIC1* promoter leads to epigenetic silencing of *HIC1* thereby causing aging. Lower HIC1 expression results in *SIRT1* up-regulation which results in excessive deacetylation and deactivation of p53 function and thus increases the cancer risk in mammals. Any such feedback inhibition of *SIR2* expression by Sir2 itself in yeast is not yet known.

1.2.2.9. ROLE OF SIRTUINS IN LONGEVITY:

In higher eukaryotes, telomere length is the measure of cellular senescence. Primary cells such as fibroblasts do not express telomerase as a result these cells show shortening of telomeres with each cell division. When telomeres become too short to meet a point of crisis, the cells either senesce or occasionally repair their telomeres through recombination-mediated alternative lengthening of telomere (ALT) mechanism which help the cells to survive. But in yeast both aging mother and daughter cell do not show shortening of telomere with cell division due to presence of constitutively expressed telomerase. Yeast cells with inactivated telomerase show progressively shortening of telomeres in mother and daughter cells which causes senescence of yeast population. Study shows that deletion of Sir2 in ⊿*est2* ⊿*rad52* strain accelerates senescence in yeast [134]. Recently it has been shown that telomere regulatory protein Rif1 is involved in maintenance of proper Sir2 balance at rDNA for replicative life-span (RLS) by limiting excessive Sir2 recruitment to telomeres and HM loci [135]. Furthermore, increase in Sir2 suppresses rDNA recombination resulting in elevated RLS in yeast [107]. In worms, genome duplication carrying SIR-2.1 extended life-span by 10% while strains lacking SIR-2.1 showed shortening of lifespan [107]. Over-expression of dSir2 increases

lifespan by 75% in fruit fly [107]. Brain specific SIRT1 over-expression increases life span by 11% in mouse model and delays cancer related death [107].

Study shows that reduction in an organism's calorie intake while maintaining proper nutrition extends life span from yeast to primates. Genetic studies in simple organisms such as yeast and fruit fly reveal the role of sirtuins in calorie restriction (CR). Specifically in yeast, CR induces NAD^+/NADH ratio because of elevated level of respiration and this increased NAD^+ helps Sir2 mediated deacetylation [136]. Most interesting result came from genetic experiments which show deletion of Sir2 in yeast can block CR meditated lifespan extension. Similar results have been found in worms and fruit flies also. In mammals, despite direct genetic tests, different evidence suggests that sirtuins are regulated in various tissues at expression level as well as in metabolic states.

1.3.1. HEAT SHOCK PROTEINS:

It is extremely important to maintain the balance between the synthesis, folding and degradation of proteins in cells. Proteins are very sensitive to stresses such as heat, oxidative stress, inflammation, irradiation, heavy metals or other toxic compounds which lead to protein unfolding, nonspecific aggregation resulting in imbalance in protein homeostasis. However, this balance is maintained through two major pathways: i) cellular degradation machinery which target proteins for proteolysis, and ii) molecular chaperones which ensure proper protein folding to their native state and prevent them from aggregation. Heat shock proteins (HSPs) mainly exist as molecular chaperones in the cell and play essential role in maintenance of protein homeostasis during cellular stress. HSPs are highly conserved and found to be up-regulated during various stressful conditions. HSPs comprise of four large and ubiquitous, ATP dependent families: Hsp100, Hsp90, Hsp70 and Hsp60 [137]. There is other ATP independent HSPs such as small HSPs. Hsp60 family helps proper folding of the mitochondrial proteins and prevent them from aggregation. Hsp90 and Hsp70 bind to the unfolded

proteins and provide correct folding. Hsp100 is involved in solubilizing aggregated proteins. HSP40 is the intermediate chaperone protein between high molecular weight proteins and small HSPs. DnaJ of *Escherichia coli* is homologous to Hsp40. J domain of Hsp40 plays an important role in binding to the unfolded client proteins and consequently transferring them to Hsp70. Small HSPs like Hsp27 and α-crystallin are ubiquitous proteins induced under different kind of cellular stresses. The main function of small HSPs is to prevent accumulation of aggregated proteins.

1.3.2. HEAT SHOCK PROTEIN 90 [Hsp90]:

The most abundant and highly conserved molecular chaperone is the heat shock protein 90 found in many organisms from prokaryotes to eukaryotes [138]. Hsp90 proteins are localized in cytosol, nucleoplasm, endoplasmic reticulum (ER), mitochondria and chloroplast [137]. This ubiquitous protein is essential for cell viability in eukaryotes [139]. In eukaryotic cell Hsp90 accounts for nearly 1% of the total cellular proteins. This protein is constitutively expressed under normal conditions and its expression is further increased by 10-fold during different stresses. Over-expression of Hsp90 is seen in cells exposed to heat shock and other stressed condition such as cancer [140]. Hsp90 plays an essential role in conformational maturation of nascent polypeptides and refolding of denatured and unfolded proteins [141]. In tumor cells, many oncogenic proteins take the advantage of Hsp90 protein folding machinery for their maturation, stability and activation. Hsp90 possesses more than 100 client proteins which include cellular signaling molecules such as steroid hormone receptors, transcription factors and signaling kinases. In association with a defined cohort of co-chaperones Hsp90 regulates its client proteins in ATP dependent manner. This molecular chaperone shows exceptional neuro-protective properties as it helps in the refolding of several aggregated proteins associated with neurodegenerative diseases.

Human Hsp90 exists as homodimer and contains three highly conserved domains: a 25 kDa N-terminal, which contains ATP-binding domain, a 35 kDa middle domain and a 12 kDa C-terminal dimerization domain. Function of Hsp90 depends on ATP binding and its hydrolyzing ability. There is an unstructured highly charged linker region which joins N-terminal domain to middle domain. The middle domain has high affinity for client proteins and co-chaperones. Structural and functional analysis of Hsp90 shows that the middle domain consists of a catalytic loop which interacts with the γ-phosphate of ATP when it is associated with the N-terminus [141]. C-terminal domain contains a conserved five amino acid stretch, MEEVD which that binds with the co-chaperones containing multiple copies of 34 amino acid long tetratricopeptide repeat (TPR). There is a second ATP binding site found in the C-terminal domain.

In human, four isoforms of Hsp90 have been identified. There are two cytosolic isoforms which include the major, inducible Hsp90α and the minor, Hsp90β which is constitutively expressed. There is almost 85% sequence identity between these isoforms. The client specificity of the two isoforms may vary due to the slight differences in their amino acid sequences. Study showed that Hsp90α has higher affinity for heat shock transcription factor and v-src than Hsp90β [142]. In contrast, some client proteins demonstrated less dependence on Hsp90 isoforms. The other two isoforms of Hsp90 are glucose regulated protein 94 (grp94), present in the ER and Hsp75/TRAP1 in the mitochondrial matrix. In contrast to the eukaryotic counterparts, bacterial high temperature protein G (HtpG) is not an essential gene for bacteria and has moderate effects on growth during high temperature. Mitochondrial TRAP family of Hsp90 is closely related to HtpG like proteins. In budding yeast, two isoforms of Hsp90 are found: constitutively expressed Hsc82 which is the human ortholog of Hsp90β and Hsp82 which is the human ortholog of Hsp90α. Hsp82 is induced whenever cells are exposed to stress conditions [141]. These isoforms are 97% identical but are regulated by different

pathways. In normal physiological conditions Hsc82 level is 20 fold higher than Hsp82; however, upon heat stress there is an up-regulation of Hsp82 by more than 20 fold [143].

1.3.3. HSP90 IN DYNAMIC CHAPERONE CYCLE:

Recognition of diverse sets of clients by Hsp90 depends on its highly dynamic conformational shifts upon binding and hydrolysis of ATP molecules. While biochemical studies suggested that binding of ATP facilitates transition of ADP-bound open conformation to ATP-bound closed conformation, structural studies and mutagenesis added that ATP hydrolysis also plays an important role in structural rearrangements [144]. These rearrangements are essential for the client protein maturation by Hsp90.

In normal condition, Hsp90 is bound to the transcription factor, heat shock factor 1 (HSF1), however, upon stress HSF1 is released from Hsp90 and translocates to the nucleus [141]. In the nucleus, HSF1 is phosphorylated and forms trimer and this activated trimer binds to the promoter of Hsps (also called heat shock response elements) to induce transcription of Hsps [146, 147].

Hsp70 in association with ATP and Hsp40 binds to the nascent polypeptides just after their release from ribosome and prevents them from aggregation. Hsp70-ADP-client protein complex is subsequently stabilized by binding of Hsp70 interacting protein (HIP). In the next step, Hsp90 binds with the Hsp70-client protein complex with the help of the Hsp90-Hsp70 organizing protein (HOP) to form a multi-protein complex [148]. With the help of immunophilins, co-chaperones and other partner proteins, client protein is then transferred from Hsp70 to Hsp90 for final maturation and subsequently Hsp70, Hip and Hop are dissociated from the complex [149]. At this point, binding of ATP molecule to Hsp90 around the client protein substrate gives rise to a closed clamp structure. Destabilization of this complex by Hsp90-inhibitors leads to the proteasomal degradation of the client protein [141]. Co-chaperone p23 binds to this ATP-bound multi-protein complex and causes

ATP hydrolysis for the final conformational maturation of client protein. p23 also helps in the release of properly folded protein from Hsp90 [150]. Once the matured protein is released, Hsp90 enters into another cycle of protein folding.

1.3.4. HSP90 IS DIFFERENT FROM OTHER MOLECULAR CHAPERONES IN TERMS OF CLIENT PROTEIN MATURATION:

In contrast to other chaperones, nucleotide binding to Hsp90 does not lock it to specific conformation. Without nucleotide binding, human HSP90 is found in open conformation where in *E. coli* and *S. cerevisiae*, it exists in both the open and closed forms [144]. This high degree of conformational transitions are regulated by different co-chaperones and binding of diverse client proteins. While Hsp40 and Hsp70 molecular chaperones are engaged in refolding of mis-folded proteins, Hsp90 is essential for many client proteins to prevent them from aggregation and for their activity. Hsp90 mainly prevents its client proteins from unfolding. Hsp90 forms complex with the majority of its clients and maintains them in an inactive state (Figure IV). The dissociation of clients from Hsp90 makes them active. For example, stress induced release of HSF1 from Hsp90 and facilitates the trimerization and activation of HSF1.

A detailed structural study of glucocorticoid receptor (GR) interpreted the chaperone activity of Hsp90. While initial folding of nascent chains involves Hsp70, final maturation is given by Hsp90 which helps GR to achieve a hormone-activated state [151]. In absence of Hsp90 GR has 100-fold low affinity for glucocorticoids (GCs). By forming a complex with GR, Hsp90 contributes un-liganded GR inactive as a transcription factor. ATP-bound Hsp90-GR complex is stabilized by p23 which opens the ligand-binding cleft of GR. Binding of steroid hormone closes ligand-binding cleft and shifts the Hsp90 interaction from stable to dynamic which causes activation of GR [151].

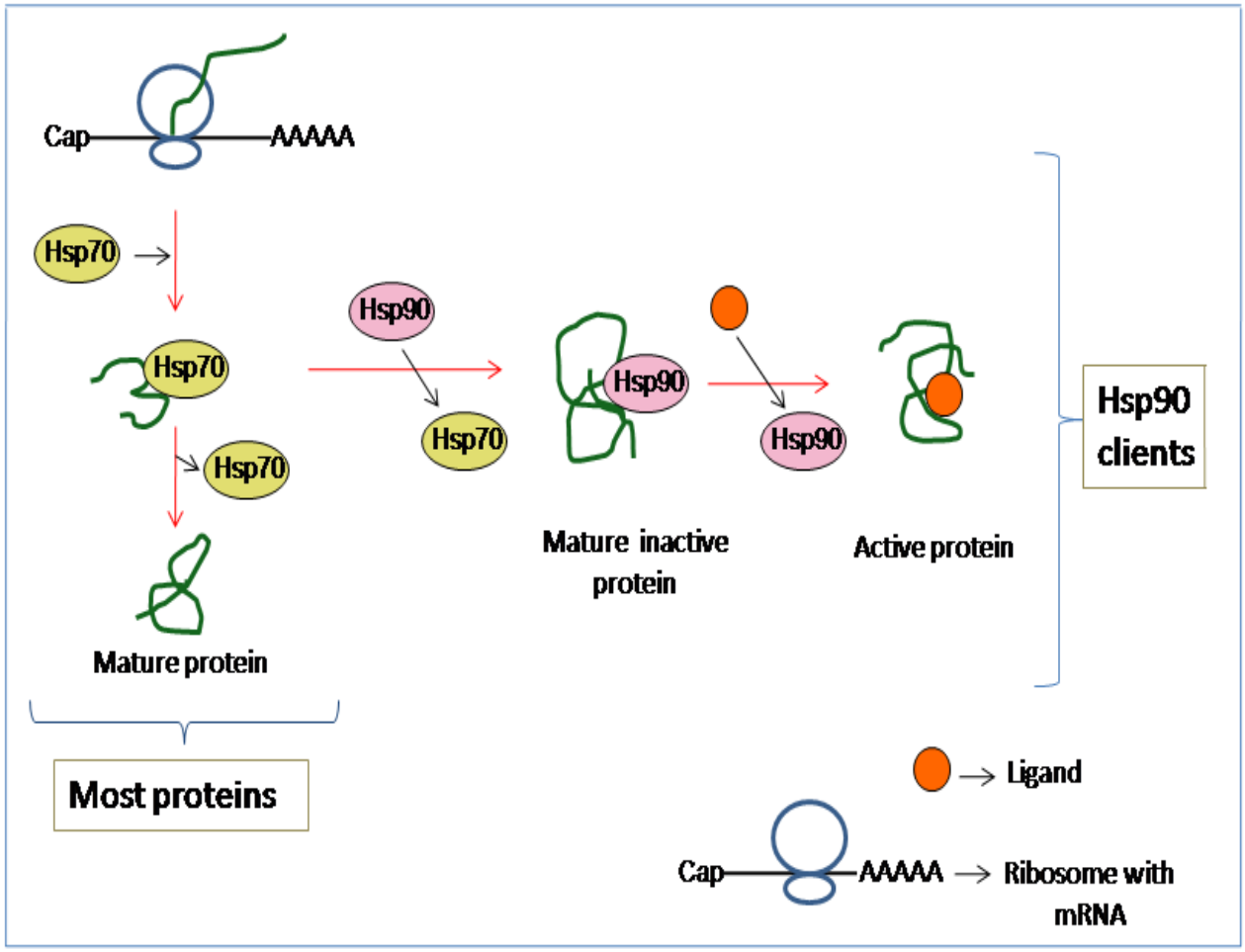

Figure IV: Role of Hsp90 in folding and regulation of protein.

Another example of Hsp90's role in influencing client protein activity is demonstrated by its interaction with glycogen synthase kinase (GSK). Once bound to Hsp90, GSK acts as an auto phosphorylating tyrosine kinase. When GSK is released from Hsp90, it becomes serine/threonine kinase [152]. These evidences suggest that Hsp90 stabilizes particular conformation of client proteins in order to maintain their functionality and thereby preventing them from unfolding and degradation.

1.3.5. REGULATION OF HSP90 FUNCTION:

Hsp90 functions are remarkably regulated by different biochemical pathways. Besides, ATP binding and hydrolysis, Hsp90 depends on diverse co-chaperones for its function. In addition, both Hsp90 and its co-chaperones undergo various post-translational modifications. The combinatorial regulations of Hsp90 by co-chaperones and post-translational modifications are discussed below.

1.3.5.1. HSP90 AND ITS CO-CHAPERONES:

In eukaryotic cell, there are more than 20 co-chaperones of Hsp90. Some co-chaperones are essential in certain organisms but absent in others. Co-chaperones modulate Hsp90 function in four different ways: 1) they functionally coordinate between Hsp90 and other chaperones; 2) the ATPase activity of Hsp90 is modulated by co-chaperones; 3) specific client proteins of Hsp90 are recruited by them; 4) they contribute to the chaperone cycle through their enzymatic activities [144]. The majority of co-chaperones contain TPR (tricopeptide repeat) domain which binds to the MEEVD region of Hsp90 [153]. Some of them recruit specific protein and some recruit multi-protein complexes. For example, Hop is involved in the recruitment of Hsp70-Hsp90 complex. Others have different enzymatic activities like phosphatase (eg. Ser/Thr protein phosphatase 5) and prolyl isomerase function (eg. Immunophilin cyclophilin 40) [154, 155, 156]. Cooperative action of Hsp40, Hsp70 and Hsp90 is dependent on TPR domain containing co-chaperones. Many co-chaperones

inhibit or facilitate the ATPase activity of Hsp90. Inhibitors of ATPase function include Hop, Cdc37 and p23 and activators of ATPase include AHA1 and peptidyl-prolyl cis-trans isomerase 6 (Cpr6) [144].

1.3.5.2. POST-TRANSLATIONAL MODIFICATIONS:

Post-translational modifications like acetylation, phosphorylation, nitrosylation also contribute to the regulation of Hsp90 function. For example, interaction of Hsp90β with aryl hydrocarbon receptor (AHR) is regulated by phosphorylation of its charged linker region. Mutational analysis suggests that phosphorylation negatively regulate HSP90-AHR complex formation [157]. In contrast, phosphorylation of Hsp90β facilitates binding of its client, endothelial nitric oxide synthase (eNOS) [158]. In yeast, phosphorylation of Hsp82 at Tyr24 is responsible for inhibition of its ATPase activity and dimerization [159]. Acetylation in different parts of Hsp90 is another regulatory mechanism. Both client protein maturation and co-chaperone binding is impaired by acetylation at Lys 294 [160]. Nitrosylation at Cys 597 inhibits functional maturation of eNOS [161]. In addition, post-translational modifications of Hsp90's co-chaperones also contribute to its functions. Both in mammals and yeast, Cdc37 is found to be phosphorylated at Ser13 (Ser14 in yeast) by casein kinase 2α (CK2α) [162]. This regulation by phosphorylation is an essential part of Hsp90's chaperone cycle.

1.3.6. CLIENTS OF HSP90:

To establish whether a protein is a client of Hsp90 it should follow three lines of evidences [144]. This protein must physically interact with Hsp90. ii) Functional inhibition of Hsp90 must result in lower level and loss of activity of this protein. iii) Inhibition of Hsp90's function must lead to proteasomal degradation of this protein.

The HSP90 chaperone network contributes to a wide variety of client proteins operating in a number of pathways required for cell viability, including telomere maintenance, signal transduction,

transcription, protein transport, chromatin remodeling, and protein stabilization (Figure V) [162, 150]. The two main classes of Hsp90 clients are protein kinases and nuclear steroid receptors. These are the best understood clients of Hsp90. Among its telomeric clients telomerase is the well established client of Hsp90. HSP90 and p23 are the first two proteins shown to interact directly with telomerase and regulates its enzymatic activity [163]. Hsp90 contributes to telomere length maintenance by promoting both DNA binding and nucleotide affinity for telomerase [164, 165]. Genetic studies have suggested numerous telomeric clients of Hsp90. Proteomic, bioinformatics, and genetic approaches unravel many more telomeric proteins which are potential clients of Hsp82 such as Cdc13, Stn1, [131, 166], Sir2 [167], Mre11 [168], Ku80, Mec1 and Est1 [166]. It has been established that Cdc13 along with Stn1 and Ten1 forms an un-extendable telomere structure which is dissociated in presence of yHsp90 [169]. Hence, Hsp90 participates in extending telomere structure.

It was observed that Hsp90 co-precipitated with the oncogene Tyr kinase v-Src. It also co-purified with other v-Src like proteins which include Fes, Fps, Yes [144]. Hsp90 was also found with several nuclear hormone receptor complexes [170, 171, 172].

The DNA repair protein Rad51 has been established as a client of Hsp90 [173]. Functional inactivation of yeast Hsp90 causes proteasomal degradation of Rad51 and DNA damage sensitivity in yeast.

In plants, Hsp90 interacts with many cytoplasmic sensor proteins like nucleotide binding Leu-rich repeat (NLR) proteins [174]. Hsp90 with help of its co-chaperone SGT1 gives stability to these proteins. Mutations in Hsp90 and inhibition of Hsp90 function reduce the cellular level of NLR proteins and compromise their functions. Hsp90 interact with mammalian NOD-like receptors as well which are functionally similar to NLR proteins [175].

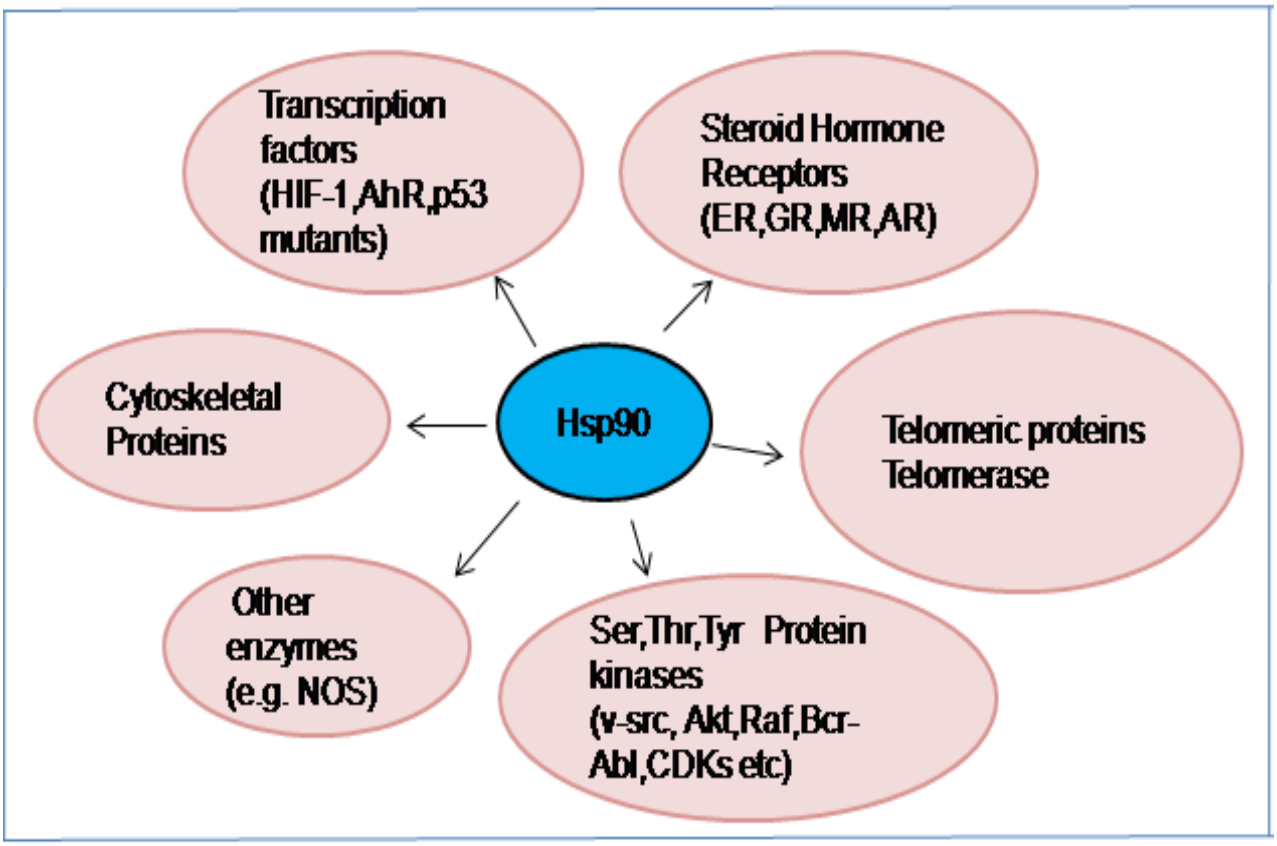

Figure V: Hsp90 clientele.

Hsp90 interacts with RhoGDIα and regulates its function which is required for recycling of numerous Rab proteins and hence it is involved in protein trafficking [176]. In both mammalian and yeast cell, Hsp90 inhibition leads to impaired protein secretion demonstrating a conserved role in protein trafficking. Recent studies illustrated Hsp90's involvement in RNA processing. In yeast, Hsp90 in association with its co-chaperone, TPR-containing protein regulates function of the R2TP complex (Rvb1, Rvb2, Tah1 and Pih1) which are involved in ribosomal RNA processing [177]. Inhibition of Hsp90 results in the disappearance of RNA processing factors. In flies, inhibition of Hsp90 reduces accumulation of pi-RNA (piwi-associated RNA) and activates numerous transposons [178].

1.3.7. THE ROLE OF HSP90 ON TRANSCRIPTION:

Though most of the clients of Hsp90 protein include signal transduction proteins, a significant number of its clients are transcription factors. Several recent findings have demonstrated the regulatory involvement of Hsp90 in transcription of certain genes. It has been well demonstrated the involvement of Hsp90 and its co-chaperone p23 in the release of glucocorticoid receptors from chromatin in absence of hormonal ligand [179]. Similarly Hsp90 is found to stabilize BCL-6 at promoters of target genes involved in B-cell lymphoma [180]. Other transcription factors which are regulated by Hsp90 include Sp1 [181], signal transducer and activator of transcription 5 (STAT5) [182] and MyoD1 [183] (Figure VI). Experiments in yeast have shown that Hsp90 also aids in removing nucleosomes from induced genes, allowing gene transcription by RNA polymerase II (pol II) such as induction of galactose genes in budding yeast [184]. According to a recent finding, Hsp90 in conjunction with Hsp70 is involved in histone turnover in cytosol via chaperone-mediated autophagy (CMA) [185]. Hsp90 also functions in the context of gene expression by regulating certain epigenetic modifiers.

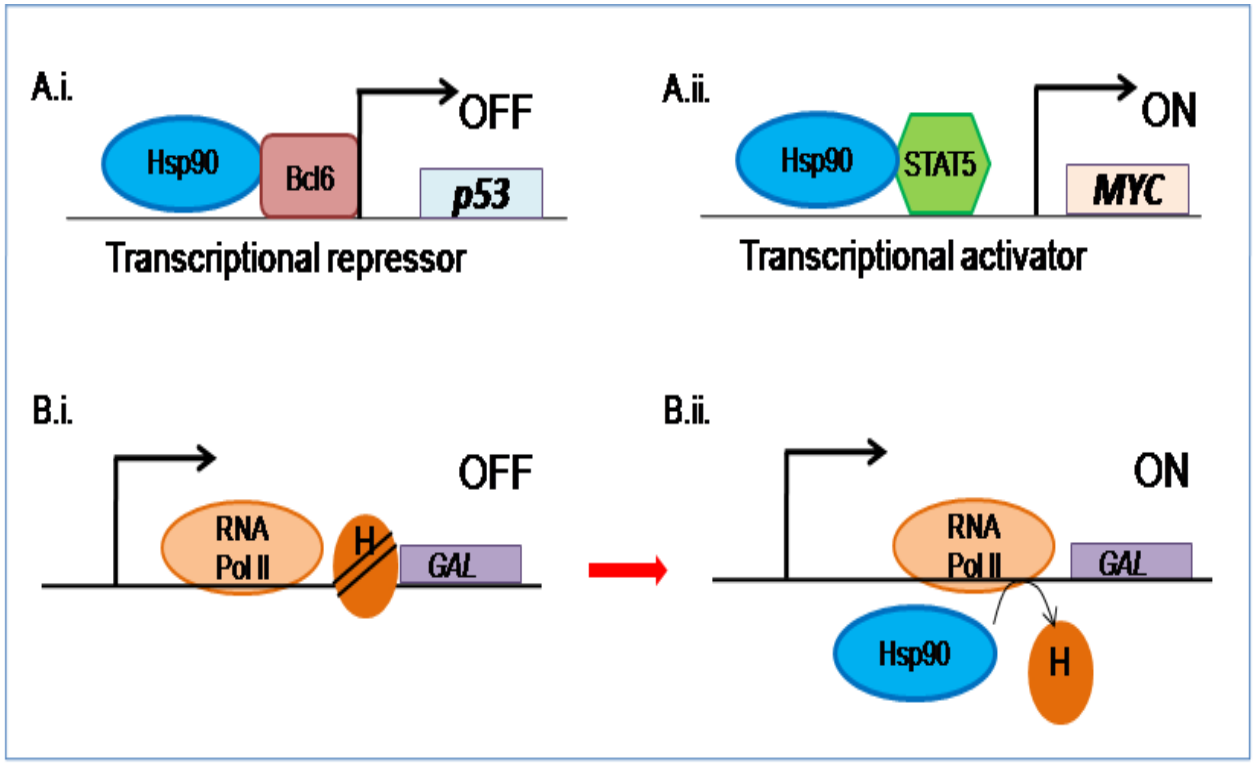

Figure VI: The role of Hsp90 on transcription: (A.) Involvement of Hsp90 in transcriptional regulation through chaperoning different transcription factors: (A.i) Hsp90 is found to stabilize Bcl-6 and repress *p53*. (A.ii.) Hsp90 regulates transcriptional activator STAT5. (B) Hsp90 is found to control eviction of histones from promoter region of *GAL* gene. (B.i.) Transcription is paused as RNA PolII is stalled (B.ii.) Removal of histone turns on transcription of *GAL*.

It has several well characterized genes which are subject to epigenetic control, such as MYC and TP53 [186]. In Drosophila, inhibition of Hsp90 results in down-regulation of Hox target genes which causes concomitant degradation of Trithorax, an epigenetic regulator of gene expression [187]. Using chromatin immunoprecipitation sequencing (ChIP-seq) of cultured fly cells, a global and high-resolution chromatin-binding profile of Hsp90 shows accumulation of this chaperone in the transcription start sites (TSSs) of one third of all protein-coding genes and several miRNA-coding genes [188]. Interestingly this study shows that all Hsp90-target genes exhibit the paused state of RNA polymerase II (pol II) and Hsp90 inhibition releases this pause.

1.4. SIGNIFICANCE OF THE STUDY

In human cell line, it has been demonstrated that SirT1 in association with HIC1 (hypermethylated in cancer 1) binds to the *SIRT1* promoter to repress its own transcription [133]. Epigenetic modification of *HIC1* promoter causes silencing of *HIC1* which results in aging. Reduction in HIC1 expression results in *SIRT1* up-regulation which results in deactivation of p53 function by excessive deacetylation and thus increases cancer risk in mammals. Any such transcriptional regulation of *SIR2* in yeast is not known. We, for the first time, demonstrated regulation of *SIR2* in yeast in response to environmental stimuli.

In recent years Hsp90 has achieved tremendous prospective as anticancer target. The total cellular pool of Hsp90 is found to increase from 1% to 10% when cells become malignant. It plays a role in functional and conformational stability of many proteins in signal transduction, some of which are oncogenic; ErbB2 and Raf-1. Our present study shows that Hsp90 also participates in epigenetic modifications. Our finding clearly demonstrates that the endogenous level and activity of the histone deacetylase is controlled by Hsp90 through two independent pathways. Firstly our work gave compelling evidence that Sir2 is a direct client of Hsp90. On the other hand under Hsp90 over-expressed condition, Sir2 undergoes transcriptional down regulation in a Cup9 dependent manner. It is thus very important to understand whether the same phenomenon also exist in mammalian cell under carcinogenic condition while Hsp90 level is found to be elevated. Understanding such mechanism will lead to better insight of epigenetic regulation in cancerous cell.

According to our findings a short period of heat shock rendered the cell as *sir2* knockdown for more than ninety generations and causes defect in yeast mating behavior which has tremendous implications on yeast physiology. This observation is very important in a broader context as Sir2 is one of the epigenetic factors that establishes silencing at subtelomeric regions in many eukaryotes.

In protozoan parasites such as *Plasmodium* and *Trypanosomes,* Sir2 mediated telomere silencing plays a major role in mutually exclusive expression of virulent multi-gene family [217, 218]. Such a mechanism controls antigenic variation and thereby involves in evasion of host immune system [219]. In the light of our findings, it will be interesting to explore whether exposure to elevated temperature (around 39˚C) as a natural consequence of *Plasmodium* infection has any correlation with derepression of subtelomeric virulent genes as a consequence of lower Sir2 activity.

Our study shows that transient heat shock leads to shortening of telomere length which is maintained through multiple generations in unicellular fungi and gradually restored to wild-type length. These findings are very significant in a way that it not only provides direct evidence for the effects of environment on telomere length but also identifies the molecular mechanism which links environmental signal to genome maintenance. This study opens up new avenue to investigate the effect of different stresses on telomere length which may be useful for the treatment for age related diseases.

1.5. OBJECTIVES OF THE STUDY

My overall research goal is to find out the link between environmental factors and the emerging phenotype due to alterations in gene expression. To that end using *Saccharomyces cerevisiae* as a genetic model I have explored whether environmental cue like heat stress has any effect on the expression and activity of the type III NAD^+ dependent histone deacetylase Sir2. In budding yeast, histone modification is one of the major chromatin modifications that lead to alterations in gene expression. Studies have shown that heat shock causes transcriptional up regulation of heat shock protein 90 which modulates significant number of proteins and gives them conformational and functional stability. Though most of the clients of Hsp90 include signal transduction proteins, a significant number of its clients are transcription factors. A two hybrid screen of yeast proteome for Hsp90 interactors uncovers that one of the major histone modifiers, Sir2 interacts with Hsp90 [167]. We hypothesize that Hsp90 might be required for the maturation and activity of Sir2. Our study shows that Sir2 level and activity is modulated by two different pathways under Hsp90 inactivated and over-expressed condition.

1.6. SPECIFIC AIMS:

I. **To study the role of Hsp90 in functional maturation of Sir2:**

Using a temperature sensitive mutant of Hsp90 (*iG170Dhsp82*) we have analyzed the cellular abundance and functions of Sir2 to understand whether Sir2 is a direct client of Hsp90.

II. **To investigate of the effect of heat shock or Hsp90 over-expression in cellular abundance and function of Sir2:**

We explored whether a transient heat shock or over-expression of Hsp90 regulates the abundance and activity of Sir2. We mimicked the Hsp90 over-expressed condition in *Saccharomyces cerevisiae* by transforming an episomal centromeric plasmid which over-expresses Hsp90 and studied its effect on Sir2 activity.

III. **Identification of the cis-regulatory and trans-regulatory elements associated with Hsp90 mediated regulation of *SIR2*:**

The results from specific aim 2 reveal that over-expression of Hsp90 causes transcriptional down regulation of *SIR2*. Using a series of genetic and biochemical experiments we have identified the cis-regulatory region as well as the trans-regulatory factor (repressor) responsible for transcriptional regulation of *SIR2* during heat shock.

CHAPTER 2

MATERIALS AND METHODS

2.1. MOLECULAR CELL BIOLOGY METHODS:

2.1.1. BACTERIAL PLASMID DNA ISOLATION BY ALKALINE LYSIS METHOD:

Bacterial colony harboring the plasmid DNA was inoculated in 5 ml of LB medium containing appropriate antibiotic. This was incubated overnight at 37°C at 200 rpm. The culture was centrifuged at 4000 rpm for 15 minutes to collect the cells. The supernatant was discarded completely, and the pellet was resuspended in 200µl of solution I (Tris 25 mM pH 8, EDTA 10 mM) and this suspension was transferred to a microfuge tube on ice. Then 200 µl of solution II (NaOH, SDS 1%) was added to the tube and mixed well by inverting the tube 4-5 times. This suspension was incubated at room temperature for 5 minutes. Then 150 µl of chilled solution III (NaOAc, 3M) was added to the tube, mixed well by inverting the tube 4-5 times, and incubated on ice for 5 minutes with intermittent mixing. The sample was then centrifuged at 12000 rpm for 15 minutes at room temperature. The supernatant was collected in another fresh microfuge tube. 2.2 volume of absolute alcohol was added to the tube, mixed well and kept at -20°C. The sample was then centrifuged at 12000 rpm, 4°C to precipitate the DNA. The supernatant was discarded and the pellet was washed by adding 70% alcohol. The pellet was air-dried, and then resuspended in 50 µl of 1X Tris-EDTA buffer. To remove RNA, RNase (10 mg/ml) solution was added to the sample and incubated at 37°C. The RNase treated sample was extracted by Phenol Chloroform Isoamyl alcohol mixture. Then it was centrifuged at 12000 for 15 minutes and the upper aqueous layer containing DNA was precipitated at -80°C. The tube was then centrifuged to collect plasmid DNA followed by washing it by 70% alcohol. The pellet was air-dried and resuspended in 30 µl 1X TE.

2.1.2. BACTERIAL COMPETENT CELL PREPARATION:

A single bacterial colony was incubated in 10 ml of LB medium containing appropriate antibiotics for overnight at 200 rpm, 37°C. The next day, 50 ml of LB medium containing appropriate antibiotic was inoculated using 500 µl of the overnight culture. The culture was then incubated at 37°C at 200 rpm till the OD_{600} reached 0.5 (approximately 2 hr). The culture was centrifuged at 8000 rpm, 4°C, for 8 minutes to harvest the cells. The supernatant was discarded and the pellet was then very gently resuspended in 25 ml of ice-cold $CaCl_2$ (0.1M). The suspension was centrifuged at 8000 rpm, 4°C for 8 minutes and the supernatant was discarded. The pellet was gently resuspended in 12.5 ml of ice-cold $CaCl_2$ (0.1M) and incubated on ice for 4-8 hours to make cells competent. The cells were harvested by centrifuging the culture at 800 rpm, 4°C, for 8 minutes. The pellet was then resuspended in 2.15 ml of ice-cold $CaCl_2$ (0.1M) and then 350µl of glycerol was added. The cell suspension was then divided into 100 µl aliquots in pre-chilled microfuge tubes, and frozen in liquid nitrogen. The frozen tubes were stored in -80°C.

2.1.3. BACTERIAL TRANSFORMATION:

25-50 ng of DNA solution was layered on the competent cells.. The cells were then incubated on ice for 30 minutes. Then heat shock was given to the cells for the required time period. The tubes were incubated on ice for 2 min immediately after heat shock. 1ml of LB was added to the cells and incubated at 37°C, 200 rpm for 1 hour. The cells were given a short spin at top speed, most of the supernatant was removed, and the pellet was resuspended in the left-over supernatant. The cell suspension was then spread on LB agar plate containing appropriate antibiotic. The plates were incubated at 37°C for 12-16 hours.

2.1.4. RNA ISOLATION FROM YEAST:

5ml of yeast growth media was inoculated by yeast cell and incubated overnight. Next day 10 ml yeast growth media was inoculated using overnight culture in such a way that after two generations cells grow up to mid log phase ($OD_{600} \approx 1$). The cells were harvested by centrifuging at 3000 rpm, 10 min. The pellet was suspended in Tris-EDTA-SDS buffer (10 mM Tris HCl, 10 mM EDTA, 0.5% SDS). Equal volumes of phenol (pre equilibrated with water) was added and vortexed for 10 seconds. The mixture was then incubated at 65^0C for 1 hour, with intermittent vortexing in every 15 minutes. The mixture was rapidly chilled on ice for 5-10 min, and centrifuged at 14000 rpm for 10 min at 4^0C. The aqueous layer was collected in a chilled and fresh micro-centrifuge tube and precipitated by adding $1/10^{th}$ volume of 3 M sodium acetate and 2.2 volume of chilled ethanol. The tube was kept at -80^0C. The tube was then centrifuged to collect RNA at 14000 rpm at 4^0C. The precipitate containing RNA was washed with 70% ethanol and the pellet was dissolved in DEPC treated water. RNA was then subjected to DNase I (Fermentas) digestion.

2.1.5. SEMI QUANTITATIVE PCR:

About 10 μg of total RNA was then reverse transcribed with oligo dT primer (Sigma Aldrich) using reverse transcriptase (Omniscript kit, Qiagen). RNase inhibitor was used in this process. Resulting cDNA was first quantified, diluted appropriately to normalize and then subjected to PCR amplification (27 cycles) using gene specific primers. The PCR products were run on 1.4% agarose gel and stained with ethidium bromide.

2.1.6. REAL TIME PCR:

For real time PCR, cDNA was diluted (1:50) and used for PCR using RT-PCR kit (Roche). The real time analysis was done using Applied Biosystems 7500 Fast Real Time PCR system. Following reaction mixture was used for Real time PCR analysis:

Total 10 μl reaction mixture for Real time PCR:

0.5 μl Forward primer (10 pmol/ μl)

0.5 μl Reverse primer (10 pmol/ μl)

5 μl of SYBR Green

2 μl cDNA

2 μl DEPC water

2.1.7. SOUTHERN BLOTTING:

Yeast cells were grown in YPD medium to a density of 1.5×10^7 cells per milliliter. Heat shock (HS) was given by exposing them to 39°C for 40 min and subsequently returning them to 30°C. Cells were continued to grow at 30°C 7 days. The genomic DNA was isolated from the control as well as from the post HS samples collected after 2 hr and those collected on the 4th day and the 6th day. Equal amount of genomic DNA from each sample was subjected to XhoI digestion. Subsequently, all the digested samples were electrophoresed on a 0.8% agarose gel for 7 hr at 100 volt. The gel was incubated first in the denaturing buffer (containing NaOH, NaCl) and then in the neutralizing buffer (NaCl, Tris base, HCL, pH 7.4) for 35 minutes followed by incubation in 10X SSC buffer (350.67g NaCl, 176.47g sodium citrate dissolved in 4L water) for 15 minutes on rocker. DNA was transferred to nitrocellulose membrane for overnight. For probe preparation, a 625 bp (poly GT/poly CA) fragment (kindly provided by Professor Arthur Lustig) was labeled with [α-^{32}P] dCTP using Deca label DNA labeling kit (Fermentas). The membrane was UV cross linked. The membrane was incubated in pre hybridization buffer (containing 10X SSC, SDS, 0.5g Ficoll70, 0.5g of Ficoll400, polyvinyl phenol, BSA are added to double distilled water) at 65°C. The probe was added into hybridization buffer (containing 10X SSC, SDS, 50g dextran sulfate are added to double distilled water) and the membrane was incubated in it for overnight at 65°C. The membrane was washed

using 100 ml of washing buffer for 1 hour (wash buffer-I: 6X SSC, SDS). The Southern blot was finally exposed to X-ray films and developed.

2.2. YEAST GENETIC METHODS:

2.2.1. YEAST COMPETENT CELL PREPARATION:

A single colony of desired yeast strain was inoculated in 5 ml of yeast growth medium and incubated for overnight. Next day, OD_{600} of the overnight culture was measured after 1:10 dilution. The culture was then incubated at 30°C till the final OD reached 0.6-0.8. The cells were harvested by centrifuging and then washed by suspending them in 10 ml sterilized water. The cells were resuspended in 300 µl Lithium solution (1X Tris-EDTA, 1X lithium acetate) and thus competent cells were prepared.

2.2.2. YEAST TRANSFORMATION:

About 1-3 µg of the sample DNA and 10 µg of carrier DNA were added in microfuge tube. 200 µl of competent yeast cells was added gently over the DNA mixture in each transformation tube. To each tube, 1.2 ml of PEG solution (10X LiOAc, 10X TE, 50% PEG 2000) was added and incubated at for 30 min in shaker incubator. Heat shock was given at 42°C followed by incubation on ice. The cells were then collected by centrifugation at 10000 rpm for 10 seconds. The pellet was resuspended in 200 µl 1X Tris-EDTA buffer and then spread on appropriate plates. The plates were incubated at 30°C till transformed colonies were seen.

2.2.3. GENOMIC DNA ISOLATION:

10 ml culture was grown overnight at 30^0C in shaker incubator. Cells were harvested by centrifuging the overnight culture at 3000 rpm at room temperature. Cells were suspended in about 0.5 ml autoclaved double distilled water, transferred into 1.5 ml microfuge tube and centrifuged at top speed for 10 seconds to collect cells. Supernatant was removed and pellet was suspended through

vortexing. 200µl breaking buffer [Triton X-100 (2%), SDS (1%), NaCl (0.1M)], glass beads and Phenol/Chloroform mixture were added. Vortexing was done for 5 min. 200µl 1X Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA pH-8.0) was added followed by vortexing and centrifuging at maximum speed for 5 min. Supernatant was transferred into a new tube and 1 ml 100% absolute ethanol was added. The DNA was precipitated and the pellet was dissolved in 1X Tris-EDTA buffer. RNase (10 mg/ml) solution was added to remove RNA. Then 4M NH_4OAc and 100% absolute ethanol was added to precipitate the DNA. Genomic DNA was collected by centrifuging at maximum speed for 10 min at room temperature. Supernatant was discarded and the pellet was washed by 70% ethanol. Finally the genomic DNA pellet was dissolved in 1X TE buffer.

2.2.4. GENE KNOCKOUT:

Gene knockout was done using homologous recombination method. The strain SLY20 is isogenic to W303a having *ADE2* marked telomere VIIL. Yeast knock out strains YSC1021-551935 (*HSP82*::*KAN*^{r}), YSC1021-552834 (*SBA1*::*KAN*^{r}), YSC1021-551520 (*HSC82*::*KAN*^{r}) were purchased from Thermo Scientific, Open Biosystems, Huntsville, AL, USA. Using the genomic DNA of YSC1021-551520 as a template and the pair of primers OSB1 and OSB2 KANMX cassette with *HSC82* flanking regions was amplified. The PCR product was then integrated into SLY20 strain and selected on G418 sulphate containing agar plates. *HSC82* gene was knocked out by homologous recombination and the resultant *hsc82* null strain is referred as SLY4 in this study. In a similar way, using genomic DNA of YSC1021-551935 (*HSP82*::*KAN*^{r}) as a template and the pair of primers OSB3 and OSB4 KANMX cassette with *HSP82* flanking regions were amplified. It was then integrated to SLY20 to generate *hsp82* null strain which is referred to as SLY5. To knock out *SBA1* gene, the genomic DNA of YSC1021-552834 was used as a template to amplify KANMX cassette with *sba1* flanking regions. Forward primer OSB5 and the reverse primer OSB6 were used for this

purpose. The PCR product was then integrated into SLY20 cells to generate *sba1* null strain SLY6. The *sir2* null strain SLY12 (*sir2::KAN*') was made by integrating KANMX cassette having *SIR2* flanking regions in SLY20. All of the knockout genotypes were confirmed by PCR analysis. To confirm gene knockout forward primer used to amplify KANMX cassette was used and the reverse primer KanB1 was used which amplify from the middle of the cassette.

Using *pFA6a-TRP1* plasmid as a template, *TRP1* cassette with *CUP9* flanking regions was amplified. The product was then integrated into SLY20 strain by following yeast transformation protocol and selected on a medium lacking tryptophan to generate *⊿cup9* strain which is referred to as SLY71 in this study. Using the same strategy we created *⊿sum1, ⊿rim101* and *⊿sok2* strains which are referred to as SLY75, SLY73 and SLY74 strain respectively. To confirm gene knockout forward primer was designed 200 base pair upstream to the gene (OSB172, OSB175 and OSB163 respectively) and reverse primer which was used to amplify *TRP1* cassette.

2.2.5. GENE TAGGING:

In order to *MYC* tag C-terminal end of *CUP9* at the chromosomal locus, *13MYC-KanMX6* cassette was amplified with C-terminal flanking regions of *CUP9*. It was then integrated into SLY20 strain by following yeast transformation protocol and selected on G418 sulphate plates to generate *CUP9 MYC* tagged strain SLY87.

2.2.6. TELOMERE POSITION EFFECT ASSAY:

ADE2 marked cells were grown overnight in appropriate yeast growth media. Next day 2 ml medium was inoculated using overnight culture and incubated at 30°C, 200 rpm for two generations (3hr). Approximately 500 cells were spread over SC- low Adenine plates and incubated for 2-3 days at 30ºC and at last kept at 4ºC for color development.

2.2.7. MATING TYPE FREQUENCY ASSESSMENT:

Wild type strain (*HSP82*), ⊿*hsp82*, ⊿*hsc82*, *YDS32 (MATa)* and ⊿*sir2* were grown in appropriate growth media to the mid exponential phase. About 400 cells from each culture were plated in YPD plate and incubated at 30^0C until colonies appeared. Also 400 cells of the wild type (*HSP82*), ⊿*hsp82*, ⊿*hsc82*, and ⊿*sir2* were mixed with 400 cells of *YDS32 (MATa)* and incubated at 30^0C shaker for 10 minutes. Then each of them was spread on SD (synthetic dextrose) plate and incubated at 30^0C incubator for 30 hours. The number of cells grown on each YPD and SD plates were counted and the mating frequency was calculated as = (No. of cells grown on SD plate/ No. of cells on YPD plate)*100. The value obtained in case of ⊿*sir2* strain was subtracted from each of the three strains.

2.2.8. PROTEIN ISOLATION:

Yeast cells were inoculated in 5 ml of appropriate medium and grown overnight at 30°C at 200 rpm. Cells were inoculated into 20 ml of appropriate medium. The culture was grown till the OD_{600} reaches 0.5. The cells were centrifuged and the cell pellet was suspended in autoclaved water and the cell suspension was transferred to a 2 ml microfuge tube and centrifuged again to remove any medium residue. This pellet was first washed with TCA, and centrifuged and supernatant was discarded. The pellet was again resuspended in TCA and glass beads were added. The sample was vortexed thoroughly. The supernatant was transferred into new 1.5 ml tube. The cell and glass bead mixture was washed with TCA by mixing thoroughly and collecting into the same tube. It was centrifuged and the supernatant was discarded and the TCA precipitated proteins were collected and dissolved in 60 µl 1X sample buffer (Tris-HCl, pH 6.8, 2% SDS and bromophenol blue). The sample was then boiled for 3-5 minutes and then centrifuged at top speed for 5 min.

2.3. BIOCHEMICAL METHODS:

2.3.1. CHROMATIN IMMUNO PRECIPITATION:

50 ml cells were grown to an O.D.$_{600}$ of 1.2 and cross-linked with 1% formaldehyde at 30˚C for 15 minutes, 100 rpm. Cells were spun down at 3000 rpm for 3 minutes at room temperatures. Cells were washed with 1X PBS buffer. Then cells were suspended in spheroplast buffer (18.2% sorbitol, 1% glucose, yeast nitrogen base, Casamino Acids, 25 mM HEPES [pH 7.4], 50 mM Tris, 1 mM dithiothreitol) along with lyticase and incubated at 30˚C for 30 minutes to generate spheroplasts. The spheroplast was first washed in 500 µl of ice cold 1X PBS buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, 150 mM NaCl) containing PMSF. Then it was resuspended in HEPES, Triton X-100 buffer (Triton X-100, 10 mM EDTA, 10 mM HEPES [pH 6.5]) containing 0.5 mM PMSF and protease inhibitor cocktail (Roche) and was centrifuged at 7000 rpm for 7 minutes. The spheroplasts were resuspended in HEPES, NaCl buffer (200 mM NaCl, 1 mM EDTA, 10 mM HEPES [pH 6.5]), containing 0.5 mM PMSF and protease inhibitor cocktail and again spun down at 7000 rpm for 7 minutes. Finally the spheroplasts were resuspended in 100 µl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris) containing PMSF and sonicated (Elma, Model-S-60H) to generate an average DNA fragment size of 0.5 to 1 kb. 1 ml of IP dilution buffer (Triton X-100, 1.2 mM EDTA, Tris, NaCl, PMSF and protease cocktail inhibitor) was added and approximately 1.1 ml of suspension was collected and left on ice for 15 minutes to form the chromatin fraction. This chromatin solution was pre-cleaned by adding 75 µl of protein A agarose beads (UPSTATE) and incubated at 4˚C for 1 hr on rocker. This was spun down at 2500 rpm for 10 minutes and the supernatant was collected. Immuno precipitation was performed with 1µg anti Myc antibodies and protein A agarose beads to precipitate Cup9. Beads were washed sequentially by TSE-150 (0.1% SDS, triton X-100, 2 mM EDTA, 20mM Tris [pH 8.1], 500 mM NaCl), lithim chloride buffer (0.25 M LiCl, 1% NP-40, 1%

sodium deoxycholate) and 1X TE buffer. The immune complex was eluted by SDS-sodium bicarbonate buffer (1% SDS and 0.1M sodium bicarbonate). Reverse cross linked the immune complex using 5 M sodium chloride followed by precipitation of chromatin by adding 100% absolute ethanol and incubating for overnight. Chromatin was centrifuged at 12000 rpm for 30 minutes, 4°C and the pellet was washed by 70% ethanol. The pellet was dissolved in 5X proeinase K buffer and 30 µg proteinase K was added to incubate at 42°C for 2 hr. Then 1X TE, PCIA was added to it and vortexed for 2 minutes. Centrifuged the sample at top speed for 15 minutes and collected the supernatant. The DNA was precipitated by 5 µg glycogen, $1/10^{th}$ volume of 3 M sodium acetate and 2.2 volume of 100% ethanol. The chromatin pellet was dissolved in 100µl of 1X TE. *SIR2*$_{UAS}$ were amplified using the primers OSB125 and OSB87 in a reaction volume of 50 µl using $1/75^{th}$ of immunoprecipitates and $1/50^{th}$ of input DNA. Samples were subjected to electrophoresis on 1.5% agarose. Cup9 binding was also measured at *ACT1* locus using the primers OSB16 and OSB14. Control antibody for CHIP was rabbit IgG.

2.3.2. WESTERN BLOTTING:

The protein samples were boiled for 3-5 minutes and appropriate volumes of samples were loaded on 10% SDS poly acrylamide gel. The gel was then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was pre treated with methanol for 20 seconds, water for 2 minutes and transfer buffer (glycine, Tris base, SDS and methanol) for 5 minutes. The membrane was blocked using blocking buffer (5 gm non fat dry milk dissolved in 1X TBST) for 2 hr and immuno blotted for Hsp82, Sir2 and Actin for overnight at 4°C. Next day, the blot was washed by TBST buffer (0.2M Tris base, 9% sodium chloride, pH 7.6, 0.1% Tween 20). The blot was incubated with secondary antibody for 2 hr followed by washing with TBST buffer. The anti Hsp90 antibody (Calbiochem), the anti Sir2 (Santa Cruz Biotechnology Inc., CA), anti Actin antibody (Abcam) were

used at 1:5000 dilution. Anti Myc antibody (Abcam) was used at 1:8000 dilution. Horseradish peroxide-conjugated rabbit IgG (Santa Cruz Biotechnology Inc., CA) was used as a secondary antibody for Sir2 and Myc at 1:10000 dilution. HRP conjugated mouse IgG (Promega) was used as a secondary antibody for Hsp82 and Actin blot at the same dilution. The Western blots were developed using chemiluminescent detection system (Pierce).

2.3.3. PROTEASOME INHIBITOR ASSAY:

SLY89 strain was grown in MG132 (Sigma Aldrich) containing YPD medium with final concentration of 50 μM at 37°C for 24 hr and proteins were isolated from this strain.

TABLE 1: YEAST STRAINS USED IN THIS STUDY

STRAINS	GENOTYPE
iG170Dhsp82	*MATa can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 HSP82::LEU2 HSC82::LEU2 HIS3::HSP82G170D*
YDS31	*MATa his1*$^{-}$
YDS31	*MATa his1*$^{-}$
YSC1021-551520	*MATa HSC82::KAN*^{r}
YSC1021-551935	*MATa HSP82::KAN*^{r}
YSC1021-552834	*MATa SBA1::KAN*^{r}
SLY20	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2*
SLY4	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 HSC82::KAN*^{r}
SLY5	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 HSP82::KAN*^{r}
SLY6	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 SBA1::KAN*^{r}
SLY12	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 SIR2::KAN*^{r}
SLY13C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2, pHCA*
SLY13	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2, pHCA/HSP82*
SLY10	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 HSP82::KAN*^{r} *pHCA/HSP82*
SLY31	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 SBA1::KAN*^{r} *pHCA/HSP82*
SLY32	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 pTA/HSP82*
SLY46	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 pHCA*
SLY56	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2, pCZ/200UAS*
SLY57	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2, pCZ/429UAS*
SLY60C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15*

	VIIL::ADE2, *pCZ/200UAS pHCA*
SLY60	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/200UAS*, *pHCA/HSP82*
SLY61C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/429UAS*, *pHCA*
SLY61	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/429UAS*, *pHCA/HSP82*
SLY64	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ*del*cyc1*
SLY71	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 cup9::TRP1*
SLY74	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 sok2::TRP1*
SLY73	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 rim101::TRP1*
SLY75	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 sum1::TRP1*
SLY77C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 cup9::TRP1*, *pHCA*
SLY77	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 cup9::TRP1*, *pHCA/HSP82*
SLY80C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sok2::TRP1*, *pHCA*
SLY80	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 sok2::TRP1*, *pHCA/HSP82*
SLY79C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 rim101::TRP1 pRS313*
SLY79	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 rim101::TRP1 pRS313/HSP82*
SLY81C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 sum1::TRP1 pRS313*
SLY81	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15*

	VIIL::ADE2 sum1::TRP1 pRS313/HSP82
SLY83	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/307UAS*
SLY84	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/370UAS*
SLY85C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/307UAS, pHCA*
SLY85	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/307UAS, pHCA/HSP82*
SLY86C	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/370UAS, pHCA*
SLY86	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pCZ/370UAS, pHCA/HSP82*
SLY87	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 CUP9-13MYC-KANMX6*
SLY88	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15,* *VIIL::ADE2 CUP9-13MYC-KANMX6, pHCA/HSP82*
SLY89	*⊿hsc82::kanMx4⊿hsp82:kanMx4/piHGpd-G170Dhsp82-HIS ⊿pdr::loxP-leu2-loxP, trp1-289, leu2-3, 112 his3-⊿200URA 3-52 ade2-101⊿c lys2-801am*
SLY90	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pESC/CUP9MYC*
SLY91	*MATa leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15* *VIIL::ADE2 pESC(MYC)*

TABLE 2: LIST OF PLASMIDS USED IN THIS STUDY

NAME OF THE PLASMID	BRIEF DESCRIPTION
pHCA/*HSP82*	A gift from Didier Picard. CEN/ARS plasmid, derived from pRS313 vector, which over expresses *HSP82* under the control of GPD promoter. HIS marker, ampicillin resistance in bacteria
pTA	Yeast expression vector with GPD promoter and TRP marker, ampicillin resistance in bacteria
pCZ	A high copy yeast expression vector having *LACZ* reporter gene under control of *CYC1* promoter. TRP marker, ampicillin resistance in bacteria
pESC-HIS	2μ C-terminal Myc-tagged vector. HIS marker, ampicillin resistance in bacteria
pFA6a-TRP1	To knock out gene. ampicillin resistance in bacteria (Longtine M S, *Yeast* 14, 953–96, 1998)
pFA6a-13MYC-KanMX6	MYC tagging vector. ampicillin resistance in bacteria (Longtine M S, *Yeast* 14, 953–96, 1998)
piHGpd/G170D	Yeast expression vector, pRS303, HSP82 with glycine 170 changed to aspartic acid; regulated by the strong constitutive promoter GPD. HIS marker, ampicillin resistance in bacteria

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TABLE 3: LIST OF PRIMERS USED IN THIS STUDY

PRIMER NAME	SEQUENCE (5' TO 3')	PURPOSE
OSB1	ACC AAG CGT TGG GTA ATG A	Forward primer to amplify KANMX cassette with *HSC82* flanking regions
OSB2	TGG TCA TTT GAC AGC TGA TG	Reverse primer to amplify KANMX cassette with *HSC82* flanking regions
OSB3	TGA CAC ACT AGA CGC GTC GG	Forward primer to amplify KANMX cassette with *HSP82* flanking regions
OSB4	TAC CAA CCA GGT CCT TCC GC	Reverse primer to amplify KANMX cassette with *HSP82* flanking regions
OSB5	TGC TAC CCG CCT TCC GAG TG	Forward primer to amplify KANMX cassette with *SBA1* flanking regions
OSB6	CAC ATA CAG TTC CAT TAC TTG AC	Reverse primer to amplify KANMX cassette with *SBA1* flanking regions
KANB1	TGT ACG GGC GAC AGT CAC AT	Reverse primer from the middle of the KANMX cassette
OSB16	TGA CCA AAC TAC TTA CAA CTC C	Forward primer used to amplify *ACT1* for real time RTPCR
OSB14	TTA GAA ACA CTT GTG GTG AAC G	Reverse primer used to amplify *ACT1* for real time RTPCR
OSB131	CTG ATT AAT CGT GAT CCC GTC	Forward primer used to amplify *SIR2* for real time RTPCR
OSB132	CTT AGA GGG TTT TGG GAT GTT C	Reverse primer used to amplify *SIR2* for real time RT-CR
OSB19	ATC ACG AGT AAG GAT CAA AG	Forward primer used to amplify *YFR057w* for real time RTPCR

OSB20	TTA TGG CTT TGT TAC GCT TG	Reverse primer used to amplify *YFR057w* for real time RTPCR
OSB62	AAT CGG CGG ATG GGT TGG	Forward primer used to amplify *HMLa* for real time RTPCR
OSB63	TCA TTC TTT CTT CTT TGC CAG	Reverse primer used to amplify *HMLa* for real time RTPCR
OSB125	ATC CTC GAG CTG CAA CTC CTC AAT GTG TC	Forward primer used to amplify 429 bp *SIR2UAS*
OSB193	ATC CTC GAG GTA TAT GCT TAT ATG CAT GCG	Forward primer used to amplify 370 bp *SIR2UAS*
OSB194	ATC CTC GAG CCA AGC TAC ATC TAG CAC TC	Forward primer used to amplify 307 bp *SIR2UAS*
OSB126	ATC CTC GAG CTT TGG CCG CCA GTT GCG	Forward primer used to amplify 200 bp *SIR2UAS*
OSB87	ATC GGA TCC GGT CAT CCA GCT TTA ATG TGC CG	Common reverse primer used to amplify all above 4 deletion constructs of *SIR2UAS*
OSB41	CAGGAAGATCGCACTCCAGCC	Common reverse primer used to amplify all above 4 deletion constructs of *SIR2UAS* by PCR
OSB121	CAA CTG ATG GAA ACC AGC C	Forward primer used to amplify *LACZ* for real time RTPCR
OSB122	TTA CGC GAA ATA CGG GCA G	Reverse primer used to amplify *LacZ* for real time RTPCR
OSB161	CAA AAT CAT CCT TAT ATA ACC CTG GTA AGG TCC TTT TGT CCG GAT CCC CGG GTT AAT TAA	Forward primer used for *SOK2* knockout
OSB162	GAT TAA AGT AAC ATA ATT ATC CAA GGA ATT CAT AGT TGT TGA ATT CGA GCT CGT TTA AAC	Reverse primer used for *SOK2* knockout
OSB163	GCA TTA GTT GTG TGT GCC TG	Confirmatory primer located around 240 base upstream of the SOK2 region

OSB164	CTT TTA TGC TAA CAA CCT TCG AGA ATA GTT ACA TTC GAA GCG GAT CCC CGG GTT AAT TAA	Forward primer used for *CUP9* knockout
OSB165	TAT AAT TAT ATG AAT ATT TAA GTA ATG CAT TGA TAA GTG AGA ATT CGA GCT CGT TTA AAC	Reverse primer used for *CUP9* knockout
OSB166	ACT GAT TTC GTC GCG CCC	Confirmatory primer located around 220 base upstream of the CUP9 region
OSB170	AAG TTT CAT ACA TAA TTA ACA AAA TTC GTT TGT TGC GGG GCG GAT CCC CGG GTT AAT TAA	Forward primer used for *SUM1* knockout
OSB171	TTT TAT CTA TTC TCG AAA CTG CCC CAA CGT ACG GAC CAG CGA ATT CGA GCT CGT TTA AAC	Reverse primer used for *SUM1* knockout
OSB172	GTC AGC AAA CAG AGC ACA AG	Confirmatory primer located around 130 base upstream of the sum1 region
OSB173	ACT GAA AAC GGT AAA GTA GGT TTG TTT AAA TTG ACT TAA GCG GAT CCC CGG GTT AAT TAA	Forward primer used for *RIM101* knockout
OSB174	GCA AAG AAA CAA CTA AGA ATA AAA TAT CCG ACA ATC CAT AGA ATT CGA GCT CGT TTA AAC	Reverse primer used for *RIM101* knockout
OSB175	GTG AGG GAT GCC AAT CTA	Confirmatory primer located around 230 base upstream of the RIM101 region
OSB189	CTA ATG ACA ACG CGA ATA ATA C	Forward primer used to amplify *CUP9* for real time RTPCR
OSB190	CAA TTC ATA TCA GGG TTG GAT AG	Reverse primer used to amplify *CUP9* for real time RT-PCR
OSB191	CAA ACA TTA AAG GAG CCA CGA	Forward primer used to amplify *SOK2* for real time RTPCR
OSB192	TTG TAT CAT CAG CAG CAA TTT C	Reverse primer used to amplify *SOK2* for real time RTPCR
OSB213	GGG TTT CTT GAC TGA CCG	Forward primer used to

		amplify *PNC1* for real time RTPCR
OSB214	GCA AGC CAC CCT AGT TCA	Reverse primer used to amplify *PNC1* for real time RTPCR
OSB203	GAC GGA TCC ATG AAT TAT AAC TGC GAA ATA C	Forward primer used to amplify Cup9 for cloning in *pESC-MYC* tagged vector
OSB204	CGA GTC GAC ATT CAT ATC AGG GTT GGA TAG	Reverse primer used to amplify Cup9 for cloning in *pESC-MYC* tagged vector
OSB21	GAC GGA TCC ATG GCT AGT GAA ACT TTT GAA TTT C	Forward primer to amplify *HSP82* having BamHI overhang
OSB22	CGG GTC GAC CTA ATC TAC CTC TTC CAT TTC GG	Forward primer to amplify *HSP82* having SalI overhang

CHAPTER 3

THE ROLE OF Hsp90 IN FUNCTIONAL MATURATION OF CLASS III HISTONE DEACETYLASE, Sir2

3.1. INTRODUCTION:

Hsp90 is the most abundant eukaryotic protein found in cytosol, nucleoplasm, endoplasmic reticulum (ER), mitochondria and chloroplast [137]. It is involved in maturation and folding of some special class of proteins which are called its clients. The clients of Hsp90 include proteins which are primarily involved in signal transduction [189, 190, 141]. With the help of several co-chaperones, it dimerises in an ATP dependent manner and provides the maturation of the target protein at a near native state [191]. Budding yeast contains two isoforms of Hsp90; Hsc82 (human ortholog of Hsp90β), which is constitutively expressed in the cell and Hsp82 (human ortholog of Hsp90α) which is induced in stressed conditions. It is shown that yeast viability depends on expression of either one of the two isoforms of Hsp90 [143]. The two isoforms comprise (1-2) % of the total cellular proteins and share 97% sequence identity. Novel roles of Hsp90 and its co-chaperone p23 have been uncovered by recent studies which show their role in stabilization of different protein DNA complexes during RNA transcription, telomere maintenance, DNA replication *etc.* [140, 192, 193, 194]. In recent years, many telomere proteins which are potential clients of Hsp82 have been identified through a plethora of proteomic, bioinformatics, and genetic approaches. These include Cdc13, Stn1, [131, 167], Sir2 [168], Mre11 [169], Ku80, Mec1 and Est1 [167]. Some of these gene products are involved in telomere position effect (TPE) [96], a phenomenon where transcription of gene is repressed due to its close proximity to telomere.

Lower eukaryote *Saccharomyces cerevisiae* genome use histone acetylation-deacetylation as one of the measures of epigenetic mechanisms to control gene expression. Sir2 is one of the major histone modifiers in budding yeast. In *Saccharomyces cerevisiae*, the initial steps of telomere silencing include recruitment of Sir2-Sir4 complex at sub-telomeric regions by telomere bound proteins such as Rap1 and Ku70/Ku80 heterodimer. Histone deacetylation activity of Sir2 helps the binding of Sir3.

Binding of Sir3 triggers the spreading of the Sir complex along the sub-telomeric regions, causing heterochromatinisation. Sir2, NAD^+ dependent histone deacetylase, is conserved across the evolution [77]. It is involved in transcriptional silencing of the silent mating type loci, *HML* and *HMR*, as well as genes near telomeres [195, 86]. It is established that length of telomere positively regulates the efficiency of silencing, *i.e.*, the longer the telomere, greater is the silencing [123]. However, the effect of Hsp90 deficiency on TPE has not been explored yet.

We aim to monitor whether Sir2 is a direct client of Hsp90. A client of Hsp90 follows three criteria: firstly it must physically interact with Hsp90, secondly, inhibition of Hsp90's function must result in a loss of activity and lower level of client protein, thirdly, inhibition of Hsp90's function must lead to degradation and clearance of client protein from cell. Genome wide study revealed a physical interaction between Hsp90 and Sir2 [167]. We aim to determine whether Hsp90 homeostasis plays a critical role in maintaining cellular pools of Sir2p. Using a temperature sensitive mutant *iG170Dhsp82* we observe a drastic reduction in the endogenous level of Sir2 activity at the restrictive temperature. Our result shows that at restrictive temperature, both the mating type silencing as well as telomere silencing function is completely lost, a phenotype similar to that observed in ⊿*sir2* cells. This conclusively proves that Sir2p function is dependent on Hsp90. We have also established that Sir2 undergoes proteasomal degradation under Hsp90 inactivated condition, which is reversed in presence of proteasome inhibitor MG132. This is the general fate of major Hsp90 clients under Hsp90 inactivated condition. Thus our data collectively concludes that Sir2 is a direct client of Hsp90.

3.2. RESULTS:

3.2.1. INHIBITION OF Hsp90'S FUNCTION RESULTS IN LOSS OF ABUNDANCE AND ACTIVITY OF Sir2 IN CELL

3.2.1.1. Effect of single knockout mutant allele Δhsp82 or Δhsc82 on Sir2 *function and abundance:*

Hsp90 has two isoforms: Hsc82 (human ortholog of Hsp90β), which is constitutively expressed in the cell and Hsp82 (human ortholog of Hsp90α) which is induced in stressed conditions. We wanted to determine whether knockout of single allele *HSP82* or *HSC82* has any effect on cellular function and abundance of Sir2. Two important silencing functions of Sir2 were investigated in ⊿*hsc82* and ⊿*hsp82* strains: telomere silencing (also called telomere position effect) and mating type silencing.

We generated two deletion mutants, Δ*hsp82* and Δ*hsc82* in SLY20 strain, which had *ADE2* gene marked at the sub-telomeric region of chromosome VIIL as shown in Figure 1. Under normal condition, when SLY20 was allowed to grow on low adenine medium at 30^0C, they developed mostly as red colored colonies due to the silencing of *ADE2*, whereas Δ*sir2* strain developed as white colored colonies owing to the de-repression of *ADE2*. We observed that single deletion mutants SLY4 (⊿*hsc82*), SLY5 (⊿*hsp82*) exhibited wild type phenotype, *i.e.* they all produced red colored colony (Fig. 2A) due to transcriptional silencing of *ADE2* gene. This result suggests that functions of Hsp82 and Hsc82 are redundant to each other. We monitored the expression of another sub-telomeric gene *YFR057w,* located within 1kb from the end of chromosome VIR telomere, to verify the activity of Sir2 in single deletion strains (Fig. 2B). *YFR057w* ORF codes for a protein of unknown function and its ORF is repressed under normal conditions as Sir2 spreads as far as 3kb length in the chromosome VIR [221]. But in ⊿*sir2* strain it is de-repressed.

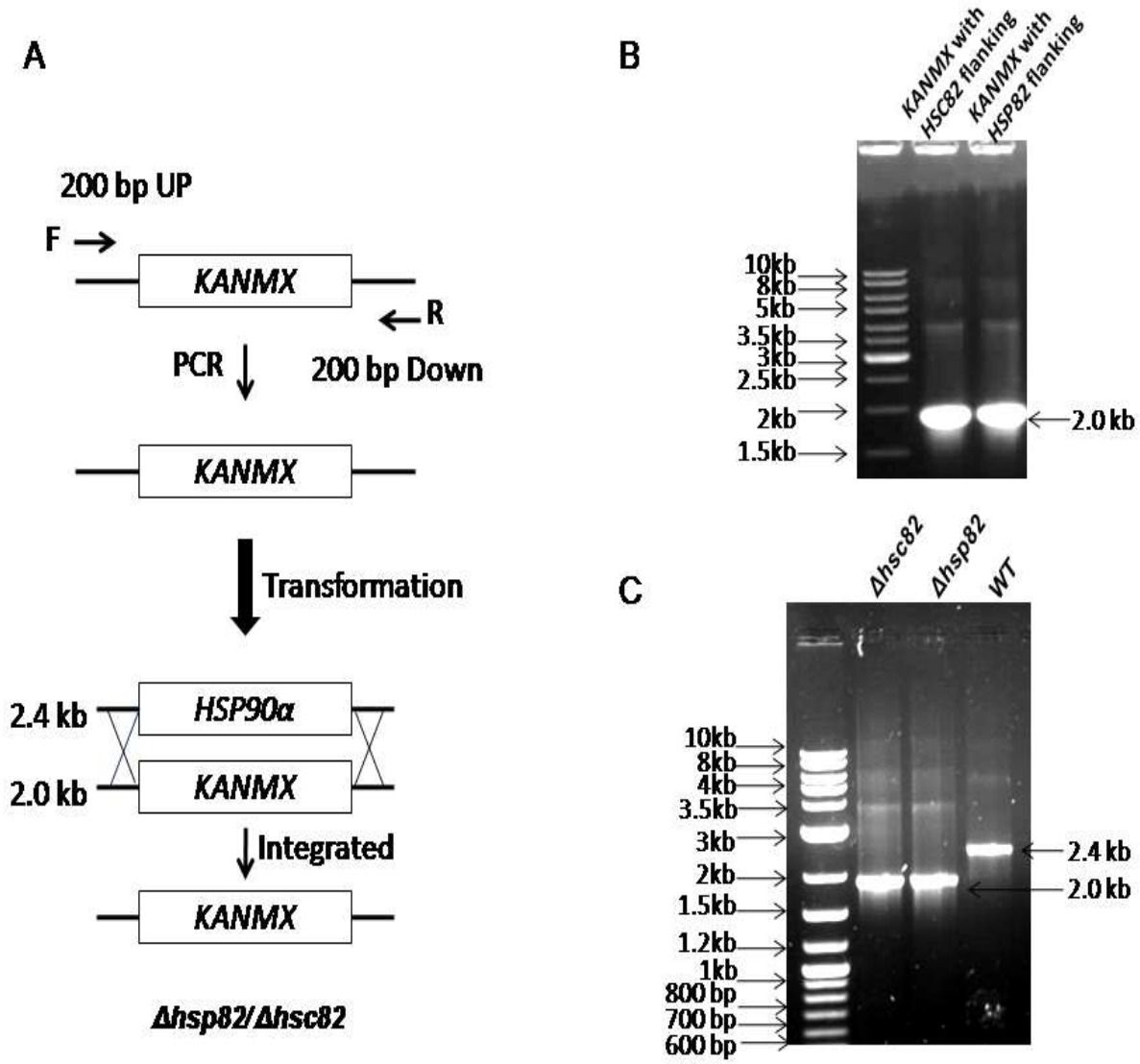

Figure 1: Generation of *Δhsc82* and *Δhsp82* strains. (A) Experimental strategy to knockout *HSC82* and *HSP82.* (B) Amplification of *KANMX* cassette. (C) Agarose gel showing confirmation of knockout.

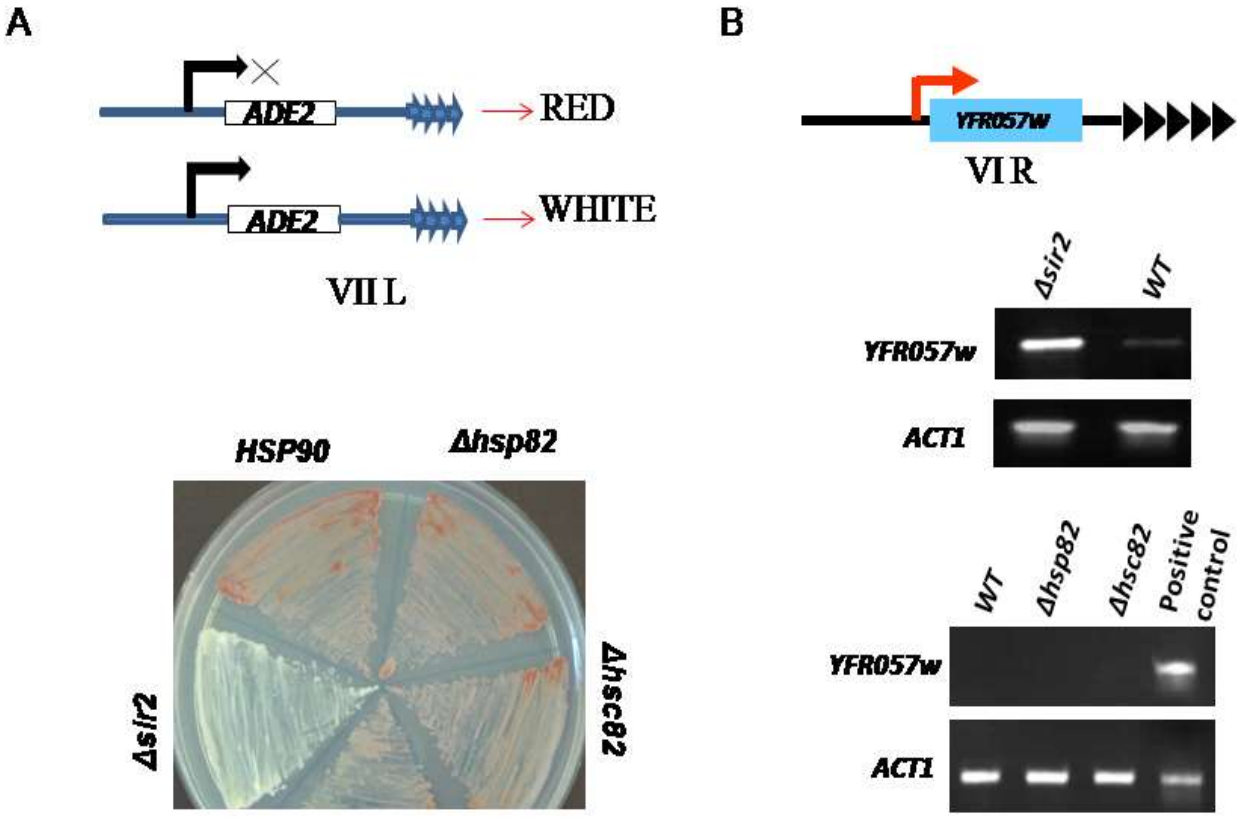

Figure 2: Effect of *Δhsc82* and *Δhsp82* on telomere silencing. (A) Telomere position effect assay shows *Δhsc82* and *Δhsp82* strains develop pink colored colonies like wild type strain where *Δsir2* strain develops white colored colonies. (B) Quantification of *YFR057w* transcript by semi-quantitative RT-PCR. *YFR057w* remains silenced in *Δhsc82* and *Δhsp82* strains, whereas it is de-repressed in *Δsir2* strain. *ACT1* is expressed equally in these mutants and in wild type. Yeast genomic DNA was taken as positive control.

Our result showed that of *YFR057w* remained silenced in single knockout strains suggesting that *⊿hsc82* and *⊿hsp82* do not affect the activity of Sir2.In order to find out the effect of single knock out (*⊿hsp82* or *⊿hsc82*) strains on mating type silencing, we measured the mating ability of these strains and compared with wild type and *⊿sir2* strains. We observed that all of them exhibited wild type mating phenotype when mated with the tester strain YDS32 (*MATa*) (Fig. 3A). The mating type frequency for each of the deletion mutants (*⊿hsp82* and *⊿hsc82*) were calculated and plotted (Fig. 3B). It was found to be comparable with that of the wild type. This observation led to the conclusion that the single knockout mutants do not affect mating type silencing.

We monitored whether there was any change in Sir2 level in *⊿hsp82* and *⊿hsc82* strains. Proteins were isolated from SLY20, SLY4 and SLY5 strains and equal amount from each protein preparation was loaded into SDS-PAGE and they were probed with anti-Hsp90 antibody as well as anti-Sir2 antibody. Our result showed that in SLY4 (*⊿hsc82*) and SLY5 (*⊿hsp82*), the level of Sir2 remained unaltered compared to that of SLY20. This result suggests that Hsc82 (which is poorly detected by the antibody) compensates for the lack of Hsp82 (in SLY5) and vice versa and thus the level of Sir2 is not affected (Fig. 4).

3.2.1.2. Effect of conditional mutant, iG170Dhsp82 on* Sir2 *function and abundance:

From our results it is clear that single deletion mutants do not affect Sir2 activity. So we wanted to check the function and abundance of Sir2 in absence of both *HSP82* and *HSC82*. As double mutants are lethal we have chosen a temperature sensitive mutant *iG170Dhsp82* strain where Hsp82 is functionally defective at restrictive temperature. In this strain both *HSP82* and *HSC82* genes are deleted and the strain harbors mutant *G170Dhsp82* which is integrated into its chromosome. This strain behaves as wild type when grown at permissive temperature 25^0C, but as mutant when grown at restrictive temperature at 37^0C [222].

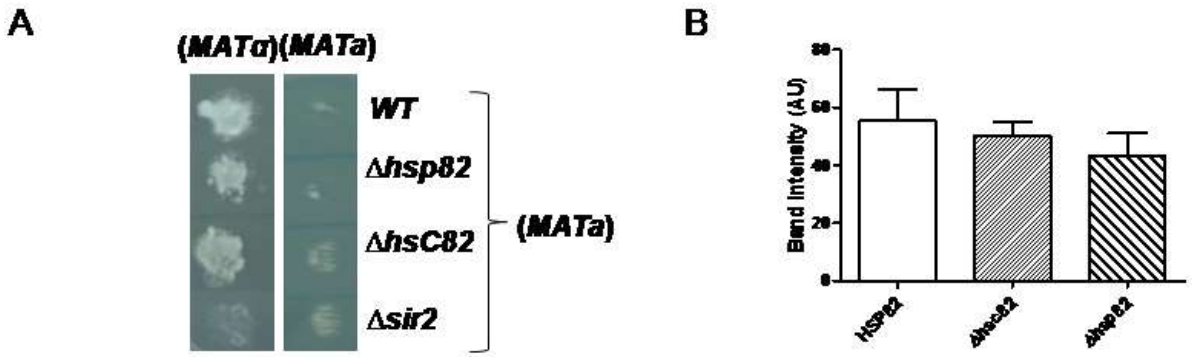

Figure 3: Mating type silencing assay in *Δhsc82* and *Δhsp82* mutants. (A) *Δhsc82* and *Δhsp82* strains can perform mating like wild type strains, where *Δsir2* strain cannot mate. (B) Mating type frequency of wild type, *Δhsc82* and *Δhsp82.* Wild type, *Δhsc82* and *Δhsp82* shows comparable values. Each bar represents mean density ± SD.

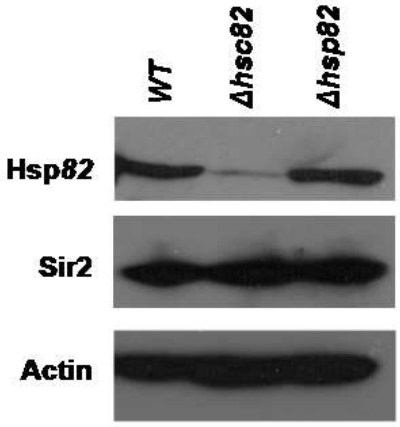

Figure 4: Western blot analysis to show Sir2 level in *Δhsp82* or *Δhsc82.* Single knockout strains, *Δhsp82* or *Δhsc82* show comparable amount of Sir2 with wild type.

It tolerates little bit higher temperature than its counterpart where *G170Dhsp82* is maintained episomally in the cell [29]. Previous study by Nathan and Lindquist showed that the mutant *G170Dhsp82* when present as an episomal copy within the cell, its growth is significantly decreased above 34^0C. However if it is integrated within the chromosome it can tolerate $(2\text{-}3)^0$C higher temperature [222].

To explore whether Sir2 function is dependent on Hsp82, we examined both telomere silencing as well as mating type silencing in this conditional mutant. We monitored the mRNA level of *YFR057w*. The steady state level of *YFR057w* transcript was quantified in *iG170Dhsp82* at both 25^0C and 37^0C and compared with *ACT1* transcript (Fig. 5A, B). The semi quantitative RT-PCR showed significant increase in *YFR057w* transcript at 37^0C compared to 25^0C, whereas the level of *ACT1* transcript was comparable at both temperatures (Fig. 5A). In order to rule out the possibility that the loss of silencing is not a mere effect due to shift in temperature from 25^0C to 37^0C we measured the *YFR057w* transcript level of wild type strain (SLY20) at both temperatures (Fig. 5A). We found there was no loss of silencing in wild type strain at higher temperature. This result is consistent with the previous finding that higher temperature causes more silencing [220]. The above experiments conclusively prove that loss of silencing at 37^0C was only due to non-functional Hsp82 in *iG170Dhsp82* strain. We aimed to explore mating type silencing in *iG170Dhsp82* strain. *iG170Dhsp82* (*MATa*) strain was allowed to mate with tester strain YDS32 (*MATa*) at 25^0C and 37^0C and compared its mating behavior with wild type (*HSP82*) and *⊿sir2* strains. We found due to loss of function of Hsp82 at 37^0C the mating ability of the strain was impaired and it behaved like *⊿sir2* (Fig. 6A).

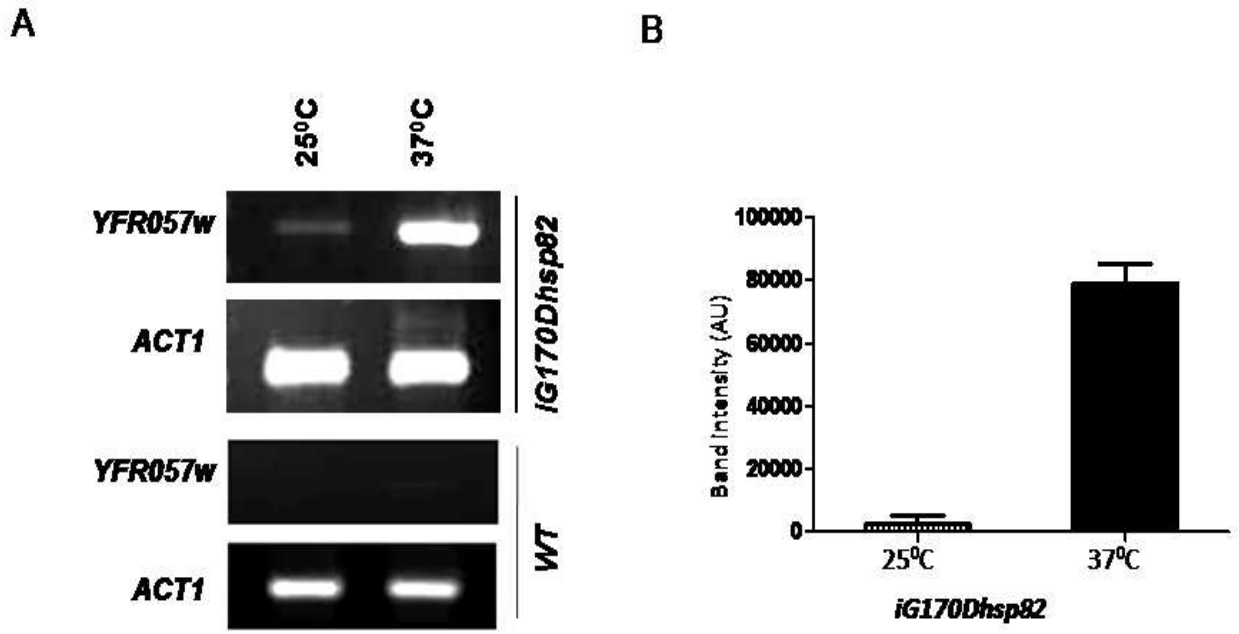

Figure 5: Sir2 protein function at telomere is dependent on Hsp82. (A) Semi quantitative RT-PCR shows increase in *YFR057w* transcript in *iG170Dhsp82* at 37^{0}C compared to 25^{0}C indicating loss of telomere silencing, whereas wild type strain shows silencing at higher temperature (37^{0}C). (B) Graphical representation of band intensity of four independent experiments of (A) is done after normalization with *ACT1*. Each bar represents mean density ± SD.

However, at 25^0C, the strain showed mating ability comparable to wild type cells. In order to rule out the possibility that the lack of silencing at restrictive temperature (37^0C) is not due to any lack of growth, we checked the viability of both temperature sensitive mutant and that of the tester strains at 37^0C, and found that all the strains were viable at 37^0C (Fig. 6B and 6C). According to earlier report, Hsp90 controls the pheromone signaling in yeast [223]. Therefore, it is important to understand whether the loss of mating type silencing of *G170Dhsp82* at restrictive temperature is due to the defect in pheromone signaling pathway alone, or also due to de-repression of *HMLa* locus in this *MATa* strain. To address this question we measured the *HMLa2* transcript level from *G170Dhsp82 (MATa)* after growing them at 25^0C as well as 37^0C. The semi quantitative RT-PCR data showed negligible amounts of *HMLa2* transcript at 25^0C, whereas at 37^0C significant amount of *HMLa2* transcript was visible (Fig. 7). This result was well corroborated with the loss of mating ability of *iG170Dhsp82* at 37^0C, since in order for a *MATa* strain to behave as a mating compatible haploid strain, *HMLa2* factor should remain silenced [225]. As a control, we had taken *⊿sir2* strain where considerable amount of *HMLa2* transcript was present, which made it sterile. This experiment concludes that in *hsp82* deficient condition, there is a loss of silencing at *HML*, which is not only due to defect in pheromone signaling pathway [223] but also due to derepression of *MATa2* transcript in *iGD170hsp82 (MATa)*. Since, Sir2 is the key protein involved in both of these silencing mechanisms; we wanted to see whether there is any change in the level of Sir2 in *iGD170hsp82* strain at different temperatures. We monitored Sir2 level by Western blot analysis which showed at non permissive temperature (37^0C) the level of Sir2 was substantially diminished (Fig. 8A), whereas the level of Hsp82 was comparable at both the temperatures, Act1 being a loading control.

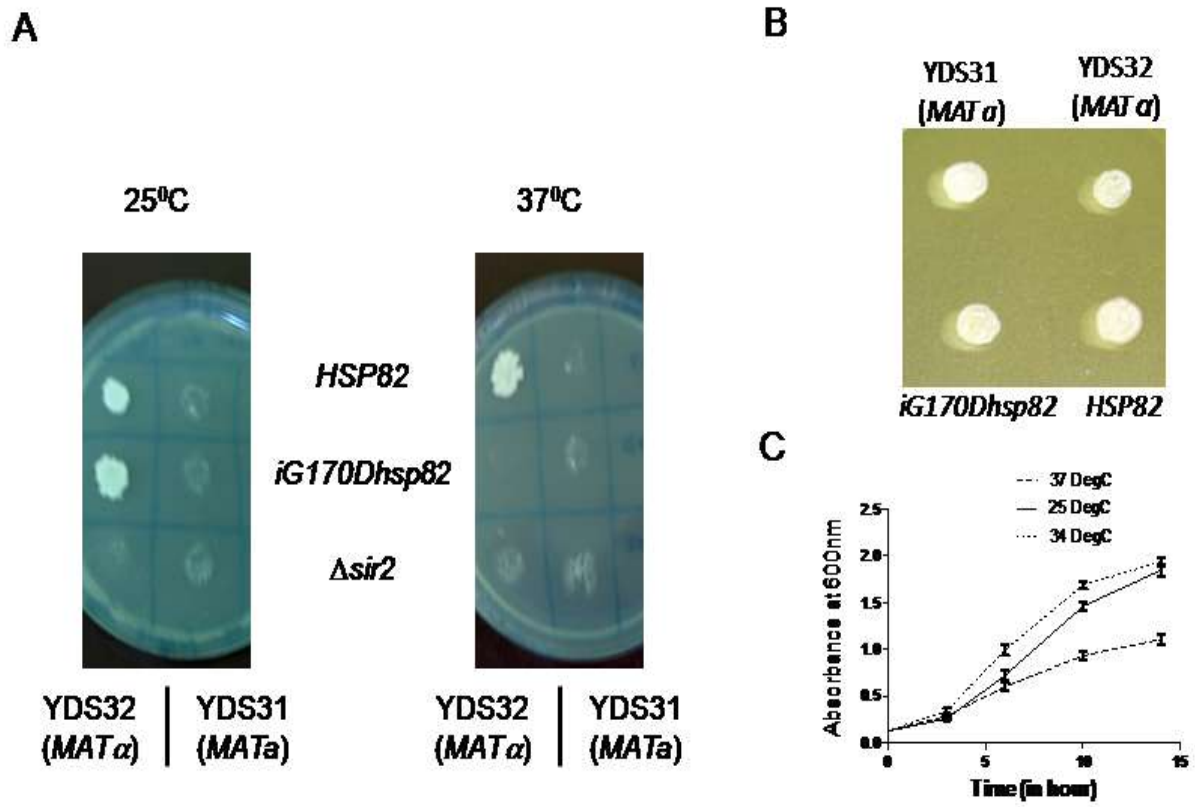

Figure 6: Sir2 protein function at mating type loci is dependent on Hsp82. (A) The temperature sensitive strain *iGD170hsp82* causes disruption of mating type silencing at restrictive temperature (37^{0}C), where Hsp82 is non functional and behaves as *Δsir2*. (B) *iG170Dhsp82* cells, wild type cells (*HSP82*), tester strains YDS32 (*MATα*) and YDS31 (*MATa*) all show comparable growth at 37^{0}C on YPD plate. (C) Growth kinetics of *iG170Dhsp82* at indicated temperatures. This strain shows slow growth phenotype at 37^{0}C.

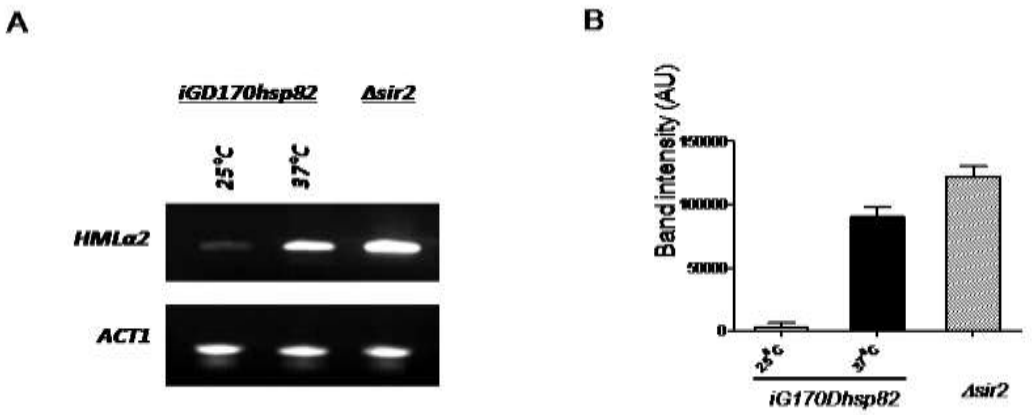

Figure 7: De-repression of *HMLα2* transcript due to non-functional Hsp82 (A) *iG170Dhsp82* at 37^{0}C shows considerable amount of *HMLα2* transcript comparable to that present in Δ*sir2* cells; whereas at 25^{0}C negligible amount of *HMLα2* is seen. (B) Graphical representation of band intensity of four independent experiments of (A) is done after normalization with *ACT1* control. Each bar represents mean density ± SD.

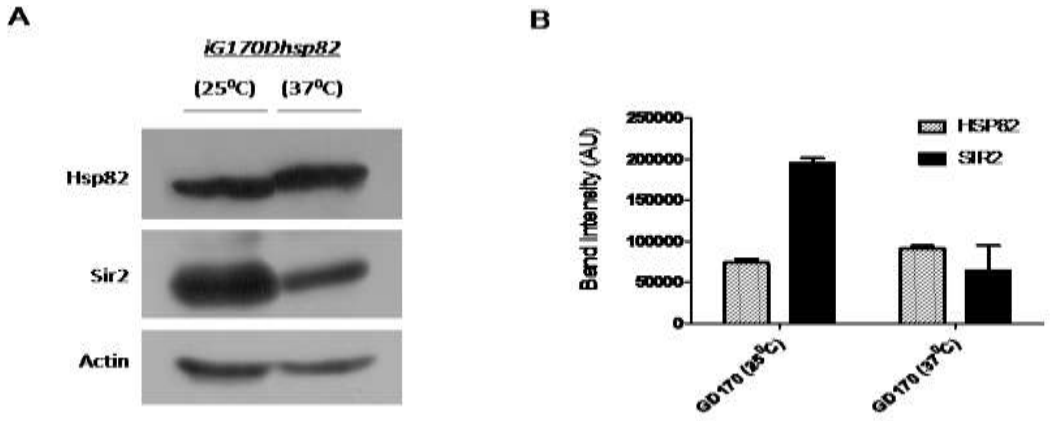

Figure 8: Cellular abundance of Sir2 decreased due to non-functional Hsp82. (A) Western blot shows the relative abundance of Hsp82 and Sir2p at two temperatures. Actin is the loading control. (B) The quantification of Western blot (from 4 independent experiments) shows more than 50% reduction in steady state level of Sir2p at higher temperature. The data are normalized with respect to the loading control Actin. Each bar ± SD.

The experiment was repeated four times and the mean value of the quantification of the band intensity on Western blot showed that there was at least 50% reduction in the level of Sir2 at 37^0C compared to 25^0C (Fig. 8B). We hereby speculate that Sir2 may be a putative client of Hsp82, whose activity inside the cell is regulated by Hsp82. When Hsp82 is nonfunctional at restrictive temperature, Sir2 level is reduced by 50% and thus at lower levels it is unable to provide mating type silencing and telomere silencing activity. Thus budding yeast requires native Hsp82 protein for activity of Sir2.

3.2.2. INHIBITION OF Hsp90'S FUNCTION LEADS TO DEGRADATION AND CLEARANCE OF Sir2 FROM CELL:

Our previous result show that Sir2 protein is diminished significantly in temperature sensitive *iG170Dhsp82* mutant strain at restrictive temperature (37^0C) compared to that observed at permissive temperature (25^0C). In order to examine whether this reduction is at transcriptional level, we isolated total mRNA of the cells growing at permissive and restrictive temperatures. Real time RTPCR result confirmed the presence of comparable *SIR2* transcript at both the temperatures (Fig. 9A). This result encourages us to investigate whether Sir2 is a putative client of Hsp82. The common fate of Hsp90 client proteins is proteasome-mediated degradation in presence of Hsp90 inhibitors [225]. We were interested to explore whether Sir2 proceeded through proteasome-mediated degradation in *hsp82* null condition. For that purpose we constructed *⊿pdr5-iG170Dhsp82* strain from parental *⊿pdr5* (p553) strain. Deletion of *PDR5* gene, which codes for a membrane associated drug export pump, ensures optimal entry of MG132 [226]. The strain was grown with 50 μM MG132 (a proteasome inhibitor) at 37°C for 24 hrs in a condition where Hsp82 was functionally inactive. Total protein was isolated from this strain and probed with anti-Sir2 antibody.

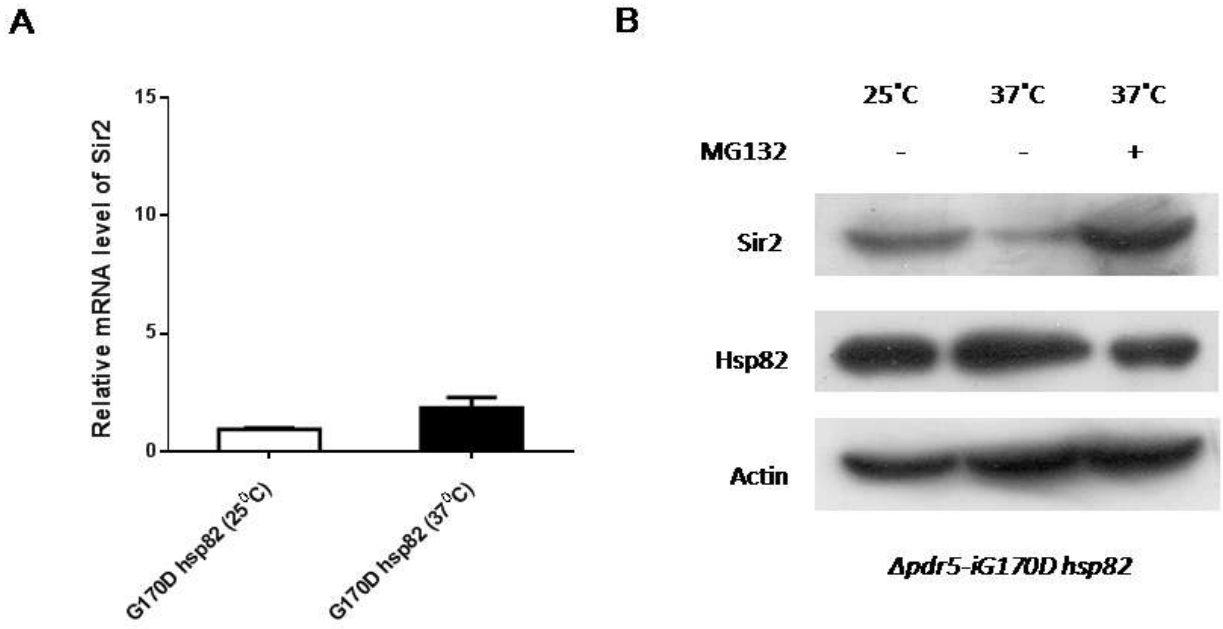

Figure 9: Hsp82 prevents Sir2 from proteasomal degradation. (A) Semi quantitative RT-PCR shows comparable amount of SIR2 transcript in *Δpdr5-iG170Dhsp82* strain at 37˚C and 25˚C. (B) Western blot analysis shows MG132 treatment increased the cellular pool of Sir2p compared to that maintained at 37˚C (*hsp82* null condition). Hsp82 and Actin are comparable in all the conditions. Each bar represents mean density ± SD.

Western blot analysis showed that MG132 treatment increased the cellular pool of Sir2p compared to that maintained at 37˚C (*hsp82* null condition) indicating inhibition of proteasomal degradation of Sir2 in the cell (Fig. 9B). This experiment exhibits that under *hsp82* null condition Sir2p is processed *via* proteasomal degradation pathway supporting Sir2 as a direct client of Hsp82.

3.2.3. ROLE OF Sba1 IN Sir2 MATURATION:

We were also interested to investigate whether Sba1 (human ortholog of p23), which is a co-chaperone of Hsp82 has any role in Sir2 maturation. To this end we created *⊿sba1* deletion strain SLY6 and performed telomere position affect (TPE) assay and mating type silencing assay. TPE assay showed SLY6 mostly developed as pink colored colonies which suggested telomere silencing was unaffected (Fig. 10A). We monitored the steady state level of *YFR057w* in this strain. The *⊿sba1* deletion strain maintain the silencing of *YFR057w* (Fig. 10B) like that of wild type. In order to find the role of Sba1 in mating type silencing function, we allowed *⊿sba1 (MATa)* to mate with tester strain YDS32 (*MATa*). We found that mating ability of *⊿sba1* strain remained unperturbed (Fig. 10C). Abundance of Sir2 was measured in *⊿sba1* strain by western blot analysis. Our result showed that Sir2 level was not altered in *⊿sba1* deletion mutant (Fig. 10D). We concluded from these results that Sba1, one of the major co-chaperones of Hsp82 does not affect Sir2 maturation.

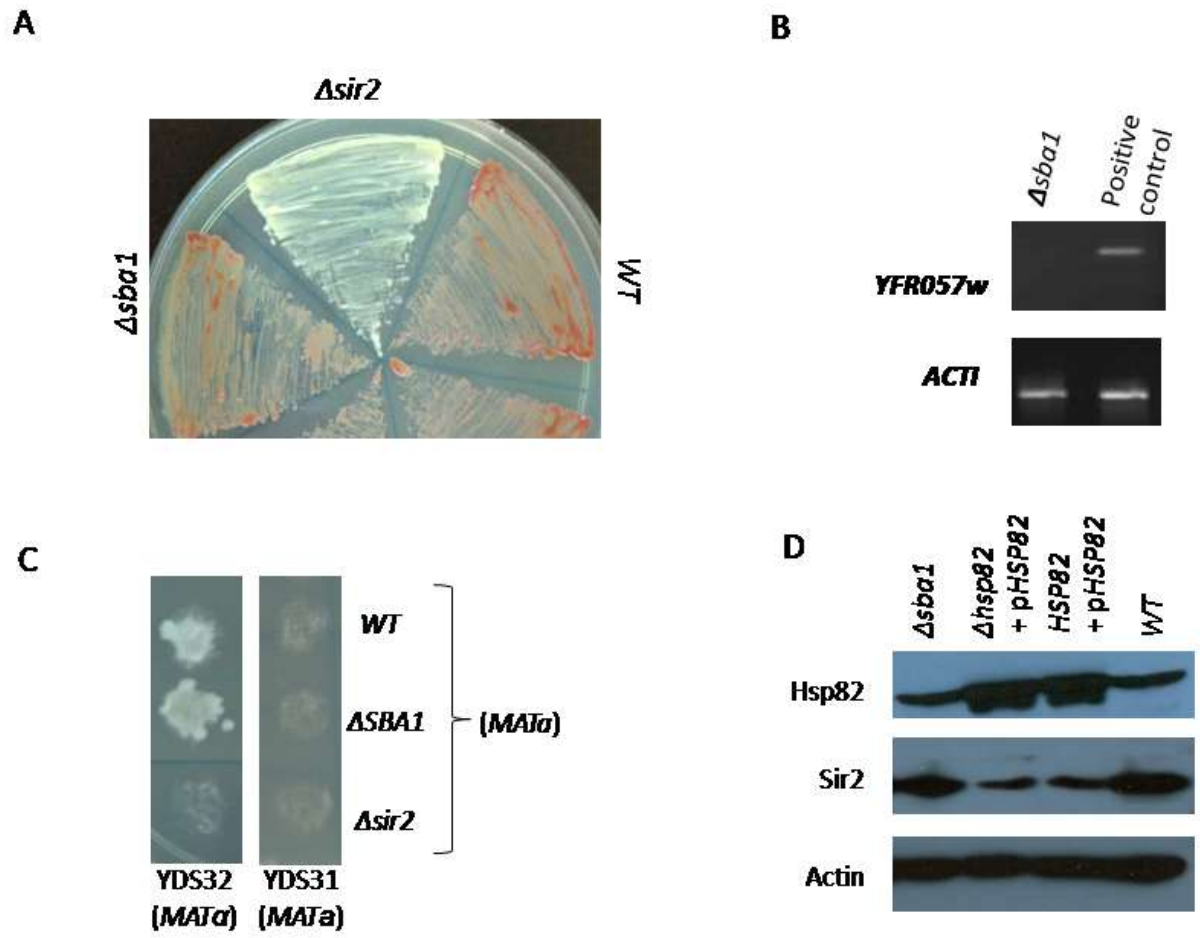

Figure 10: Sba1 has no effect on function and abundance of Sir2. (A) TPE assay shows *Δsba1* develops pink colour colonies like wild type strain. (B) Semi quantitative RT-PCR shows *YFR057w* remains silenced. (C) *Δsba1* does not show any defect in mating type silencing. (D) Western blot analysis shows *Δsba1* does not affect cellular abundance of Sir2.

3.3. DISCUSSION:

This study establishes Sir2 as a direct client of Hsp90 through several important findings. Firstly, our study shows for the first time that Sir2 function is dependent on Hsp90, and its cellular abundance is also controlled by Hsp90. We observe that there is a significant reduction of Sir2 in *iGD170hsp82* temperature sensitive mutant, where Hsp82 is nonfunctional at restrictive temperature. This reduction of Sir2 is associated with loss of mating type silencing as well transcriptional silencing at telomere. As Hsp82 controls pheromone signaling pathway in yeast, we wanted to decipher whether the loss of mating type silencing at restrictive temperature is due to the derepression at the *HMLa* locus in *iGD170hsp82* mutant which is *MATa* strain. According to our result there is significant amount of *MATa2* transcript at 37^0C, which is well correlated with the sterile phenotype of *iG170Dhsp82 (MATa)*. Our result shows that considerable amount of Sir2p is still present at the restrictive temperature (albeit at 50% reduced level); however functional studies demonstrated that these Sir2 proteins are functionally inactive at hidden mating locus. According to earlier reports in yeast, silencing is dependent on temperature [220]. Increase in the temperature leads to stronger TPE and mating type silencing in yeast. As an explanation of this effect it has been shown that at higher temperature Sir2-Net1 complex is destabilized whereas Sir2-Sir4 complex remains unaffected in high temperature [202]. Thus at elevated temperature more Sir2 is available to bind with Sir4 which leads to an increase in telomere and mating type silencing in yeast. In our system the loss of transcriptional silencing both at telomere and *MAT* locus at higher temperature is directly linked with the lack of Hsp82/Hsc82 activity.

Secondly, our work establishes that Hsp82 provides conformational maturation to Sir2 level by preventing it from degradation. Under normal condition Sir2 acts as a client of Hsp82 as inhibition of *HSP82*'s function results in degradation and loss of Sir2 activity. Using *iG170Dhsp82* strain at

37^0C we have shown that Sir2 follows proteasomal degradation as treatment of MG132, proteasome inhibitor, reverses its degradation. Earlier yeast two hybrid experiment with *E33Ahsp82* displayed its physical association with Sir2 [168]. Thus our work for the first time establishes Sir2 as a direct client of Hsp82.

Finally, our genetic experiments show that Hsp82 mediated maturation of Sir2 is independent of its one of the major co-chaperones, Sba1. This observation does not fit with classical steroid hormone receptor chaperone model, where Sba1 is found to be required at the last stages during client release.

CHAPTER 4

THE ROLE OF HEAT SHOCK OR Hsp90 OVER-EXPRESSION IN EXPRESSION AND ACTIVITY OF Sir2

4.1. INTRODUCTION:

There are rising number of evidences which suggest that environmental factors can lead to stable alterations in gene expression by modifying chromatin structure without any deviation in genome sequence. Histone tail modifications lead to up-regulation of many stress specific genes. Changes at the gene expression are brought by different environmental factors such as diet, temperature changes, chemical pollutants and other external stresses. But the mechanistic links between environmental trigger and observed epigenetic modifications are yet to be understood.

In lower eukaryote *Saccharomyces cerevisiae,* histone acetylation-deacetylation is one of the epigenetic mechanisms to control gene expression. Histone deacetylases (HDACs) are the transcriptional repressors which cause localized regions of repressed chromatin by deacetylation of histones. There are three groups of HDACs based on their homology to yeast proteins: RPD3 (Class I), HDA1 (Class II) and Sir2 (Class III) [63, 196]. Sir2 deacetylates histone H3 (at K9, K14 and K56) and H4 (particularly K16) to regulate telomere heterochromatin structure in yeast [197, 198]. In yeast, Sir2 is one of the major histone modifiers and mediates silencing at the silent mating-type loci HML and HMR, telomeres, and ribosomal DNA locus through a series of protein-protein interactions. Sir2 plays a crucial role during telomere silencing by contributing its deacetylation activity. Telomere silencing initiated with recruitment of Sir2/Sir4 complex by telomere binding proteins Rap1 and Ku70/80 hetero-dimer [199]. Sir2p deacetylates neighboring nucleosomes and facilitates the binding of Sir4 and Sir3 to hypo acetylated H3 and H4 [200, 201]. Sir3 and Sir4 recruit additional Sir2 and thus the renewal of this cycle causes the spreading of the Sir complex along the sub-telomeric region [202]. As Sir2 is NAD+ dependent histone deacetylase, mutation at the NAD+ binding pocket of Sir2 makes it severely defective in telomere silencing [203]. Nicotinamide (NAM), a byproduct of the enzymatic reaction, acts as a non-competitive inhibitor of Sir2 [93]. It has been demonstrated that

PNC1 which codes for nicotinamidase acts positively regulates Sir2 activity by causing deamidation of NAM and thus increasing the replicative lifespan of yeast [204]. Surprisingly, how *SIR2* gene is regulated at the transcription level under normal condition or in response to different environmental cues is not understood at all.

In this study we were interested to find out the link between environmental factors and the emerging phenotype occurring through alterations in gene expression. To that end using *Saccharomyces cerevisiae* as a genetic model we aimed to explore whether environmental cues like heat stress has any effect on the expression and activity of the type class III NAD^+ dependent histone deacetylase Sir2.

Studies have shown that heat shock causes up regulation of a molecular chaperone heat shock protein 90. Hsp90 is responsible for the overall stability of cellular proteins at elevated temperature. Hsp90 level is significantly increased in the cell upon exposure to stress, including temperature, non physiological pH, nutrient deprivation and malignancy [140]. Heat shock and other proteotoxic stress trigger over-expression of Hsp90 due to the activation of the transcription factor Hsf1. This transcription factor homo-trimerises and translocates to the nucleus from the cytoplasm and causes the transcriptional activation of Hsp90 [205, 206]. Our previous studies in budding yeast have established Sir2 as a client of Hsp90. The temperature sensitive mutant (*iGD170hsp82*) not only leads to the reduced level of Sir2 at the restrictive temperature, but also results in the inactivation of Sir2 proteins. These interesting findings led us to explore the role of over-expression of yeast Hsp90 in Sir2 regulation.

In this specific aim, we have shown that Hsp82 (the yeast ortholog of Hsp90) homeostasis controls the abundance as well as activity of Sir2. Our results demonstrate that heat stress (or over-expression of Hsp82) leads to transcriptional down-regulation of *SIR2* which is inherited through successive

generations before it returns to the normal level. As a result the sub-telomeric genes remain de-repressed for about 90 generations. Also the *HMLa* transcript level remains de-repressed up to four days post heat shock in *MATa* cell indicating a defect in its mating behavior which is has tremendous implications in yeast physiology.

4.2. RESULT:

4.2.1. HEAT SHOCK INDUCES DEREPRESSION OF TELOMERE SILENCING IN YEAST BY DOWN REGULATING Sir2 PROTEIN:

Our findings conclude that the level and function of Sir2 is dependent on active Hsp90 in the cell. This observation led us to explore whether heat shock induced over-expression of Hsp90p leads to alteration of Sir2 protein level and consequently its function. For this purpose we exposed wild type yeast cells (SLY20) to 39^0C heat shock (HS) for 40 minutes which is the optimum condition for induction of heat shock gene *HSP82*. We isolated protein from these HS treated cells. The Western blot analysis showed (Fig. 11A) a significant reduction in Sir2 level in heat stressed condition compared to that growing at normal condition (30^0C). The experimental data were plotted (Fig. 11B) and the graphical representation demonstrated that heat shock increased the expression of Hsp82 which in turn reduced about 50% of the total cellular pool of Sir2.

We aimed to find out whether heat shock induced down regulation of Sir2 causes any change in its activity. To monitor the telomere silencing activity we studied the transcript level of the *YFR057w* at HS condition. The total RNA from the wild type cells grown under normal (30^0C) as well as heat stressed condition (exposing at 39^0C for 40min) was isolated. The semi-quantitative RTPCR showed the derepression of *YFR057w* transcript as a result of heat shock, indicating loss of telomere silencing (Fig. 11C). This result establishes that heat shock leads to loss of Sir2 function at telomere.

4.2.2. EFFECT OF Hsp82 OVER-EXPRESSION ON SILENCING ACTIVITY OF Sir2:

As Hsp82 level is elevated during heat stress, we were interested to determine whether over-expression of Hsp82 led to similar decrease in Sir2 level and its activity. We transformed a centromeric plasmid pHCA/hsp82 in SLY20 to generate SLY13 strain to mimic Hsp82 over-expressed condition within the cell. Hsp82 was expressed under a strong promoter GPD in SLY13.

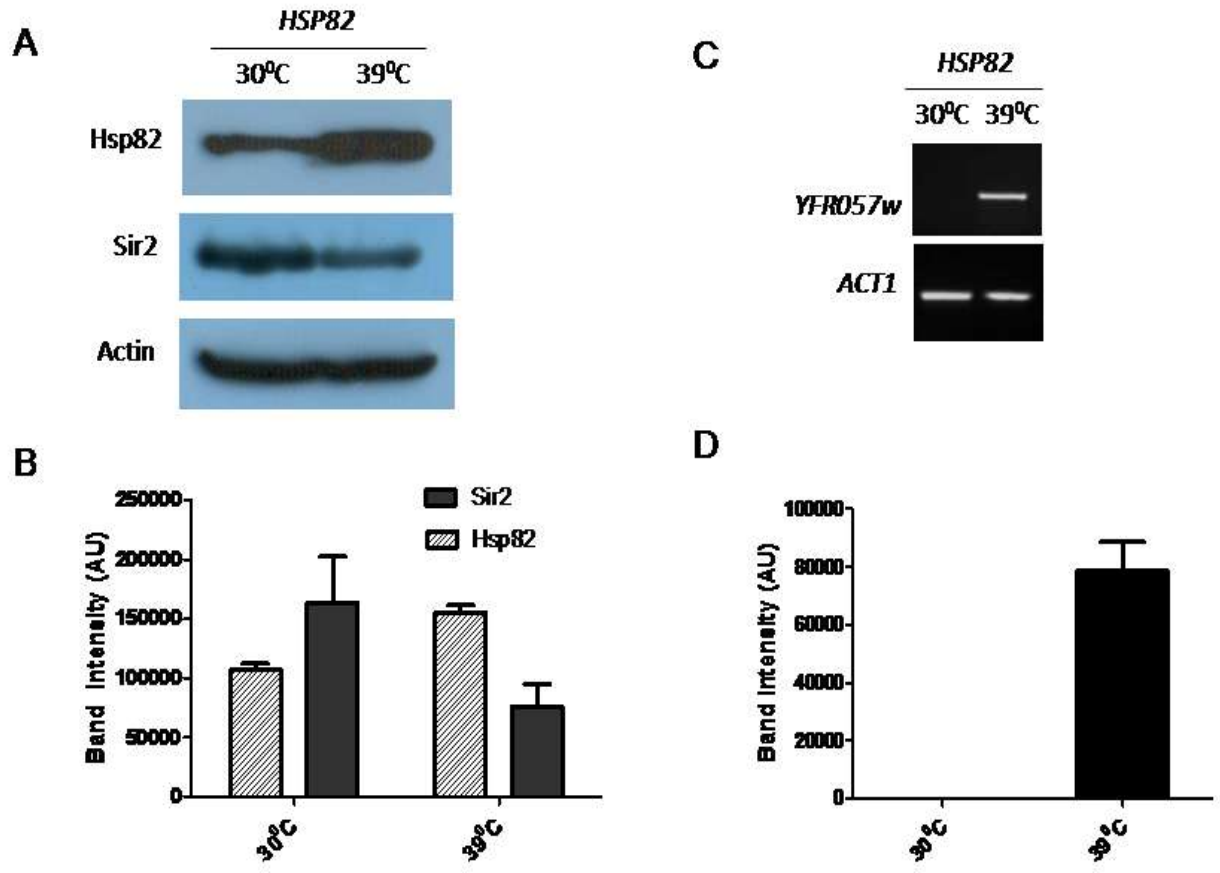

Figure 11: Heat shock induces derepression of transcriptional silencing at telomere. (A) The Western blot shows the relative levels of Hsp82, Sir2 and Act1 in wild type (SLY20) strain at normal (30˚C) *vs.* heat shock condition (39˚C). (B) Three independent experimental data are normalized with respect to Actin level and plotted. Each bar represents mean density ± SD. (C) Heat shock induces the derepression of sub-telomeric gene *YFR057w*, *ACT1* being the loading control. (D) Three independent experiments are performed and the data are plotted after normalizing with *ACT1*. Each bar represents mean density ± SD.

Our results showed that SLY13 cells resulted mostly as white colored colonies indicating a loss of TPE (Fig. 12A), however SLY46 strain containing the blank vector showed pink color colonies. In order to test whether this Hsp82 over-expression mediated loss of TPE is restricted to chromosome VII alone or other sub-telomeric genes as well, we monitored the mRNA level of *YFR057w* gene. We measured the steady state level of *YFR057w* transcript in Hsp82 over-expressed cell (SLY13) and compared with wild type cell (SLY20). The semi quantitative RT-PCR exhibited significant increase in *YFR057w* transcript in Hsp82 over-expressed cells compared to the wild type, whereas *ACT1* transcript remained same in both the conditions (Fig. 12B). From this data we concluded that over-expression of Hsp82 led to de-repression of transcriptional silencing at telomeres.

We also monitored the effect of Hsp82 over-expression on mating type silencing. The mating ability of SLY10 and SLY13 was measured and compared with wild type and *⊿sir2* strains. We observed that all of them exhibited wild type mating phenotype when they were allowed to mate with the tester strain YDS32 (*MATa*) (Fig. 13A). We also quantified the level of *MATa2* transcript in *HSP82* over-expressing cell (SLY13) and compared its level with wild type (SLY20) and *⊿sir2* (SLY12) strains. SLY13 showed negligible amount of *MATa2* transcript, whereas *⊿sir2* strain showed abundant levels of *MATa2* as expected (Fig. 13B).

We, therefore, conclude that over-expression of Hsp82 does not affect mating type silencing. We also over-expressed Hsp82 in *sba1* null background (SLY31) and determined Sir2 activity. TPE assay result showed similar results. We observed while Δ*sba1* mostly developed as pink colored colonies, SLY31 showed white colored colonies (Fig. 12A). However, the mating type silencing function of both Δ*sba1* and SLY31 remained unperturbed (Fig. 13A). These results indicate that Hsp82 over-expression in wild type as well as Δ*sba1* causes the reduction in telomere silencing but not mating type silencing.

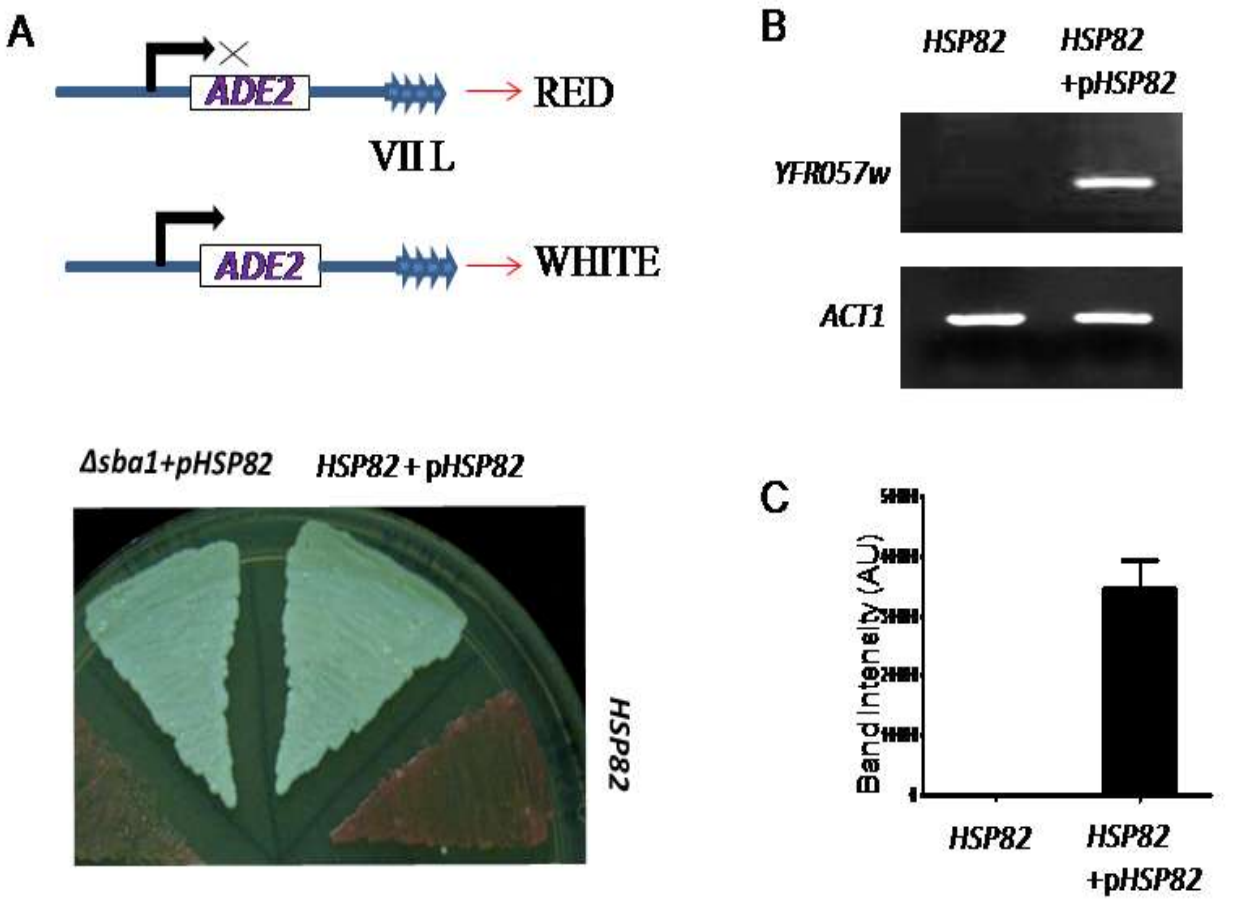

Figure 12: Over-expression of *HSP82* negatively regulates TPE. (A) ADE2 color phenotype of various strains *i.e.*, *HSP82* (wild type), *Δsir2*, over-expressing *HSP82* (*HSP82 + pHSP82*). Red colored colony represents telomere silencing and white colored colony represents de-repression of telomere silencing. (B) Over-expression of *HSP82* de-represses the expression of *YFR057w*. *ACT1* serves as loading control. (C) Quantification of *YFR057w* transcript is done from 4 independent experiments in *HSP82* over-expressed cells. The band intensity values are normalized against *ACT1*. Each bar represents mean density ± SD.

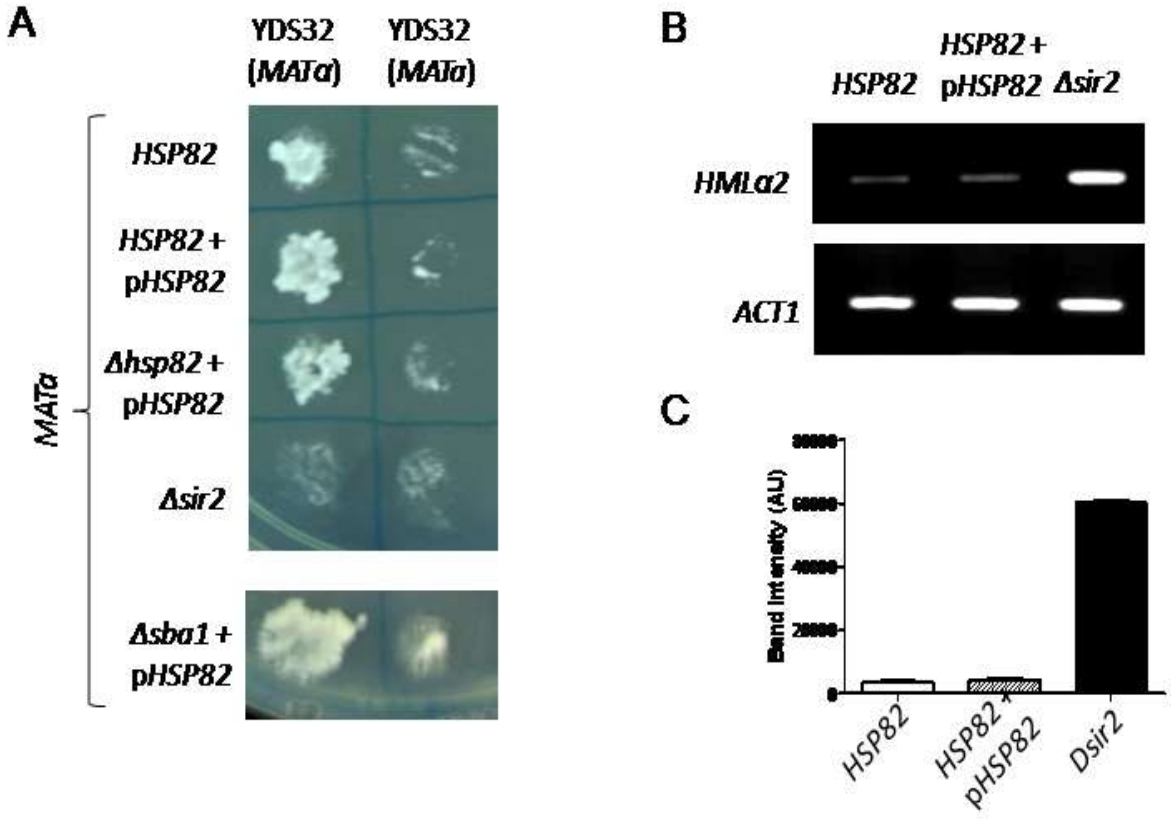

Figure 13: Over-expression of *HSP82* does not alter mating type silencing. (A) Mating type silencing phenotype of different strains (as indicated) when mated with the tester strain YDS32 (*MATα*). *Δsir2* acts as a negative control. (B) RT-PCR shows *HMLα2* transcripts in different strain backgrounds (as indicated) shows that *HMLα2* transcript levels are similar in *HSP82* over-expressing cells and wild type cells, *ACT1* serves as loading control. (C) Quantification of *HMLα2* transcript from three independent experiments are normalized with *ACT1* and plotted. Each bar represents mean density ± SD.

4.2.3. OVER-EXPRESSION OF Hsp82 REDUCES THE CELLULAR LEVEL OF Sir2 PROTEIN IN A DOSE DEPENDENT MANNER:

Our study showed that over-expression of Hsp82 leads to down regulation of transcriptional silencing at telomeres. This result led us to monitor whether there is any change in Sir2 protein under Hsp82 over-expressed condition. Equal amount of protein was isolated from SLY10, SLY13 and SLY20 strains loaded and they were probed with anti-Hsp90 antibody as well as anti-Sir2 antibody. Our result showed that in Hsp82 over-expressing cells (SLY10 and SLY13 strains) the level of Sir2 was considerable decreased (Fig. 14A). The quantification of the bands from at least three independent experiments were done which showed that in SLY13 strain while Hsp82 amount was increased by 50% compared to the wild type, the level of Sir2 was reduced by more than 50% in some cases (Fig. 14B). Since cellular pool of Hsp82 varies in response to different stressed condition, it is important to know whether Sir2 level is dependent on Hsp82 in a dose dependent manner or not. In order to address this question, SLY32 strain was generated, which over-expresses Hsp82 from a multi-copy 2μ plasmid (pTA/hsp82) under strong *GPD* promoter. The level of Hsp82 and Sir2 were compared among SLY20, SLY13 (CEN plasmid harboring *HSP82*) and SLY32 (2μ plasmid harboring *HSP82*) strains by Western blot analysis. Gradual increment in Hsp82 protein levels was observed from SLY20 to SLY13 to SLY32 and this increment was in accordance with increase in copy number of episomal plasmid. However, Sir2 protein level showed a reverse trend. The most drastic reduction in Sir2 level was observed in SLY32 cells, while SLY13 exhibited a moderate decrement compared to the wild type cells (SLY20) (Fig. 14C). Taking the average from independent three sets of experiments, the quantification of the bands were done which showed that while the increase in Hsp82 level is 4 times in SLY32 strain compared to the wild type, the decrease in Sir2 level was at least by 75% compared to the wild type and by 50% compared to the SLY13

strain (Fig. 14D). Thus, it appears that under stressed condition the over-expression of Hsp82 results in concomitant reduction of the total cellular pool of Sir2, which led us to speculate that such adaptation in Sir2p abundance in response to Hsp82 homeostasis could be responsible for the reversible nature of transcriptional silencing at telomere.

4.2.4. HEAT SHOCK OR Hsp82 OVER-EXPRESSION INDUCES DOWN-REGULATION OF *SIR2* TRANSCRIPTION:

To investigate whether the reduction of Sir2 is merely at the protein level or whether it extends to the transcript level as well, we analyzed *SIR2* transcript under heat stress conditions. To this end, SLY20 cells were exposed to 39°C for 40 minutes, a condition that over-expresses Hsp82, and compared the level of *SIR2* mRNA with the wild-type cells. We also compared the levels of *SIR2* mRNA within the strains containing the empty expression vector (SLY13C) and that containing over-expression of *HSP82* plasmid (SLY13). In both conditions, we estimated the levels of Hsp82 protein which were higher compared to that of the control cell (Fig. 15A, bottom panel). The semi-quantitative RT-PCR showed that Hsp82 over-expression (artificially or in heat stressed condition) caused down regulation of *SIR2* (Fig. 15A). Quantitative analysis by real time RT-PCR revealed about five-fold reduction in *SIR2* transcript in heat shock/Hsp82 over-expressed condition compared to that of control cells (Fig. 15B).

4.2.5. HEAT SHOCK (HS) LEADS TO TRANS-GENERATIONAL REGULATION OF *SIR2:*

We intended to observe whether heat shock induced decrement of *SIR2* transcript was transmitted to successive generations. We exposed SLY20 strain to heat stress at 39°C for 40 minutes and subsequently returned them to 30°C. Those heat stressed cells were maintained for several generations (up to 10 days) at 30°C with regular medium change at every 24 hours.

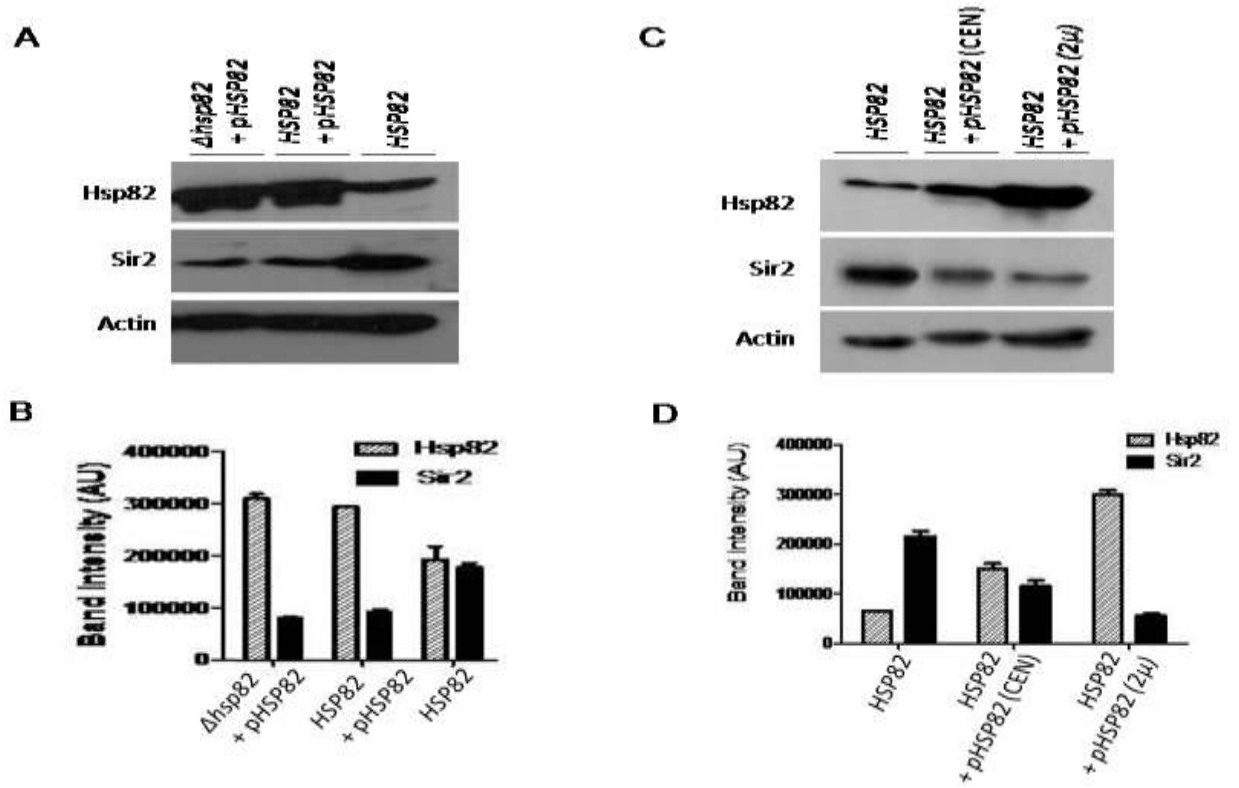

Figure 14: Western blot analysis shows over-expression of *HSP82* down regulates Sir2 in a dose dependent manner. (A) Different lanes are marked with the respective genotypes. p*HSP82* implies over-expression of *HSP82* from a CEN plasmid. (B) Graphical representation of (A), where densitometric measurements of the bands from four experiments are plotted, after normalizing with Actin band intensities. Each bar represents mean density ± SD. (C) Multiple levels of Hsp82 over-expression is shown using high (2μ) and low copy plasmids (CEN), to demonstrate dose response between Hsp82 and Sir2 levels. (D) The graphical representation of Western blot as in figure (C) shows over expression of Hsp82 from a high copy plasmid confers more reduction of Sir2p level. Each bar represents the mean density ± SD from 4 independent experiments. The data are normalized with respect to Actin.

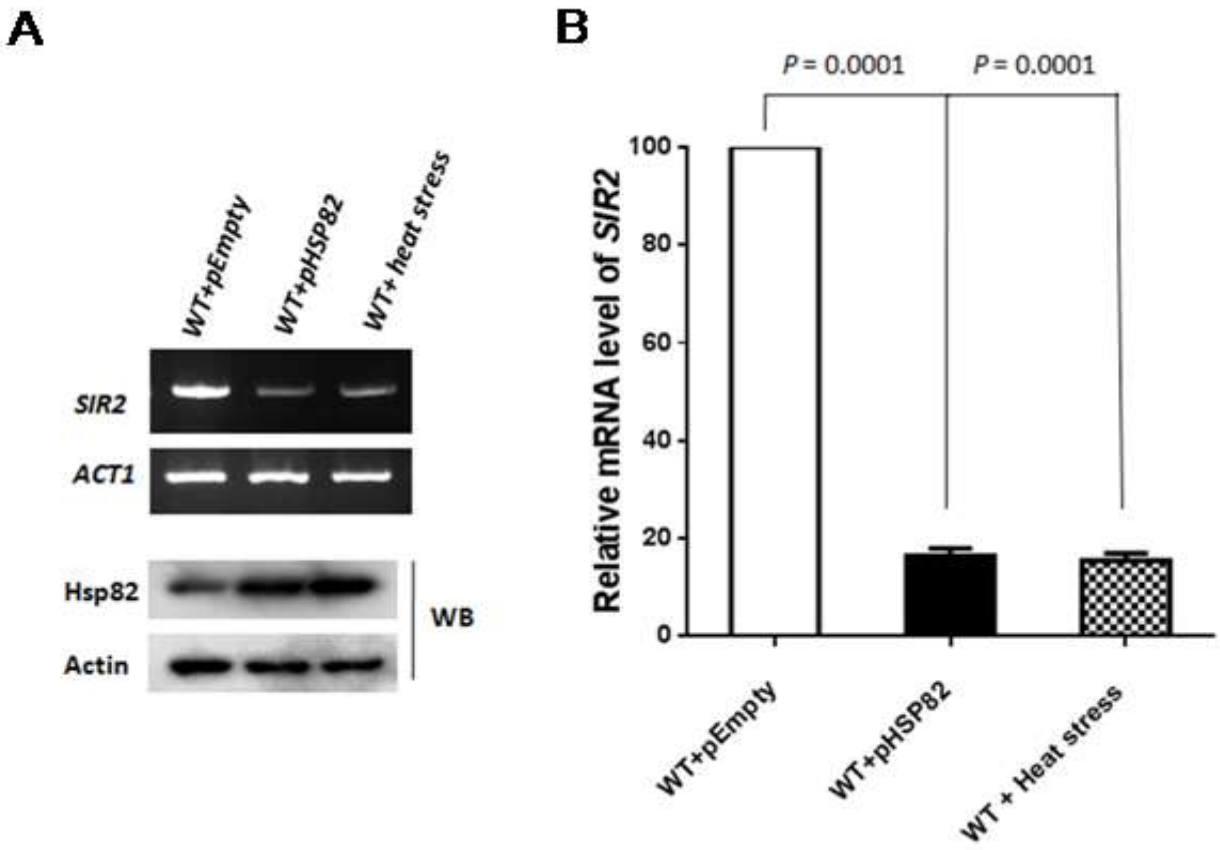

Figure 15: Heat shock or Hsp82 over-expression causes transcriptional down-regulation of *SIR2*. (A) Semi-quantitative RTPCR shows *SIR2* transcript in cells exposed to heat shock (39˚C for 40 minutes) and in Hsp82 over-expression cells compared to that present in wild-type cells. Bottom: Western Blotting was done with anti-Hsp82 and anti-Actin antibodies (B) The real time PCR shows relative mRNA levels of *SIR2* in above mentioned conditions (indicated on the X-axis were plotted after normalization with *ACT1* mRNA. In each case, the mean value (±SD) from three independent experiments with three independent harvests of cells was calculated and was plotted using Graph Pad prism6 software. *P* values were calculated using two-tailed Student's t-test.

In parallel, we maintained wild-type culture which was not subjected to heat stress. We isolated the total RNA before HS (0 hour), 2 hours post HS and thereafter at an interval of every 24 hours. The semi-quantitative RTPCR was repeated three times with independent harvests of cells and one of the representative experiments is presented (Fig. 16A). We observed that HS-mediated reduction in *SIR2* transcript continued through successive generations. *SIR2* transcript was barely observed in the 4th and the 6th day HS samples. However, it was regained to the level comparable to that of the unstressed cell on the 7th day post HS. Quantitative analysis by real time RTPCR result showed that the relative level of *SIR2* mRNA had reduced nearly twenty five-fold and fifteen-fold in the 4th day and the 6th day HS cultures respectively. However *SIR2* transcript of the 7th day HS culture was comparable to that of the control (Fig. 16B). Our observation was supported by Western Blotting with anti-Hsp82 and anti-Sir2 antibodies. Hsp82 over-expression under heat stress led to a significant reduction in Sir2 protein in the 2 hr and the 4th day post HS. It is important to note that in the 7th day HS sample, Sir2p returned to that of the unstressed cell along with a significant reduction of Hsp82 (Fig. 16C).

The quantification of the relative band intensity from three independent experiments showed that Hsp82p level increased by 2.2 times in the 2hr post HS sample and remained 2.1 times higher in the 4 days post HS compared to the level of control and finally returned to the level of control after 7 days (Fig. 16D). Similarly, the relative band intensity of Sir2 decreased by half in the 2 hours post HS and remained at that level up to 4 days and then returned to the level of the control cell on the 7th day (Fig. 16E). Our previous work showed that Hsp82 over expression led to derepression of telomere silencing but did not alter mating type silencing in yeast. We wanted to explore whether a transient heat shock leads to any trans-generational mating type silencing defect in yeast. To test this, we monitored *HMLa* transcript at various time intervals in the post HS sample and compared it to

that of the control cells which were never exposed to heat shock. The real time RTPCR result demonstrated that the relative levels of *HMLa* in the 2 hours post HS sample are slightly higher than those in the control cells, however they increased significantly (5 times) on the 4th day post HS and ultimately returned to the normal level on the 7th day (Fig. 17A).

To investigate the telomere silencing activity of Sir2 in HS samples, we quantified the transcription of sub-telomeric gene *YFR057w* by real time RTPCR analysis. Under normal conditions, Sir2 causes silencing of *YFR057w* transcription by spreading through the sub-telomere ends of chromosome. Our result showed that there was no significant change in the *YFR057w* mRNA level in the 2 hr post HS sample compared to that of the control. However in the 4th, 6th and 7th day post HS samples, *YFR057w* transcript up-regulated 4.5-fold, 3.6-fold and 3-fold respectively and it was repressed again from the 8th day onwards (Fig. 17B). We observed the growth of control and HS culture for 7 days and the kinetics showed that they were dividing at the same rate (Fig. 17C). It was earlier reported that prolonged heat shock (at 37°C) causes shortening of telomere in yeast [131]. To understand whether telomere shortening is responsible for the derepressed sub-telomeric chromatin due to HS, we wanted to find out the effect of transient heat shock in the telomere structure. For that purpose, we monitored the length of the telomere for 7 days in post HS samples. Our southern blot analysis showed that transient heat shock leads to shortening of telomere length (Fig. 18). Telomere length remained short up-to the 4th day post HS and then returned to the wild-type length on the 6^{th} day HS.

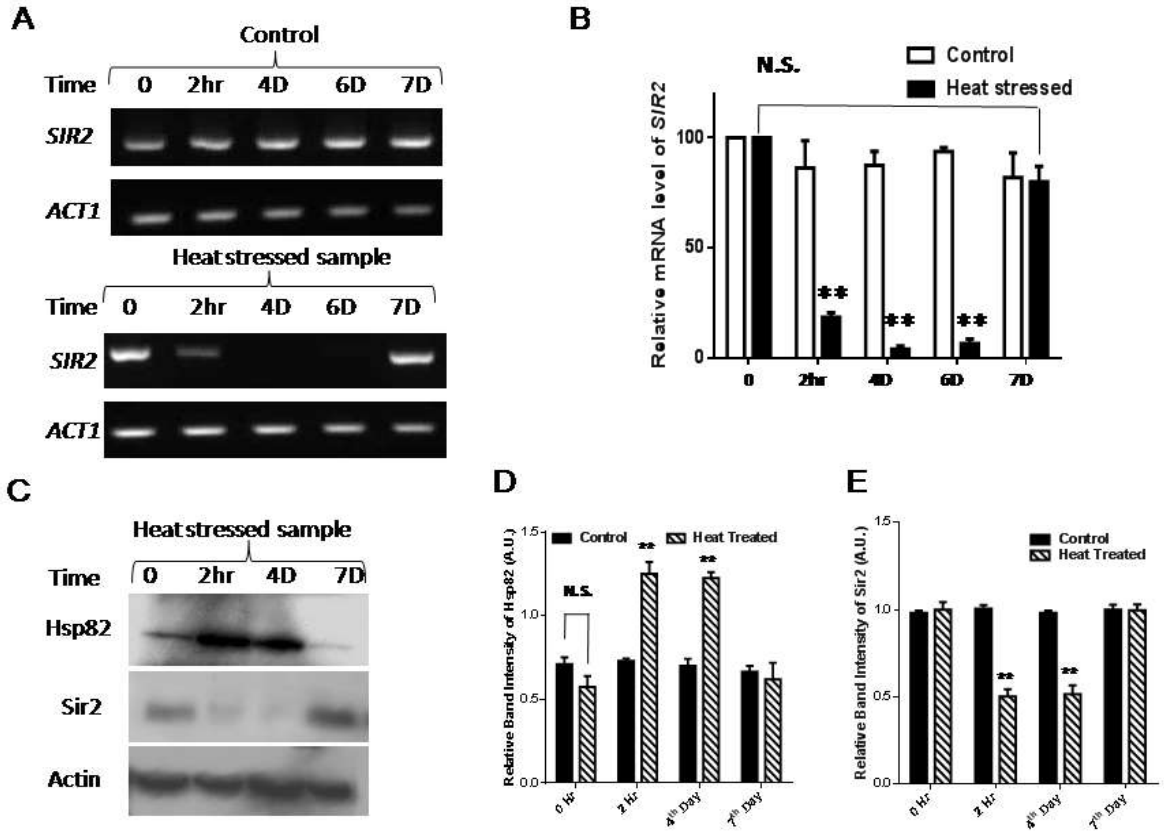

Figure 16: Heat shock results in trans-generational transmission of down regulated ***SIR2.*** **(A) Wild-type cells were exposed to heat shock (39˚C) for a period of 40 minutes and then returned to 30˚C. *SIR2* transcript profile was monitored after 2 hr, and on the 4th day, 6th day and the 7th day post HS. The experiment was repeated three times and one of the representative semi-quantitative RTPCR is presented. (B) In normal and heat stressed cell relative mRNA levels of *SIR2* from real time RTPCR at different time points (as indicated in X-axis) were plotted. Error bar indicates ±SD; n=3 experiments; asterisks indicate values significantly different from control; **p<0.01; *p<0.05; N.S., not significant: *ACT1* was used as the normalization control. (C) Western Blot analysis was done taking control and heat stressed samples, at different time intervals using anti-Sir2, anti-Hsp82 and anti-Actin antibodies (D) Densitometric measurement of Hsp82 from three independent Western blots**

was plotted with control and heat treated sample at the indicated time points; Error bar indicates ±SD. (E) Densitometry measurement of Sir2 from three independent Western blot experiments was plotted with control (before heat shock) and heat stressed sample at the indicated time points; Error bar indicates ±SD.

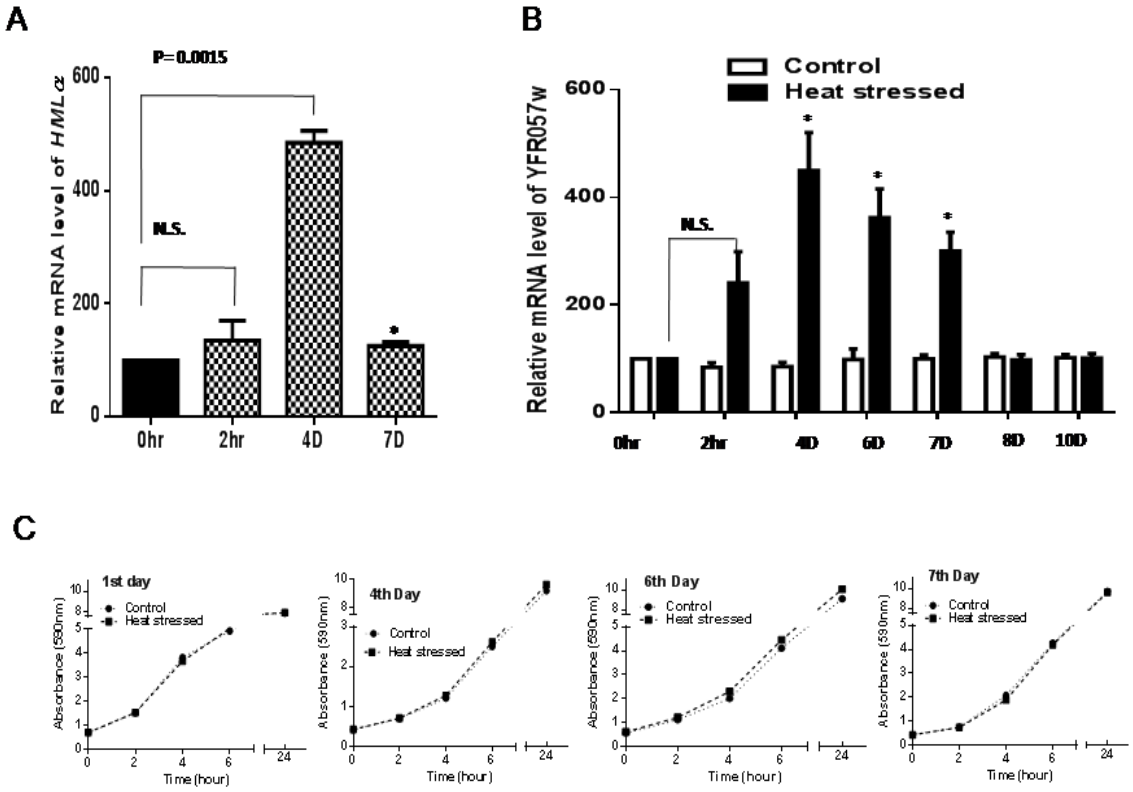

Figure 17: Heat shock leads to trans-generational transmission of de-repressed chromatin **(A) Relative mRNA levels of *HMLα* in MATa haploids are plotted at different time points given in the X-axis, before and after heat shock. Each bar represents the mean ±SD; n=3, *p<0.05; N.S. not significant (B) Relative mRNA levels of *YFR057w* in wild type cells and cells exposed to heat shock (39˚C for 40 minutes) at indicated time points are shown. Each bar represents the mean ±SD; n=3, *p<0.05; N.S. not significant. (C) Growth kinetics of**

wild-type and heat stressed cells were monitored for seven days. The graph represents a comparison between their growths on the 1st, the 4th, the 6th and the 7th day.

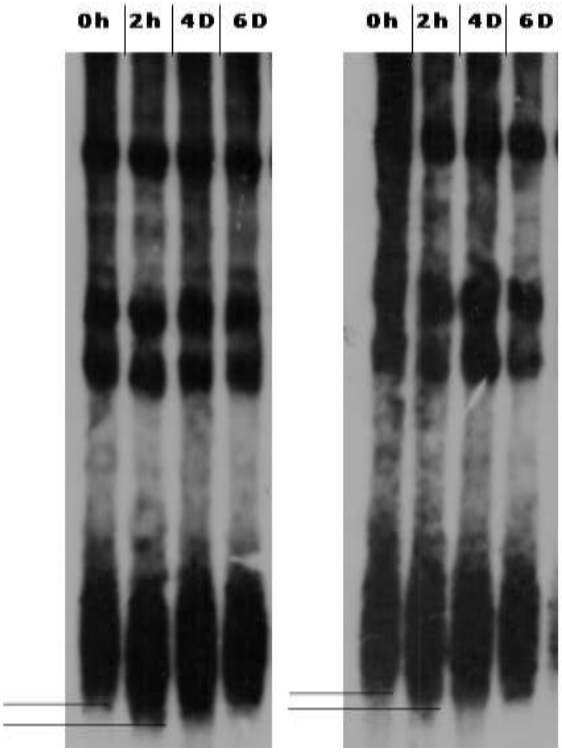

Figure 18: Transient heat shock contributes to telomere shortening in wild-type cells.

Wild type strain was subjected to heat shock at 39°C for 40 minutes and then was grown at 30°C for 6 days. Genomic DNA was isolated at the indicated time intervals (as marked on the top) subjected to XhoI digestion and telomere length was measured using Southern Blot hybridization. Two telomere blots are represented from three independent experiments. The difference between telomere lengths grown at 30°C and the 2hr post HS is represented by solid lines.

4.3. DISCUSSION:

In this specific aim, we provide very interesting link between the environmental factor, heat stress and expression of epigenetic modifier Sir2 in yeast. We have demonstrated that Hsp90 homeostasis regulates Sir2 function in the cell. Our experimental results establish that Hsp90 regulates the transcription of *SIR2* under heat stress conditions and thereby controls cellular abundance of Sir2 protein. We show that heat shock treatment as well as Hsp90 over-expression caused drastic reduction in the endogenous level of Sir2. The reduced pool of Sir2 is functionally active, as it is adequate enough to silence hidden mating type loci. However, it is insufficient to establish silencing across all the 32 sub-telomere regions in yeast. Studies have shown that dietary restriction, an environmental factor, increases longevity from yeast to mammals [136]. NAD^+ dependent histone deacetylase activity of Sir2 is involved in increased longevity during starvation and this effect is not observed in the *sir2* mutant strain [136]. Our result shows that under heat shock conditions (39°C), elevated level of Hsp82 leads to repression of *SIR2* transcription thereby mimicking *sir2* knockdown condition in yeast which is inherited for many generations. Thus, it will be interesting to predict that Sir2 may not influence longevity under heat stress conditions. This hypothesis is supported by the following evidence. ⊿*sir2* mutant strain does not show increase in yeast life span and according to our finding, upon heat treatment the steady state level of Sir2 decreased drastically and remained almost undetectable up-to 6 days in our Western blot analysis. Another study in *Caenorhabditis elegans* [226] also documented that lower temperature lengthened the life span of worms. It will be interesting to explore whether such kind of Hsp90 mediated regulation of mammalian sirtuins also results in their suboptimal activity.

We show that transient heat shock results in heritability of derepressed sub-telomeric chromatin. It had been reported earlier that telomere structure regulates the heritability of silenced sub-telomere

[228]. Elongated telomere contributes to the increased inheritance of silenced sub-telomeric state and it is independent of the yeast chromatin assembly factor 1 (yCAF-1). In another study, it was reported that prolonged exposure to heat stress (37°C) as well as Hsp82 over-expression resulted in telomere shortening in wild-type cell [131]. Thus, it is important to find out whether transient heat shock causes any change in telomere structure and can thereby influence telomere silencing. Our study shows that transient heat shock causes shortening of telomere length which is gradually returned to wild-type length at the end of 6th day. The restoration of telomere length might be one of the factors for the reappearance of the *YFR057w* transcript or telomere position effect (TPE). As the timings of Sir2 re-appearance and the re-establishment of TPE coincide very closely, we speculate that restoration of Sir2 in the cell level is also likely to be the reason behind regaining of wild-type like silencing at the telomere. Thus it may be possible that in yeast, heat stress driven re-establishment of TPE might be multi factorial and a time of 7-8 days is required for the full re-establishment of telomere silencing. Our work showed that transient heat shock rendered the cell as *⊿sir2* for more than ninety generations. We found de-repression of *HMLa* transcript upon transient heat shock and this leads to a likely defect in yeast mating behavior which has tremendous implications on yeast physiology.

TPE model in budding yeast *S. cerevisiae* have demonstrated the transitions between the two states of chromatin. However, the reversible nature of such heterochromatin-euchromatin transition and *vice versa* remains poorly understood. The regulation at the DNA-protein interface is not clear whereas, the epigenetic marks (especially H3 and H4 modifications) associated with such changes are well established. This study leads us to speculate that may be under *in vivo* condition Hsp82 is required to maintain a dynamic equilibrium of total amount of silencing proteins. This idea has been proven under heat stressed condition which alters Hsp82 levels in the cells. We have observed that heat

stress causes reduction in total Sir2 abundance by more than 50% which is associated with complete lose of silencing at telomere. Thus the adaptation in Sir2 abundance in response to the Hsp82 homeostasis could be associated with the reversible nature of transcriptional silencing at telomere.

CHAPTER 5

IDENTIFICATION OF CIS-REGULATORY AND TRANS-REGULATORY ELEMENTS ASSOCIATED WITH Hsp90 MEDIATED REGULATION OF *SIR2*

5.1. INTRODUCTION:

Sir proteins mediate silencing at telomere, mating locus and ribosomal DNA locus by a series of protein-protein interactions; Rap1, Orc1 and Abf1 recruit Sir1, Sir2 along with Sir4 and Sir3 to the silencer causing the spreading of Sir-complex along the chromosome. Sir2 deacetylates lysine 9 and 14 on histone H3 and lysines 5, 8, 12 and 16 on histone H4 generating binding sites for Sir3 and Sir4 [200, 201]. Sir2, NAD^+ dependent protein deacetylases, are sensitive to changes in intracellular concentration of NAD^+. In yeast, NAD^+ is synthesized from tryptophan and there are three vitamin precursors of NAD^+ which are nicotinic acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR). Nicotinic acid phosphoribosyl transferase (Npt1) is the rate limiting enzyme in NAD^+ biosynthesis and deletion of Npt1 results in inhibition of Sir2 mediated transcriptional silencing and ultimately shortening of replicative life span (RLS). NR gives rise to NAM which is converted into NA by deamidation acitivity of Nicotinamidase 1 (Pnc1). Presence of NAM in the growth medium inhibits Sir2 activity leading to *⊿sir2* phenotype [93]. Nicotinamide (NAM), a byproduct of the enzymatic reaction, acts as a non-competitive inhibitor of Sir2 [93]. Over-expression of PNC1 suppresses this phenotype by scavenging NAM by deamidation and hence it has been demonstrated as positive regulator of Sir2 activity [204]. As Sir2 is NAD^+ dependent histone deacetylase, mutation at the NAD^+ binding pocket of Sir2 makes it severely defective in telomere silencing [203]. Surprisingly, how *SIR2* gene is regulated at the transcription level under normal condition or in response to different environmental stresses is not understood at all.

Cup9 was originally identified as the gene that helps the cell to tolerate very high doses of copper which are otherwise toxic for cell. However, the mechanism of such effect was not discovered [207]. It was revealed from wide array of published microarray experiments, *CUP9* transcription increases many-fold when cells are exposed to hypoxia, osmotic stress and when grown in the presence of

alternate carbon sources [208, 209]. Cup9 is a homeodomain transcriptional repressor containing a high degree of identity with human PBX proteins (Pre-B cell leukaemia transcription factor) that are crucial for embryonic development [210]. It also shows identity with the *MATa2* locus in *S. cerevisiae* [211]. About thirty six targets of Cup9 have been documented so far. Among these the most characterized target is the master peptide transporter (dipeptide and tripeptide) *PTR2*. It is been shown that Cup9 along with the co-repressors Tup1 and Ssn6 [213] represses *PTR2* transcription and thus reduce peptide import in cell [214, 144].

Experiments in previous specific aims showed that Hsp82 (the yeast ortholog of Hsp90) homeostasis controls the abundance as well as activity of Sir2. Hsp82 null condition not only leads to the reduced abundance of Sir2, but also results in the loss of Sir2 protein activity. On the other hand, heat shock as well as Hsp82 over-expression down regulates *SIR2* transcription. In the third specific aim, we wanted to provide mechanistic insights into transcriptional regulation of *SIR2* by heat shock or Hsp82 over-expression. We have identified the cis regulatory region as well as trans-regulatory elements (repressor) responsible for transcriptional down regulation of *SIR2* during heat shock. We have effectively mapped the cis regulatory element of *SIR2* gene and have identified a repressor binding site in the region spanning (-429 to -369) base pair of $SIR2_{UAS.}$ We have found that (-429 to -369) base pair of $SIR2_{UAS}$ is crucial for Hsp82 mediated transcriptional down regulation of *SIR2*. Using a series of genetic experiments we have identified a transcriptional repressor Cup9 which is responsible for transcriptional down regulation of *SIR2*. Our study shows that Hsp82 over-expression (or heat shock) causes up regulation of *CUP9* both at the transcript as well as at the protein level and ChIP experiment confirms that Cup9 binds to the UAS_{SIR2} under Hsp82 over-expressed condition. Deletion of *CUP9* abrogates Hsp82 over-expression mediated decrement of *SIR2* transcript. Conversely, Cup9 over-expression leads to down-regulation of SIR2 and TPE.

5.2. RESULTS:

5.2.1. MAPPING THE CIS-REGULATORY REGION OF *SIR2* AFFECTED BY OVER-EXPRESSION OF Hsp82:

To characterize the transcriptional down regulation of *SIR2*, we cloned the upstream regulatory element of *SIR2* (- 429 to -1) into a reporter plasmid carrying *LACZ* and named it as 429_{UAS} (Fig. 19A). Similarly, three more constructs 370_{UAS}, 307_{UAS} and 200_{UAS} were generated which spanned across the $SIR2_{UAS}$ (-370 to -1), (-307 to -1) and (-200 to -1) regions as shown in the figure (Fig. 19A). These $SIR2_{UAS}$ regions were individually cloned as the exclusive promoter region of *LACZ* expression cassette into the *LACZ* reporter plasmid (Fig 19 B). We generated a reporter plasmid without any promoter to provide a negative control (SLY64). Eventually all these constructs were transformed to SLY20 to generate isogenic strains SLY57 (429_{UAS}), SLY84 (370_{UAS}), SLY83 (307_{UAS}), SLY56 (200_{UAS}) and SLY64 (negative control) respectively. *LACZ* gene expression from these constructs directly correlated with the transcriptional activity of different regions of *SIR2* upstream regulatory element. We sought to identify the cis-regulatory element of *SIR2* which is regulated by heat stress or Hsp82 over-expression. To test this, we compared *LACZ* transcription of 429_{UAS} in the presence and the absence of Hsp82 over-expression. Semi-quantitative RTPCR revealed that over-expression of Hsp82 significantly reduced *LACZ* transcript in the strain carrying both 429_{UAS} and *pHSP82* (SLY61) compared to the strain carrying 429_{UAS} and pEmpty (SLY61C) (Fig. 19C). Western blot in the bottom panel confirmed over-expression of Hsp82 in SLY61 compared to SLY61C. Quantitative analysis by real time RTPCR revealed that *LACZ* mRNA level is reduced 2.4-fold in Hsp82 over-expressed condition compared to the wild type (Fig. 19D).

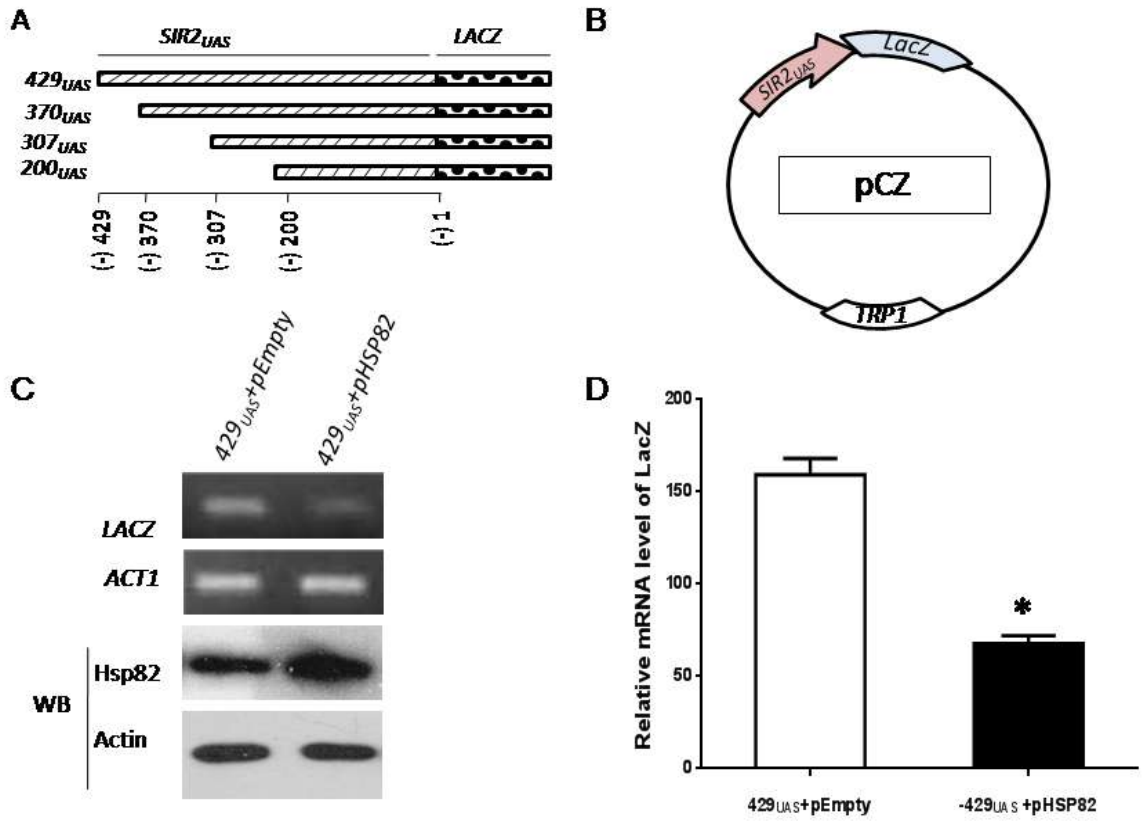

Figure 19: *429_{UAS}* of Sir2 is affected by Hsp82 over-expression. (A) 429, 370, 307 and 200 base pair upstream activator sequences of *SIR2* were cloned in the upstream region of *LACZ* to generate four reporter plasmids, namely *429_{UAS}*, *370_{UAS}*, *307_{UAS}* and *200_{UAS}*. (B) These *$SIR2_{UAS}$* regions were individually cloned as the exclusive promoter region of *LACZ* expression cassette into the *LACZ* reporter plasmid. (C) *429_{UAS}* reporter plasmid was transformed into wild-type cells and cells over-expressing Hsp82. Semi-quantitative RTPCR shows the relative levels of *LACZ* transcript between wild-type and Hsp82 over-expressing cells. Bottom: Western Blotting was done with anti-Hsp82 and anti-Actin antibodies. (D) Real time RTPCR shows relative levels of *LACZ* mRNA between wild-type and cells harboring Hsp82 over-expression plasmid. Error bar indicates ±SD; n=3 experiments; asterisks indicate values significantly different from control; *p<0.05.

We aimed to find out the repressor binding site in $SIR2_{UAS}$. We quantified the relative levels of *LACZ* mRNA in cells harboring various deletion constructs. The transcription of *LACZ* in SLY64 (no promoter control) was considered as the base line. Our result showed about eighty-fold increment in *LACZ* in the cells carrying full length (429_{UAS}) upstream activator sequence of *SIR2* compared to that of negative control (no promoter). However, there was further increase in *LACZ* transcription by seven-fold in 370_{UAS} and in 307_{UAS} (Fig. 20A). It provides the evidence of a repressor binding site in the region spanning (-429 to -369) bp of $SIR2_{UAS}$. 200_{UAS}, however, displayed about four-fold reduction in *LACZ* mRNA compared to the 429_{UAS}. Next, we aimed to narrow down the region of cis-regulatory element of *SIR2* that is regulated *via* Hsp82 over-expression. We transformed Hsp82 over-expression plasmid in the cells carrying individual constructs to mimic the heat shock condition. As a control, we transformed the empty plasmid in cells carrying different *LACZ* fusion constructs. Quantitative analysis by real time RTPCR showed about two-fold reduction in *LACZ* transcription in cells carrying 429_{UAS} along with Hsp82 over-expression (Fig. 20B). However Hsp82 over-expression in 370_{UAS}, 307_{UAS} and 200_{UAS} showed no effect on the relative mRNA level of *LACZ* compared to that having the empty plasmid. Western blotting showed over-expression of Hsp82 in each of the four constructs (Fig. 20B: Bottom panel). Altogether these data demonstrated that the region spanning (- 429 to -369) of *SIR2* is crucial for its regulation during Hsp82 over-expression.

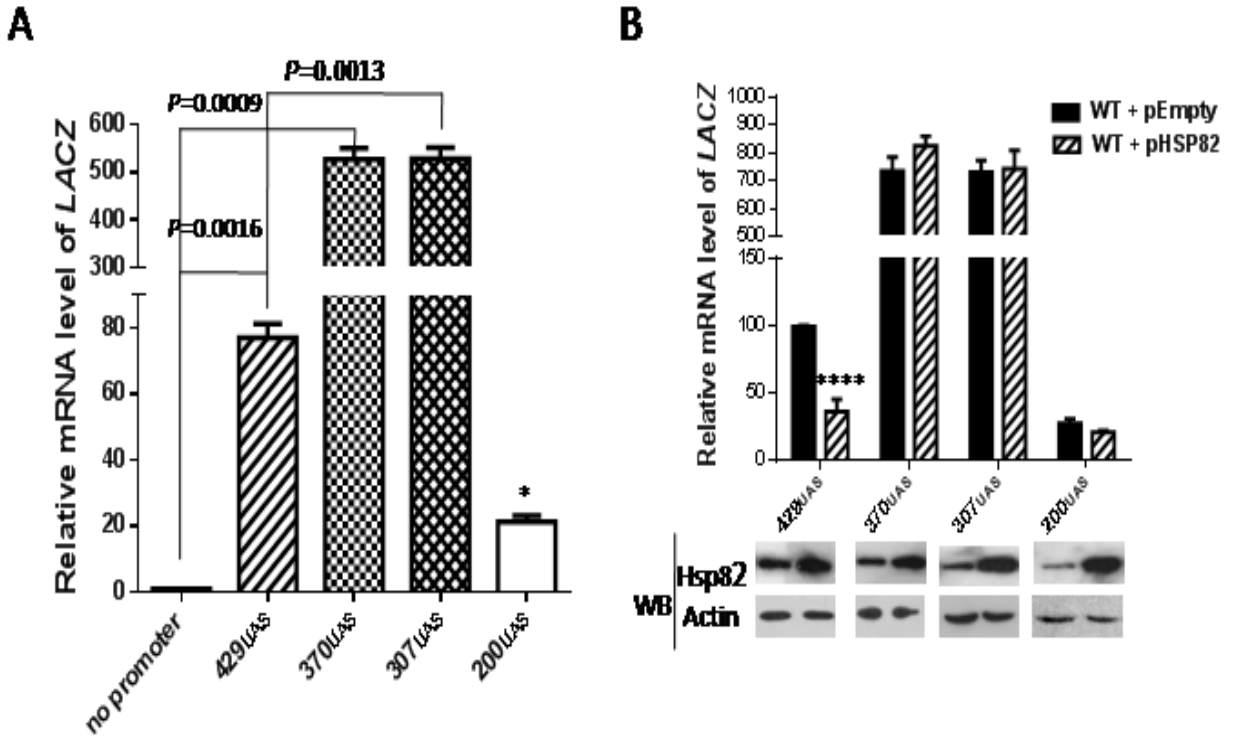

Figure 20: Mapping the cis regulatory region of Sir2 that is affected by Hsp82 over-expression (A) *LACZ* reporter plasmid without any promoter was transformed in wild-type cell as negative control. Real time RTPCR compares the relative mRNA levels of *LACZ* in cells bearing four different reporter plasmids along with the cell having no promoter. *P* values are calculated using the two-tailed Student's t-test. (B) Hsp82 over-expression plasmid was transformed in each of the four reporter plasmid harboring cells. Real time RTPCR shows how the relative mRNA level of *LACZ* is affected in the presence of *HSP82* over-expression plasmid. The relative abundances of *LACZ* from these constructs are plotted after normalization against *ACT1* mRNA. Each bar represents mean mRNA level (±SD) from three independent experiments. Bottom: Western Blot was done using anti-Hsp82 and anti-Actin antibody.

5.2.2. BIOINFORMATICS PREDICTION OF TRANSCRIPTION FACTOR THAT BINDS TO $SIR2_{UAS}$:

The 429 bp upstream regulatory region of *SIR2* was analyzed for the transcription factor binding sites in *Saccharomyces cerevisiae*. The analysis for finding transcription factor binding site was performed by the statistical method [205] employed in the most widely used TRAP (Transcription Factor Affinity Prediction) Web Tool. Based on TRAP, eight transcription factors were found to have high binding affinity, namely, Cup9, Rim101, Sok2, Tod6, Phd1, Tec1, Dot6 and Sum1, out of which four are transcriptional repressors. The databases TRANSFAC /and JASPAR show that Cup9 has the highest binding affinity (p value 0.007). Its binding sequence belongs to -411 to -402 regions [212].

5.2.3. KNOCKOUT OF *CUP9* REVERSES *HSP82* OVER-EXPRESSION MEDIATED PHENOTYPE:

Based on the bioinformatics analysis, we aimed to characterize all four repressors, namely, Cup9, Sum1, Rim101 and Sok2, to identify the putative repressor of *SIR2* transcription. We generated four deletion strains, namely, *⊿cup9*, *⊿rim101*, *⊿sok2* and *⊿sum1* using homologous recombination (Fig. 21) and screened each of them using various genetic experiments in Hsp82 over-expression background.

Semi-quantitative RTPCR showed that there was no significant reduction in *SIR2* transcript in *⊿cup9* strain (SLY77C) carrying the empty vector or the Hsp82 over-expression plasmid (SLY77). On the other hand, we observed significant reduction in *SIR2* transcription in *⊿sok2*, *⊿sum1* and *⊿rim101* strains upon Hsp82 over-expression (Fig. 22A). Real time RTPCR also showed no significant reduction in *SIR2* mRNA levels in *⊿cup9* strain with and without *HSP82* over-expression plasmid. However, other deletion strains exhibited considerable reduction in *SIR2* transcript in presence of

p*HSP82* over-expression compared to that of empty plasmid (Fig. 22B). This result was similar to the phenotype observed in wild-type strain in Hsp82 over-expression condition. From western blot analysis, we observed the presence of a comparable amount of Sir2 in both *cup9* deleted strain harboring Hsp82 over-expression plasmid and that having empty vector (Fig. 22C). However, there was considerable reduction in Sir2 under Hsp82 over-expression in *⊿sum1*, *⊿rim101* and *⊿sok2* cells compared to those carrying the empty plasmid. Relative band intensity (with respect to Actin) revealed about 50% reduction of Sir2 in the *⊿sum1*, *⊿rim101* and *⊿sok2* strains carrying Hsp82 over-expression plasmid, however, it remained unaltered in the *⊿cup9* strain with Hsp82 over-expression (Fig. 22D). These results indicate that Cup9 may be the mediator through which Hsp82 regulates *SIR2* transcription.

We wanted to monitor which of the deletion strains abrogates de-repression of sub-telomeric gene due to Hsp82 over-expression. We performed two independent functional assays of Sir2 taking two different sub-telomeric genes. Firstly, we observed the Sir2 function by color assay scoring expression of sub-telomeric gene *ADE2*. The *⊿cup9, ⊿sum1, ⊿rim101* and *⊿sok2* strains were constructed in isogenic background of SLY20 where telomere region of chromosome VIIL was marked with *ADE2*. Wild-type and *⊿cup9* deleted strains both developed pink color colonies due to the silencing of *ADE2* gene whereas *⊿sir2* exhibited white color phenotype correlating with de-repression of *ADE2*. However, in Hsp82 over-expression condition *⊿cup9* strain retained the pink color phenotype as opposed to the white color phenotype observed during Hsp82 over-expression in the wild type as well as in the *⊿sum1, ⊿rim101* and *⊿sok2* strains (Fig. 23A). In order to find out whether the maintenance of hetero-chromatinization is locus specific or not, we compared the expression of another subtelomeric gene *YFR057w,* located near chromosome VIR telomere.

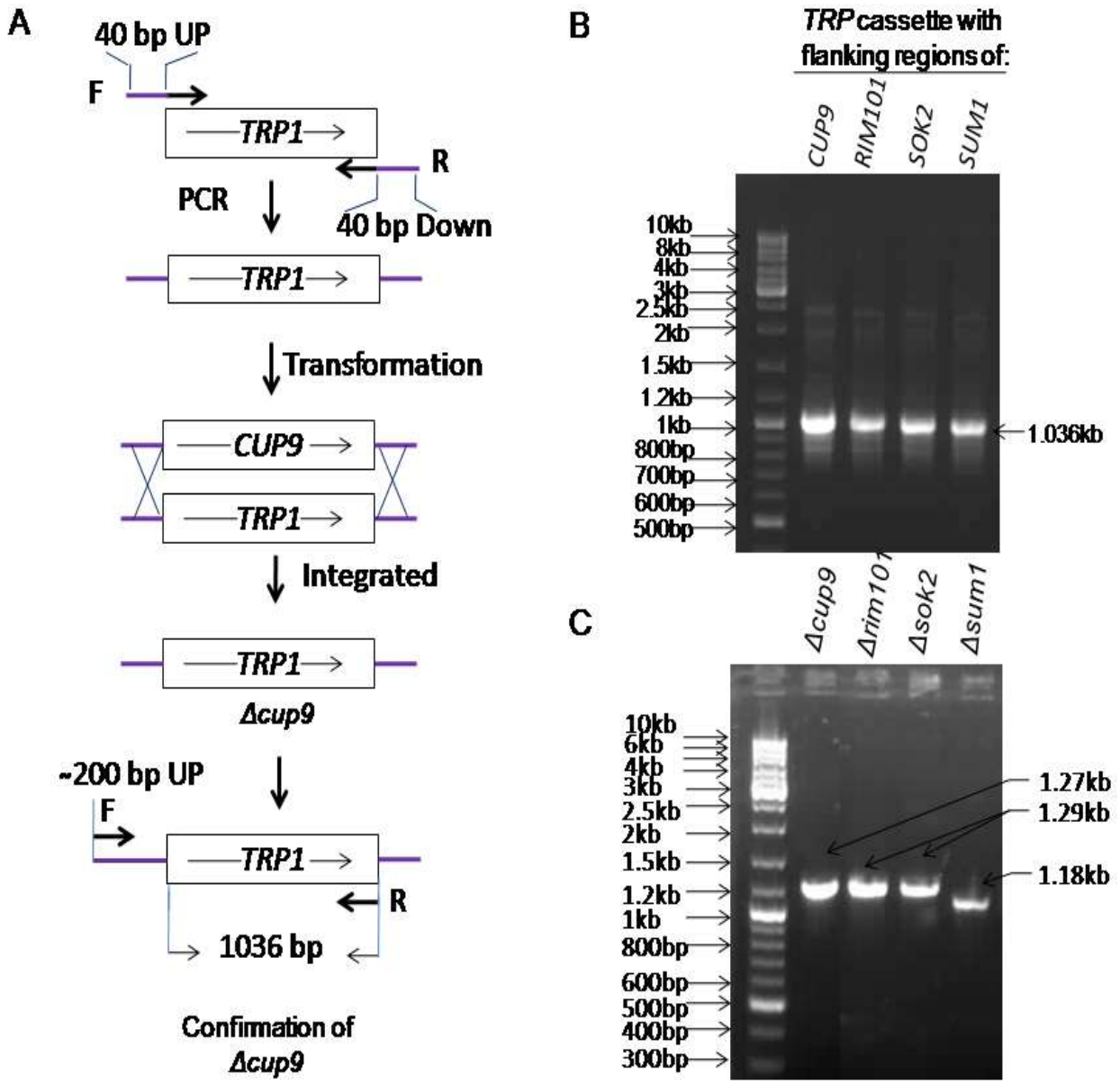

Figure 21: Generation of *Δcup9*, *Δrim101*, *Δsok2* and *Δsum1* strains. (A) Experimental strategy to knockout *CUP9, SUM1, RIM101* and *SOK2* (discussed in materials and methods). (B) Amplification of *TRP1* cassette. (C) Agarose gel showing confirmation of knockout.

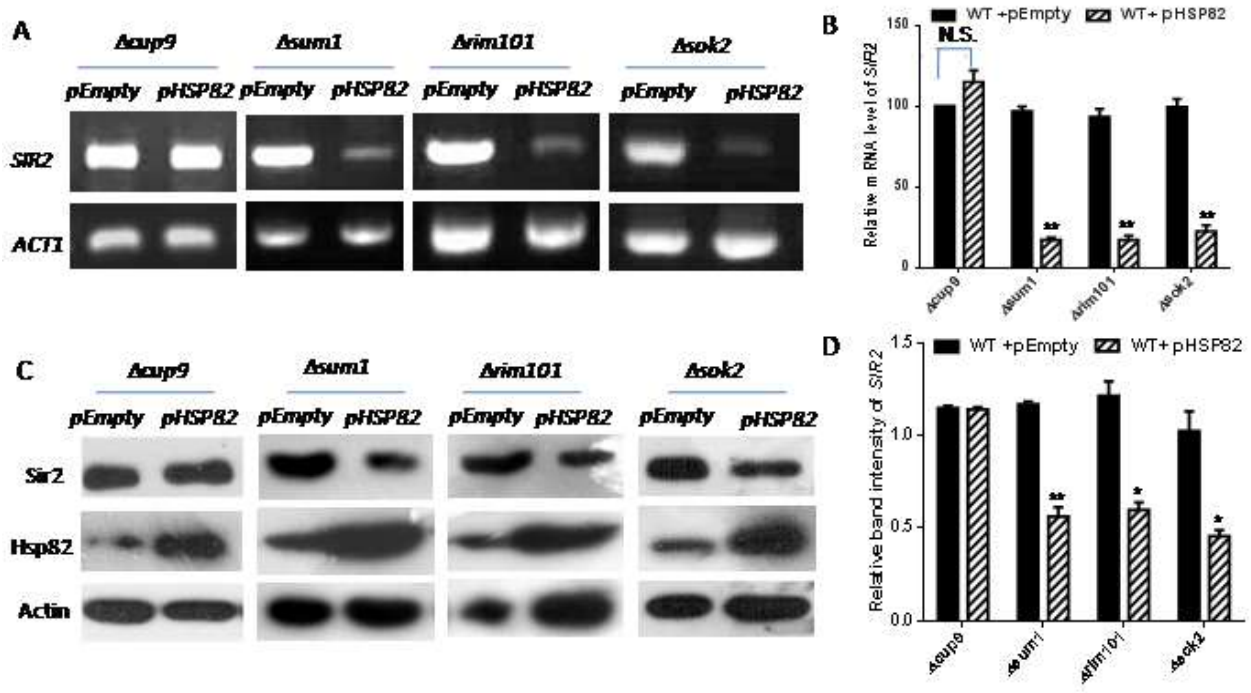

Figure 22: Reversal of heat shock/Hsp82 over-expression mediated down regulation of Sir2 in *cup9* deletion strain (A) Hsp82 over-expression plasmid was transformed in each of the four deletion strains and semi-quantitative RTPCR displays no alteration in *SIR2* transcript in *Δcup9* deletion strain bearing *HSP82* over-expression; *ACT1* acts as the normalization control. (B) Real time RTPCR data shows the relative quantity of *SIR2* mRNA between the above mentioned strains (presented in X-axis). Each bar represents the mean mRNA level (±SD) from three independent experiments. *P* values are calculated using the two-tailed Student's t-test; $^{**}p<0.01$; N.S. means not significant. (C) Western blot analysis was done with the protein isolated from the above mentioned strains using anti-Actin, anti-Hsp82 and anti-Sir2 antibodies. (D) Densitometry measurement of Sir2 (after normalization with Actin) from three independent experiments was plotted for indicated strains as given in X-axis; Error bar indicates ±SD, $^{**}p<0.01$; $^{*}p<0.05$.

Our data showed that although Hsp82 over-expression resulted in de-repression of *YFR057w* in the wild-type cell, *⊿cup9* strain did not display any defect in *YFR057w* silencing even in the presence of Hsp82 over-expression (Fig. 23B). To score the silencing activity of Sir2 quantitatively, we performed real time RTPCR analysis which showed no significant alteration in *YFR057w* mRNA level in *⊿cup9* strain with and without Hsp82 over-expression (Fig. 23C). Our result suggests that out of the four transcriptional repressors, deletion of only *CUP9* restores wild-type like Sir2 function in Hsp82 over-expressed condition. In other words, we observed increased Sir2 expression specifically in the *cup9* knockout strain during Hsp82 over-expressed condition which correlated well with the maintenance of Sir2 silencing function.

5.2.4. HEAT SHOCK AND Hsp82 OVER-EXPRESSION LEADS TO INCREASE IN Cup9 EXPRESSION AND ITS BINDING TO $SIR2_{UAS}$:

From the previous experiment, it was evident that Hsp82-mediated transcriptional down-regulation of Sir2 is Cup9 dependent. We were interested to estimate the steady state level of Cup9 under heat stressed condition. We grew the wild type cells at three different temperatures, 30˚C, 37˚C and 39˚C respectively and monitored that there was up regulation of *CUP9* transcript at 39˚C (Fig. 24A). Real time RTPCR data showed that there was no alteration in *CUP9* transcript with increase in temperature from 30˚C to 37˚C. However, at 39˚C, *CUP9* transcript was increased by 2.5-fold (Fig. 24B). We also estimated the level of *CUP9* transcript in the Hsp82 over-expressed condition and observed that it had significantly increased compared to the wild-type (Fig. 24C). Western Blotting showed while, there was very low levels of Cup9-Myc in normal cells, an increased expression of Cup9-Myc was associated with heat stress (Fig. 24E) as well as found under over-expression of Hsp82 conditions (Fig. 24F).

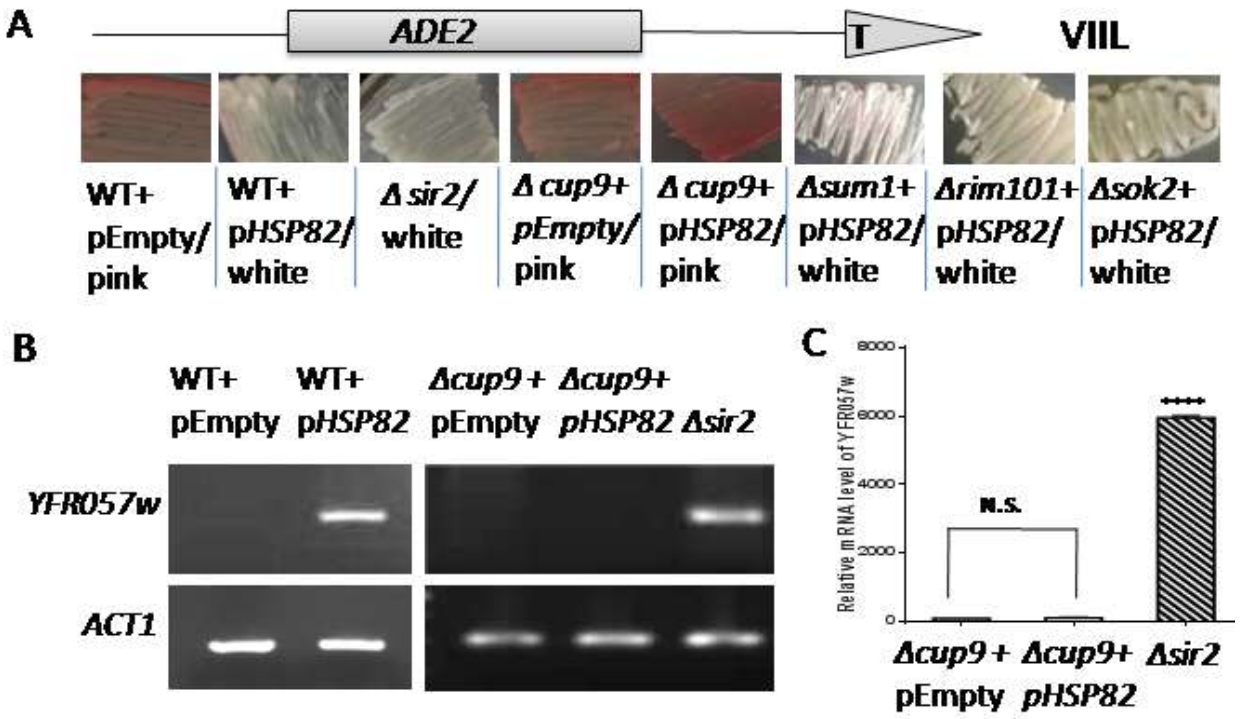

Figure 23: Reversal of heat shock/Hsp82 over-expression mediated loss of Sir2 function in *cup9* deletion strain (A) Wild-type, wild-type cells carrying Hsp82 over-expression plasmid, *Δsir2*, *Δcup9* and *Δcup9*, *Δsum1*, *Δrim101*, *Δsok2* each carrying Hsp82 over-expression plasmid were used to perform telomere silencing assay each carrying *ADE2* reporter gene located at chromosome VIIL. (B) Telomere silencing assays was done with *YFR057w* localized adjacent to telomere VI-R. Semi-quantitative RTPCR was done to study the expression of *YFR057w* in wild-type cell with and without Hsp82 over-expression plasmid and *Δcup9* strain with the presence and the absence of Hsp82 over-expression plasmid. *Δsir2* strain was used as a control. *ACT1* transcript was used as a loading control. (C) Real time RTPCR was done to quantify relative abundance of *YFR057w* transcript in *Δcup9* strain with or without Hsp82 over-expression plasmid and compare it with the same in *Δsir2*.

Next, we performed chromatin immunoprecipitation (ChIP) to analyze whether Cup9 is recruited to the upstream regulatory region of *SIR2* in the presence of Hsp82 over-expression. For ChIP, we used Hsp82 over-expressing strain where Cup9 (Cup9-Myc) was abundantly present and used anti-Myc antibody to immune precipitate chromatin bound Cup9.In Hsp82 over-expression condition, there was a bright signal of Cup9 specifically at the upstream regulatory element of *SIR2* which was absent on wild type cell (Fig. 25).

5.2.5. Cup9 OVERPRODUCTION REDUCES ACTIVITY AS WELL AS ENDOGENOUS LEVEL OF Sir2:

In order to investigate whether the endogenous level and activity of Sir2 are directly regulated by Cup9, we analyzed them in the Cup9 over-expression condition. For that, Cup9 over-expression plasmid *pESC-CUP9* and empty vector *pESC* were transformed into wild-type strain SLY20 strain to construct SLY90 and SLY91 strains respectively. In this vector Myc tagged Cup9 is over-expressed under the control of a galactose inducible promoter. We quantified *SIR2* transcript in those backgrounds by semi-quantitative RTPCR. Results demonstrated that Cup9 over-expression entirely diminished *SIR2* transcript in SLY90 compared to the SLY91 strain (Fig. 26A). Real time RTPCR analysis quantitatively showed that there was nearly ten-fold reduction of *SIR2* transcript in Cup9 over-expression background (Fig. 26B). Our results were further confirmed by Western Blot analysis which displayed that Cup9 over-expression caused modest reduction in Sir2 level specifically without any alteration to Hsp82 or Act1 (Fig. 26C). We subsequently explored the silencing function of Sir2 under Cup9 over-expression condition using three independent assays. We observed that SLY90 cells having reduced level of Sir2 showed white color phenotype indicating de-repression of subtelomeric *ADE2* gene whereas SLY91 strain containing the empty vector developed as pink colored colonies due to the silencing of *ADE2* (Fig. 26D).

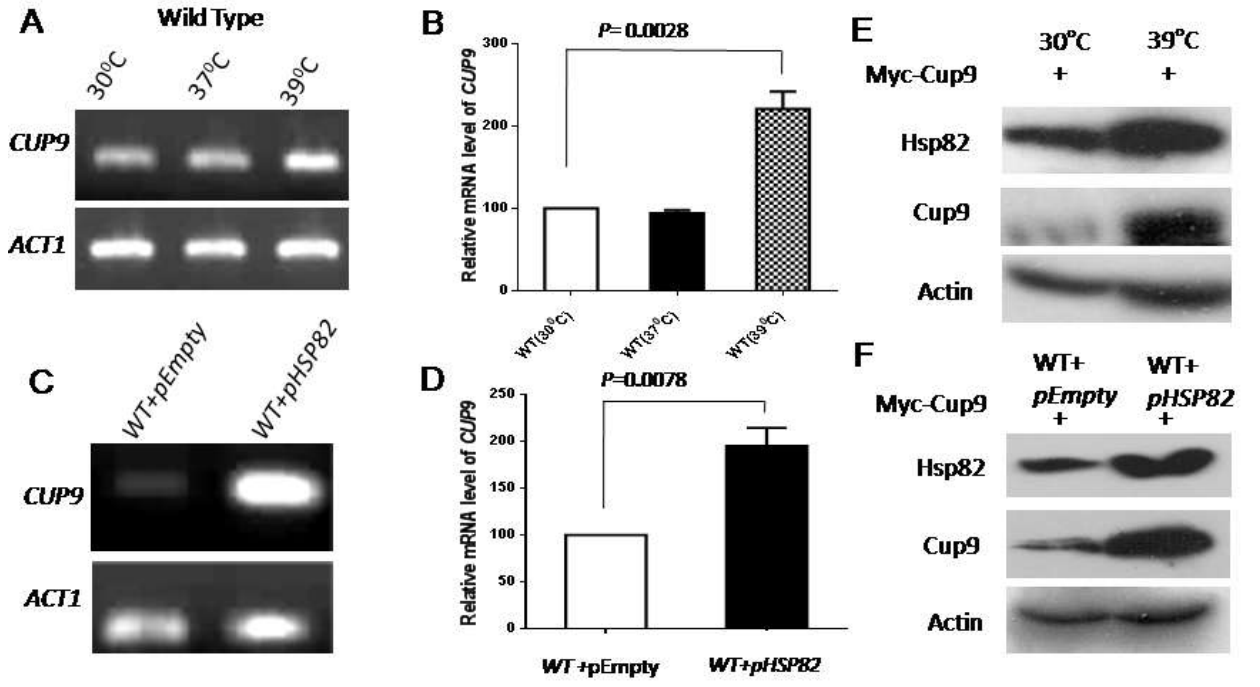

Figure 24: Heat shock and Hsp82 over-expression increases Cup9 expression. (A) Wild-type cells were divided into three parts: one part grown at 30°C, another exposed to 37°C for 2 hours and the other exposed to 39°C for 40 minutes. Semi- quantitative RTPCR result shows the *CUP9* transcript profile at three different temperatures. *ACT1* acts as the normalization control. (B) Real time RTPCR shows the relative mRNA of *CUP9* at 39˚C compared to 30˚C. Each bar represents mean mRNA level (±SD) from three independent experiments. *P* values were calculated using the two-tailed student's t-test. (C) Wild-type cells and Hsp82 over-expressing cells were used to estimate *CUP9* transcript by semi-quantitative RTPCR. (D) Real time RTPCR reveals the quantitative abundance of *CUP9* mRNA level in cell bearing *HSP82* over-expression plasmid compared to wild-type. (E and F) Western Blot analysis was done using anti-Myc (Cup9), anti-Hsp82 and anti-actin antibodies at heat treated and untreated conditions as well as in presence and absence of *HSP82* over-expression plasmid.

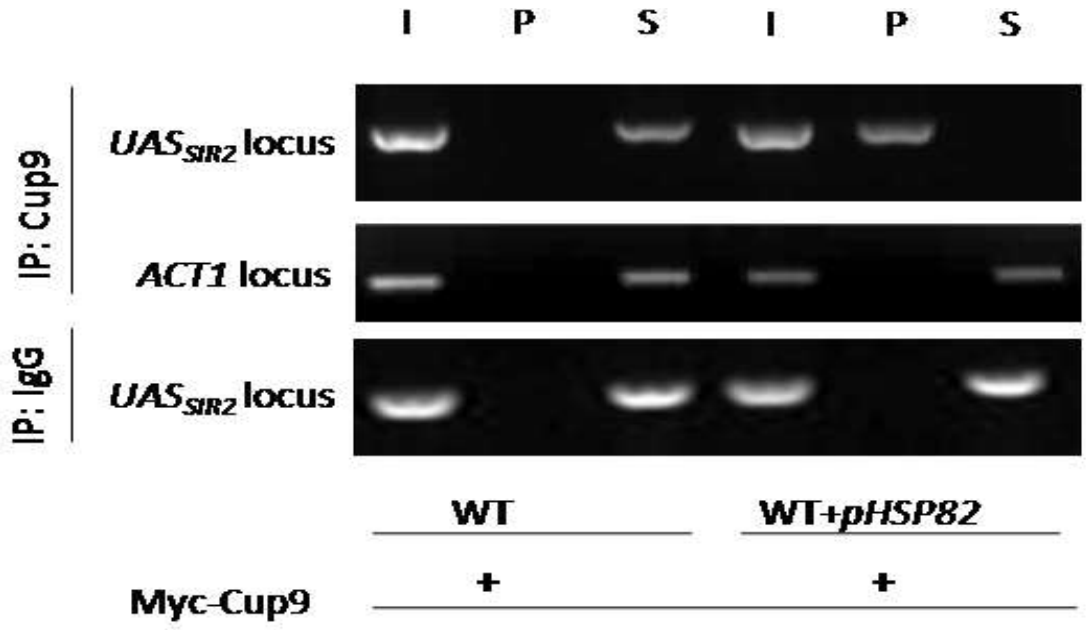

Figure 25: Hsp82 over-expression leads to association of Cup9 with $SIR2_{UAS.}$ CHIP assays were performed using *CUP9* MYC tagged cells in the absence and presence of Hsp82 over-expression plasmid. Anti-Myc antibodies were used with control IgG (immunoglobulin G). Input (I), immunoprecipitated DNA (P) and supernatant (S) were amplified by semi-quantitative RTPCR with $SIR2_{UAS}$ specific primers. The experiment was repeated two times and one of the representative data from that is presented here. I, P and S DNA were also amplified using *ACT1* specific primers which acts as a negative control.

Also the quantification of *YFR057w* transcript by real time RTPCR showed nearly an eight-fold up-regulation in transcripts in Cup9 over-expression strain compared to the WT (Fig. 26E). We also compared the mRNA level of *HMLa* by real time RTPCR in Cup9 over-expression background but observed no significant change. These results conclude that Cup9 over-expression leads to moderate reduction in the Sir2 level as a result of which the silencing activity of Sir2 across the 32 sub-telomeric regions is diminished although silencing at the hidden mating locus is maintained.

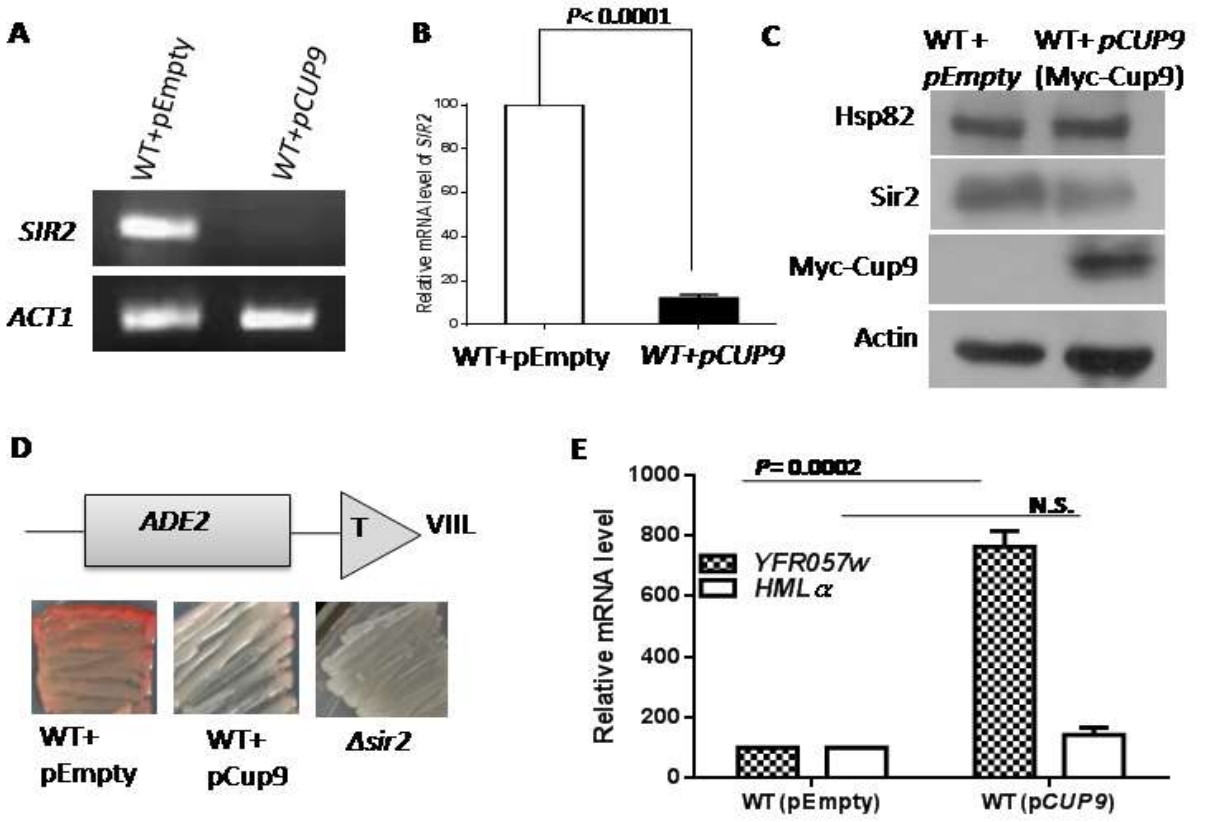

Figure 26: Cup9 over-expression reduces endogenous level as well as function of Sir2: (A) Cup9 was produced under galactose induction (to a final concentration of 2%). The overnight culture grown in galactose medium was used for secondary inoculums in galactose containing medium and was grown for additional 5 hours (until the mid log phase) and isolated RNA. The effect of Cup9 over-production on *SIR2* transcription was measured by semi-quantitative RTPCR. (B) Real time RTPCR shows *SIR2* mRNA levels in the Cup9 over-expressed cell relative to those of the wild-type cells is shown as the average of three experiments. Error bars indicate SD; *P* values were calculated using the two-tailed student's t-test. The *ACT1* mRNA was used as the normalization control. (C) Protein from overnight culture grown in galactose medium was used for Western Blot analysis. Immunoblot shows the reduction of endogenous Sir2 protein in Cup9 over-expression

condition. Cup9 over-expression vector harbors C-terminal Myc tagged Cup9. (D) *ADE2* reporter gene at telomere VIIL was used for telomere silencing assay. Wild-type, Cup9 over-expressed cell and *Δsir2* were used for telomere position effect assay. Pink colored colonies indicate a silenced *ADE2* gene and white colonies indicate de-repression of *ADE2*. (E) Cup9 over-expression induces the de-repression of another subtelomeric gene *YFR057w*. However, silencing at the hidden mating type loci is maintained as demonstrated by comparable *HMLα* transcript. *ACT1* was used as the normalization control. Error bars indicate SD; n=3; NS not significant.

5.3. DISCUSSION:

Molecular players involved in transcriptional regulation of *SIR2* gene have remained unknown. Here, we report for the first time the identification of cis regulatory element and a transcriptional repressor that regulates *SIR2* gene transcription. We provide several lines of evidence that unequivocally recognize that Cup9 is the transcriptional repressor of *SIR2* gene expression. Firstly, in Δ*cup9* background, neither heat shock nor over-expression of Hsp82 had affected *SIR2* transcription. Secondly, Cup9 over-expression drastically reduced *SIR2* transcription resulting in the derepression of subtelomeric genes and this effect was independent of heat stress. Thirdly, according to our bioinformatics analysis, Cup9 binding sequence was predicted within (-419 to -399) upstream regions of *SIR2*. Finally, chromatin immunoprecipitation assay further demonstrated that under Hsp82 over-expression condition, endogenously expressed Cup9 was recruited at the 5'end on yeast *SIR2* promoter. Our current data show that under heat shock induces Cup9 expression in cell. Under such conditions, Cup9 binds to the $SIR2_{UAS}$ resulting in transcriptional down-regulation of *SIR2* thereby causing derepression of subtelomeric genes. Reporter gene analysis using various deletion constructs of $SIR2_{UAS}$ also proved this. We showed that the effect of Hsp82 on transcriptional down-regulation of reporter gene was abolished when the Cup9 binding region was deleted from $SIR2_{UAS}$. Currently, it is unknown how Hsp82 regulates Cup9 expression. Earlier, it was observed that copper stress causes up-regulation of *CUP9* transcription when grown on lactose medium [207]. Our study reveals that expression of *CUP9* is also up regulated during heat shock treatment. It was previously reported that high temperature (37˚C) strengthens mating and telomere silencing [220]. Our finding corroborates well with this report since *CUP9* transcription remains unaltered between 30˚C-37˚C. However, at 39˚C, there is nearly a 2.5-fold up regulation of *CUP9* transcript. It has been observed that expression of *HSP82* increases many-fold not only during heat

shock but also in response to several other environmental stresses [229]. Thus, it will be interesting to know whether up-regulation of *CUP9* is a specific or general stress response phenomenon. In yeast, transcriptional regulation of *SIR2* in response to environmental stimuli has never been identified before. However, in human cell line, it has been demonstrated that SIRT1 protein associates with HIC1 (hyper-methylated in cancer 1) and the complex binds to the *SIRT1* promoter to negatively regulate its own transcription [133]. Hyper-methylation of *HIC1* promoter leads to epigenetic silencing of *HIC1* thereby causing aging. Lower HIC1 expression results in *SIRT1* up-regulation which results in excessive de-acetylation and deactivation of p53 function and thus increases the cancer risk in mammals. Any such feedback inhibition of *SIR2* expression by Sir2 itself in yeast is not yet known. To the best of our knowledge, Cup9 is the only transcriptional repressor identified so far that regulates *SIR2* gene expression in yeast.

It had been shown earlier that in normal conditions, Cup9 is a short lived protein having approximately 5 minutes as its half life. The presence of imported di/tri peptides causes the activation of E3 ubiquitin ligase Ubr1 and accelerates the Ubr1 dependent ubiquitylation of Cup9 [230]. Thus, under normal conditions, due to the unstable nature of Cup9, Sir2 can be transcribed optimally. However, under heat shock conditions (39°C), elevated level of Cup9 leads to down regulation of *SIR2* transcription.

CHAPTER 6

DISCUSSION

6. DISCUSSION:

Our work establishes that Hsp82 regulates Sir2 following two independent pathways. Under normal cellular condition Sir2 acts as a client of Hsp82. The inhibition of *HSP82*'s function results in degradation and loss of Sir2 activity. We observe that in *iGD170hsp82* temperature sensitive mutant, where Hsp82 is non functional at restrictive temperature (37^0C), there is a significant reduction of Sir2 level which is associated with complete loss of its function both at the mating type loci as well as at the telomere. It has been earlier shown that Hsp82 controls pheromone signaling pathway in yeast. This led us to decipher whether the loss of mating type silencing at restrictive temperature is due to the derepression of *HMLa* locus in *iGD170hsp82 MATa* mutant or due to the inactivation of signaling kinases which are the clients of Hsp82. Our experimental result shows considerable amount of *HMLa2* transcript at 37^0C, which can be correlated to the inactivation of Sir2 function. The Western blot shows the presence of considerable amount of Sir2 at the restrictive temperature (albeit at 50% reduced level); however functional studies at hidden mating locus showed that these Sir2 proteins are not functionally active. According to an earlier report, silencing in yeast is dependent on temperature [220]. Increased temperature is associated with increased TPE and mating type silencing in yeast. As an explanation of this effect it has been shown that at elevated temperature Sir2-Net1 complex is destabilized whereas Sir2-Sir4 complex is having no effect on temperature [202]. Thus at higher temperature there is more Sir2 available to bind with Sir4 leading to a stronger telomere and mating type silencing in yeast. In our system at higher temperature the derepression of transcriptional silencing both at telomere and *MAT* locus is directly linked with the lack of Hsp82/Hsc82 activity.

We have also observed that Sir2 undergoes proteasomal degradation at 37^0C in the temperature sensitive mutant *iGD170hsp82* strain and treatment of MG132 reverses its degradation. Earlier yeast

two hybrid experiment with *E33Ahsp82* displayed its physical association with Sir2 [96]. Thus our work for the first time establishes Sir2 as a direct client of Hsp82.

Over-expression of Hsp82 in the cell results in reduction of Sir2 protein level in a dose dependent manner. This has important consequence as occasionally the amount of Hsp82 increases within the cell when they are exposed to heat shock or any kind of stressed condition. Our study demonstrates, in a condition where Hsp82 is over-expressed, there occurs a gradual decrement of Sir2 which makes it unable to perform transcriptional silencing at telomere, although silencing at *HML*/*HMR* locus remains unperturbed. This data suggests that under heat shock condition, though Sir2 level is diminished, the remaining Sir2 level is adequate enough to perform other cellular activities. We speculate that although Sir2 is the key silencing protein to maintain both mating type silencing and telomere silencing, the threshold cellular concentration of Sir2 for these two different functions may be different. The reduced cellular level of Sir2, which is the consequence of Hsp82 over-expression, may be sufficient to maintain the silenced state at *MAT* locus, however, it may be limiting for silencing the stretch of subtelomeric regions spread over 32 chromosomal ends in yeast. The question remains that why at low concentration, Sir2 dissociates from the subtelomeric region, but not from the mating type loci. Telomere silencing is a reversible phenomenon which states that the silencing complex is in a dynamic equilibrium between DNA bound and unbound states; and mating type silencing is not reversible under normal condition. This information provokes us to speculate that the binding affinity of silencing complex towards the silencer sequences at the cryptic mating type loci might be much stronger than that for the subtelomeric regions. Thus, binding of Sir2 protein at the silent mating loci may be achieved at much less cellular concentration.

It is important to understand how Hsp82 deficient condition as well as over-expression both leads to derepression of TPE. It appears that in Hsp82 over-expression, the low abundance of active Sir2

(as low as $1/4^{th}$ of the usual amount) is good enough to ensure mating type silencing (as observed in SLY32 cells). The absence of *MATa2* transcript in *HSP82* over-expressing cells also confirms the same. We reason that these *HSP82* over-expressing cells are capable of mating, because they still possess wild type *HSC82* and *HSP82* genes due to which Sir2 maturation and functional activation remain unaltered. Taking all these together, we propose that loss of TPE under *HSP82* over-expression condition and *HSP82* knockout condition are mechanistically distinct.

Our result demonstrates that Hsp82 over-expression leads to euchromatinisation of chromosome ends. The transitions between the euchromatinisation and heterochromatinization are very well studied in TPE model in budding yeast *S. cerevisiae* [96]. However, the reversible nature of such heterochromatin-euchromatin transition and *vice versa* remains poorly understood. Although, the epigenetic marks (especially H3 and H4 modifications) associated with such changes are well established [201], the regulation at the DNA-protein interface remains elusive. It is known to a greater extent the nucleation and then spreading of silencing proteins along the heterochromatin [199]. However, the molecular events occurring during the exit of the silencing complex from the heterochromatin giving rise to euchromatin formation is mostly unknown. We speculate from the findings from this work that may be under *in vivo* condition Hsp82 is required to maintain a dynamic equilibrium of total amount of silencing proteins. This idea has been proven under stressed conditions such as heat shock that increases Hsp82 levels in the cells. We have exposed the cells to heat shock and under that conditions observed that cellular abundance of Sir2 is also reduced by more than 50% which is associated with complete lose of silencing at telomere. Thus the adaptation in Sir2 abundance in response to the Hsp82 homeostasis could be associated with the reversible nature of transcriptional silencing at telomere. We speculate that when the chromatin does not need to be maintained at the heterochromatin state Hsp82 modulates interaction of silencing complex

(Sir2-Sir3-Sir4) with DNA in such a way that it facilitates the removal (or degradation) of the major silencing proteins. Similar effect is also observed at telomere end, where Hsp82 acts as a releasing factor for Cdc13 to control the telomere protein dynamics, and helps in maintaining the extendable and unextentable states of telomere [169]. Our genetic analysis shows that maturation of Sir2 by Hsp82 is independent of its cochaperone Sba1. This observation does not fit with classical steroid hormone receptor chaperone model, where Sba1 is found to be associated with the last stages during client release. Thus, apart from traditional chaperone role, more interesting roles of Hsp82 are emerging, where it acts as a regulator of various DNA protein interactions occurring inside the cell. Our report also provides evidence along the same line.

We have explored the role of environmental cues like heat stress as well as Hsp82 over-expression on the expression and activity of the type III NAD^+ dependent histone deacetylase Sir2 which is one of the major epigenetic modifiers in yeast. Our study identified a cryptic pathway of Sir2 regulation under heat stressed condition. We report for the first time that heat shock as well as Hsp82 over-expression cause transcriptional down-regulation of *SIR2*. We have identified the cis regulatory element and a transcriptional repressor that regulates *SIR2* gene transcription. We provide several lines of evidence that unequivocally recognize that Cup9 is the transcriptional repressor of *SIR2* gene expression. Firstly, in Δ*cup9* background, neither heat shock nor over-expression of Hsp82 had affected *SIR2* transcription. Secondly, Cup9 over-expression drastically reduced *SIR2* transcription resulting in the derepression of subtelomeric genes and this effect was independent of heat stress. Thirdly, according to our bioinformatics analysis, Cup9 binding sequence was predicted within (-419 to -399) upstream regions of *SIR2*. Finally, chromatin immunoprecipitation assay further demonstrated that under Hsp82 over-expression condition, endogenously expressed Cup9 was recruited at the 5'end on yeast *SIR2* promoter. Our current data show that heat shock induces Cup9

expression in cell. Under such conditions, Cup9 binds to the $SIR2_{UAS}$ resulting in transcriptional down-regulation of *SIR2* thereby causing derepression of subtelomeric genes. Reporter gene analysis using various deletion constructs of $SIR2_{UAS}$ also proved this. We showed that the effect of Hsp82 on transcriptional down-regulation of reporter gene was abolished when the Cup9 binding region was deleted from $SIR2_{UAS}$. Currently, it is unknown how Hsp82 regulates Cup9 expression. Earlier, it was observed that copper stress causes up-regulation of *CUP9* transcription when grown on lactose medium [207]. Our study reveals that expression of *CUP9* is also up regulated during heat shock treatment. It was previously reported that high temperature (37˚C) strengthens mating and telomere silencing [220]. Our finding corroborates well with this report since *CUP9* transcription remains unaltered between 30˚C-37˚C. However, at 39˚C, there is nearly a 2.5-fold up regulation of *CUP9* transcript. It has been observed that expression of *HSP82* increases many-fold not only during heat shock but also in response to several other environmental stresses [229]. Thus, it will be interesting to know whether up-regulation of *CUP9* is a specific or general stress response phenomenon.

In yeast, transcriptional regulation of *SIR2* in response to environmental stimuli has never been identified before. However, in human cell line, it has been demonstrated that SIRT1 protein associates with HIC1 (hypermethylated in cancer 1) and the complex binds to the *SIRT1* promoter to negatively regulate its own transcription [133]. Hypermethylation of *HIC1* promoter leads to epigenetic silencing of *HIC1* thereby causing aging. Lower HIC1 expression results in *SIRT1* up-regulation which results in excessive deacetylation and deactivation of p53 function and thus increases the cancer risk in mammals. Any such feedback inhibition of *SIR2* expression by Sir2 itself in yeast is not yet known. To the best of our knowledge, Cup9 is the only transcriptional repressor identified so far that regulates *SIR2* gene expression in yeast.

Dietary restriction is proven to be an environmental factor that causes increase in longevity from yeast to mammals. Sir2 mediated NAD^+ induced histone deacetylase activity is required for increased longevity during starvation and this effect is not observed in the *sir2* mutant strain [136]. It had been shown earlier that in normal conditions, Cup9 is a short lived protein having approximately 5 minutes as its half life. The presence of imported di/tri peptides causes the activation of E3 ubiquitin ligase Ubr1 and accelerates the Ubr1 dependent ubiquitylation of Cup9 [230]. Thus, under normal conditions, due to the unstable nature of Cup9, Sir2 can be transcribed optimally. However, under heat shock conditions (39˚C), elevated level of Cup9 leads to down regulation of *SIR2* transcription thereby mimicking *sir2* knockdown condition in yeast which is inherited for many generations. Thus, it is tempting to predict that under heat stress conditions, Sir2 may not influence longevity.

These findings highlight the mechanism by which environmental factors amend the epigenetic configuration of chromatin that is inherited in successive generations. Our work showed that transient heat shock rendered the cell as *⊿sir2* for more than ninety generations. We found that upon transient heat shock, the de-repression of *HMLa* transcript is maintained for 4 days post HS. This has tremendous implications on yeast physiology as it shows a likely defect in yeast mating behavior. In a broader context, this observation is very important as Sir2 is an epigenetic modifier which establishes silencing at sub-telomeric regions in many eukaryotes. In protozoan parasites such as *Plasmodium* and *Trypanosomes,* Sir2 mediated telomere silencing plays a major role in mutually exclusive expression of virulent multi-gene family [217, 218] and controls antigenic variation. This causes evasion of host immune system [219]. It will be interesting to explore the effect of exposure to febrile temperature (around 39˚C) as a natural consequence of *Plasmodium* infection on expression Sir2 and sub-telomeric virulent genes.

In recent years Hsp90 has achieved tremendous prospective as anticancer target. The total cellular pool of Hsp90 is found to increase from 1% to 10% when cells become malignant. It plays a role in function and stability of many proteins in signal transduction, some of which are oncogenic; ErbB2 and Raf-1. Our present study shows that Hsp90 participates in epigenetic control of the cell. Our finding clearly demonstrates that the endogenous level and activity of the histone deacetylase is controlled by over-expression of Hsp90 in *S. cerevisiae*. It is thus very important to understand whether the same phenomenon also exist in mammalian cell under carcinogenic condition while Hsp90 level is found to be elevated. Understanding the mechanism by which Hsp90 is involved in chromatin remodeling will lead to better insight of epigenetic regulation in cancerous cell.

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APPENDIX

AI.1. Cloning of *HSP82* in pRS313 vector [pRS313/*HSP82*]:

Taking yeast genomic DNA as template, *HSP82* gene was amplified using forward primer, OSB21 having *BamHI* overhang and reverse primer, OSB22 having *SalI* overhang. The PCR product of size 2.1 kb was cloned into pCR2.1TOPO (3.9 kb) vector. The pCR2.1TOPO/*HSP82* clone was confirmed by *BamHI* digestion, which released the insert of 2.1 kb and the empty vector backbone of 3.9 Kb (Figure A1.B.). Then the *HSP82* gene was released by *BamHI* and *SalI* and sub cloned into the yeast expression vector, pRS313 (CEN, 5.9 kb) into *BamHI* and *SalI* sites under GPD promoter. The pRS313/*HSP82* clone was confirmed by *BamHI* and *SalI* digestion which released 2.1 kb (*HSP82*) and 5.9 kb (pRS313) (Figure A1.C.).

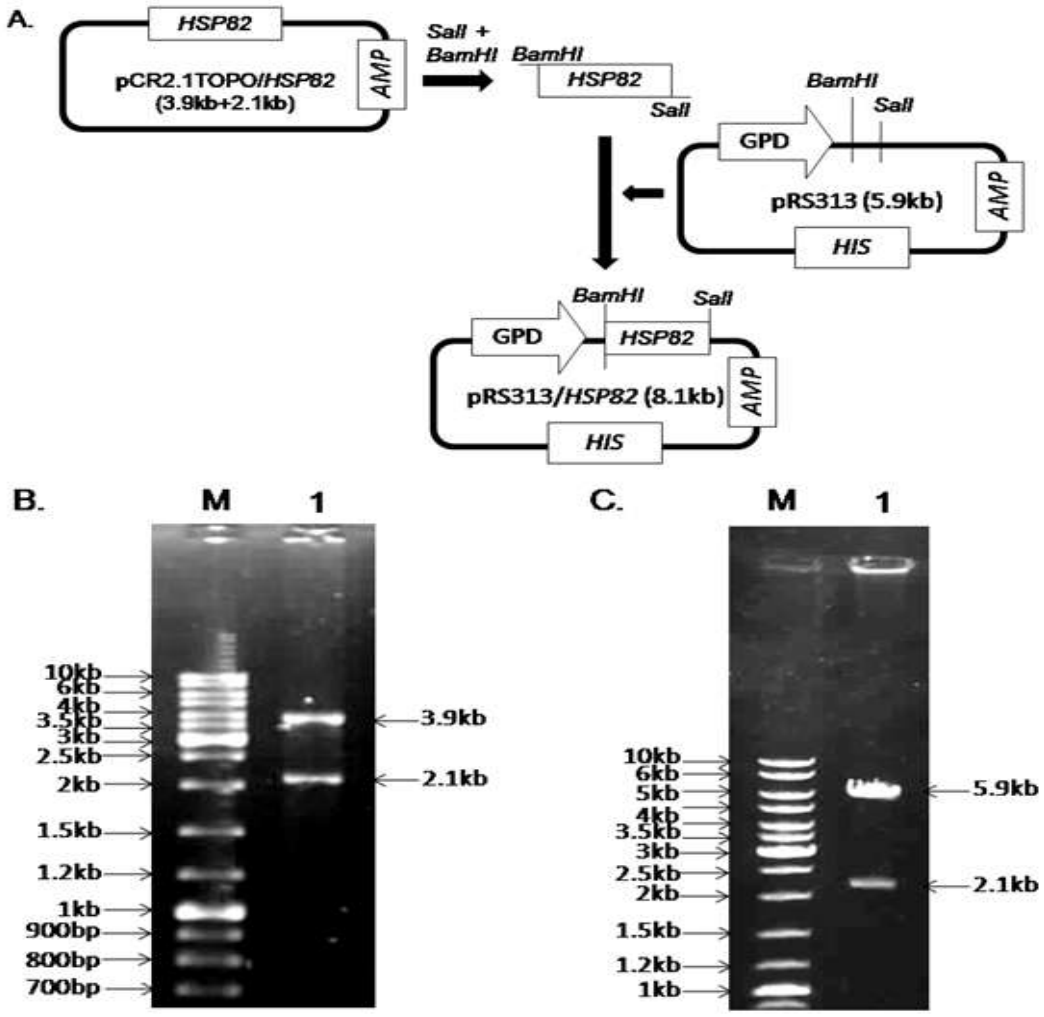

Figure A1: Cloning of *HSP82* in pRS313 vector. (A) Strategy of cloning *HSP82* in pRS313 vector. *HSP82* was cloned first in pCR2.1TOPO vector and then sub cloned into pRS313 vector in *BamHI* and *SalI* sites. (B) Screening of pCR2.1TOPO/*HSP82*: Lane 1 shows *BamHI* digestion, which released the insert of 2.1 kb and the empty vector backbone of 3.9 Kb. (C) Screening of pRS313/*HSP82*: Lane 1 shows digestion by *BamHI* and *SalI* digestion which released 2.1 kb (*HSP82*) and 5.9 kb (pRS313).

AI.2. Cloning of *HSP82* in pTA vector [pTA/*HSP82*]:

The strategy of the cloning is presented schematically (Figure A2A). The *HSP82* gene was released from pCR2.1TOPO/HSP82 by *BamHI* and *SalI* as described above and sub cloned into the yeast expression vector, pTA (2μ, 6.28 kb) into *BamHI* and *SalI* sites under GPD promoter. The pTA/*HSP82* clone was confirmed by *BamHI* and *SalI* digestion which released 2.1 kb (*HSP82*) and 6.28 kb (pTA) (Figure A2.B.).

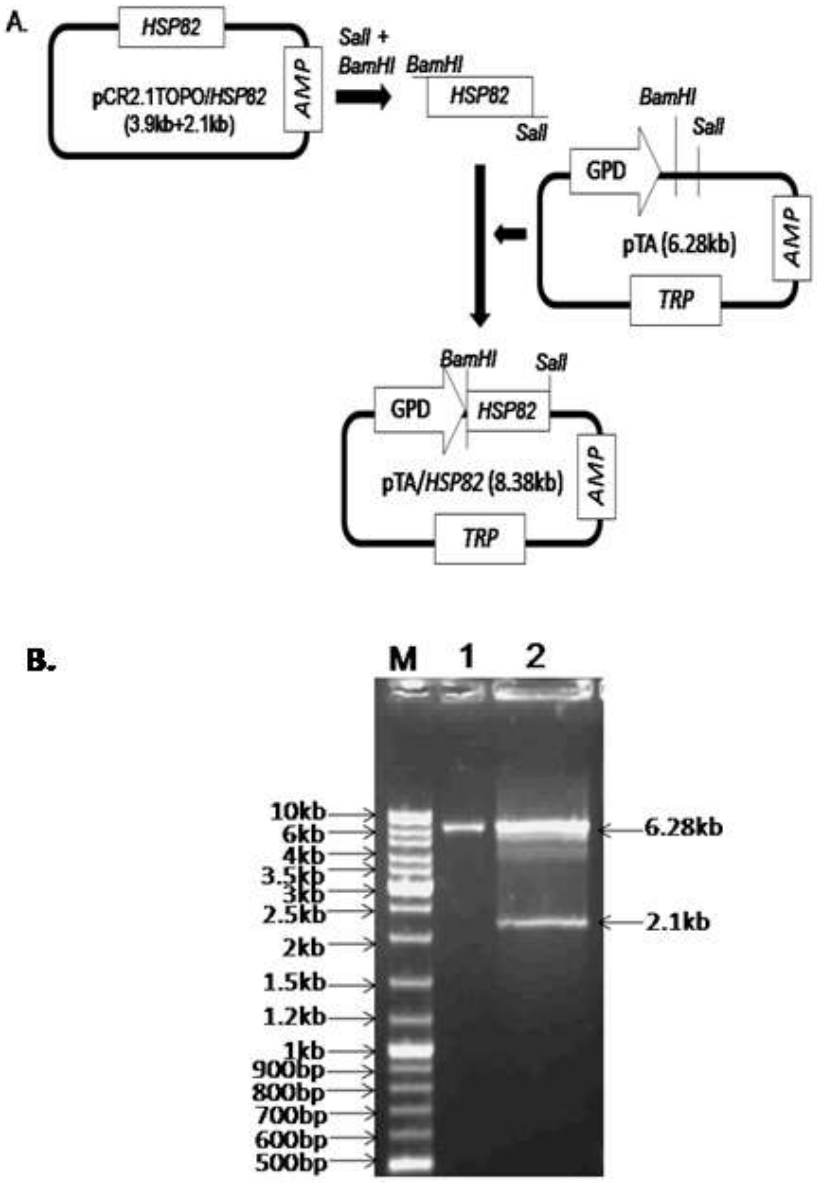

Figure A2: Cloning of *HSP82* in pTA vector. (A) Strategy of cloning *HSP82* in pTA vector. *HSP82* was cloned first in pCR2.1TOPO vector and then sub cloned into pRS313 vector in *BamHI* and *SalI* sites. (B) Screening of pRS313/*HSP82*: Lane 1 shows undigested plasmid and lane 2 shows digestion by *BamHI* and *SalI* which released 2.1 kb (*HSP82*) and 6.28 kb (pTA).

AI.3. Cloning of 429 base pair of upstream activator region of *SIR2* in pCZ vector [429_{UAS}]:

The strategy of the cloning is schematically presented in the figure A3.A. Genomic DNA isolated from wild type yeast strain was taken as template to amplify 429 base pair (bp) of upstream activator region (UAS) of *SIR2* using forward primer OSB125 and reverse primer OSB87 both having *BamHI* overhang. 429 bp of $SIR2_{UAS}$ was then cloned into pTZ vector. The pTZ/429 $SIR2_{UAS}$ clone was confirmed by *BamHI* digestion which excised 429 bp of $SIR2_{UAS}$ and the 2.9 Kb pTZ vector (Figure A3.B.). This 429 bp of $SIR2_{UAS}$ was then gel eluted by digesting with *BamHI* and cloned into pCZ vector (7.5 kb) in *BamHI* sites. Confirmation of pCZ/429 $SIR2_{UAS}$ (or 429_{UAS}) was done by *BamHI* digestion which excised 429 bp of $SIR2_{UAS}$ (Figure A3.C.). Further the orientation of the insert was confirmed by PCR amplification of 429 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp) using forward primer OSB125 and reverse primer OSB41 (total size of the amplified product is 713 bp) (Figure A3.D.).

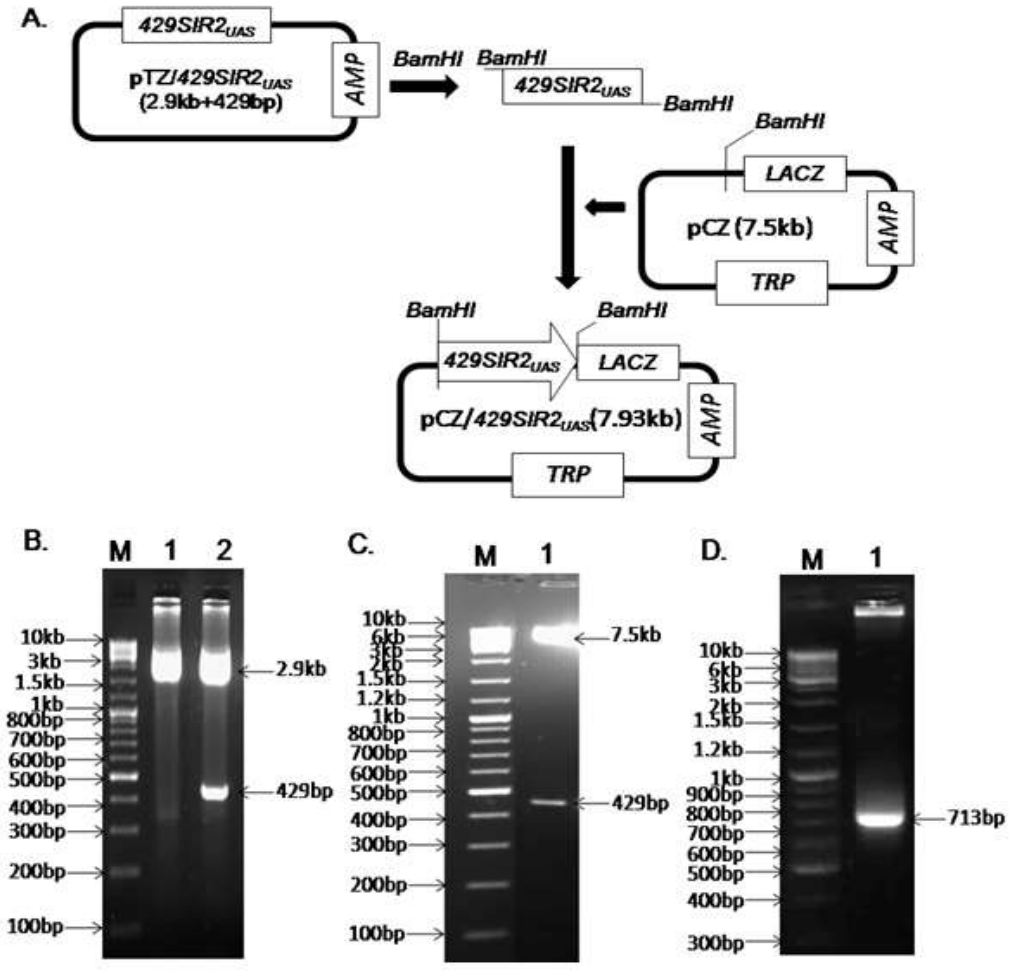

Figure A3: Cloning of 429 base pair of upstream activator region of *SIR2* in pCZ vector. (A) Strategy of cloning 429 bp *UAS* of *SIR2* in pCZ vector: 429 bp *UAS* of *SIR2* was cloned first in pTZ vector and then sub cloned into pCZ vector in *BamHI* sites. (B) Screening of pTZ/429 bp *UAS* of *SIR2*: Lane 1 shows undigested plasmid and lane 2 shows *BamHI* digestion, which released the insert of 429 bp *UAS* of *SIR2* and the empty vector backbone of 2.9 Kb. (C) Screening of pCZ/429 bp *UAS* of *SIR2*: Lane 1 shows digestion by *BamHI* which released 429 bp *UAS* of *SIR2* and 7.5 kb (pCZ). (C) Screening of pCZ/429 bp *UAS* of *SIR2* by PCR: Lane 1 shows 713 bp PCR amplified product which comprises 429 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp).

AI.4. Cloning of 370 base pair of upstream activator region of *SIR2* in pCZ vector [370_{UAS}]:

Genomic DNA isolated from wild type yeast strain was taken as template to amplify 370 base pair (bp) of upstream activator region (UAS) of *SIR2* using forward primer OSB193 and reverse primer OSB87 both having *BamHI* overhang. 370 bp of $SIR2_{UAS}$ was then cloned into pTZ vector. The pTZ/370 $SIR2_{UAS}$ clone was confirmed by *BamHI* digestion which excised 370 bp of $SIR2_{UAS}$ and the 2.9 Kb pTZ vector (Figure A4.A.). This 370 bp of $SIR2_{UAS}$ was then gel eluted by digesting with *BamHI* and cloned into pCZ vector (7.5 kb) in *BamHI* sites. Confirmation of pCZ/370 $SIR2_{UAS}$ (or 370_{UAS}) was done by *BamHI* digestion which excised 370 bp of $SIR2_{UAS}$ (Figure A4.B.). Further the orientation of the insert was confirmed by PCR amplification of 370 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp) using forward primer OSB193 and reverse primer OSB41 (total size of the amplified product is 654 bp) (Figure A4.C.).

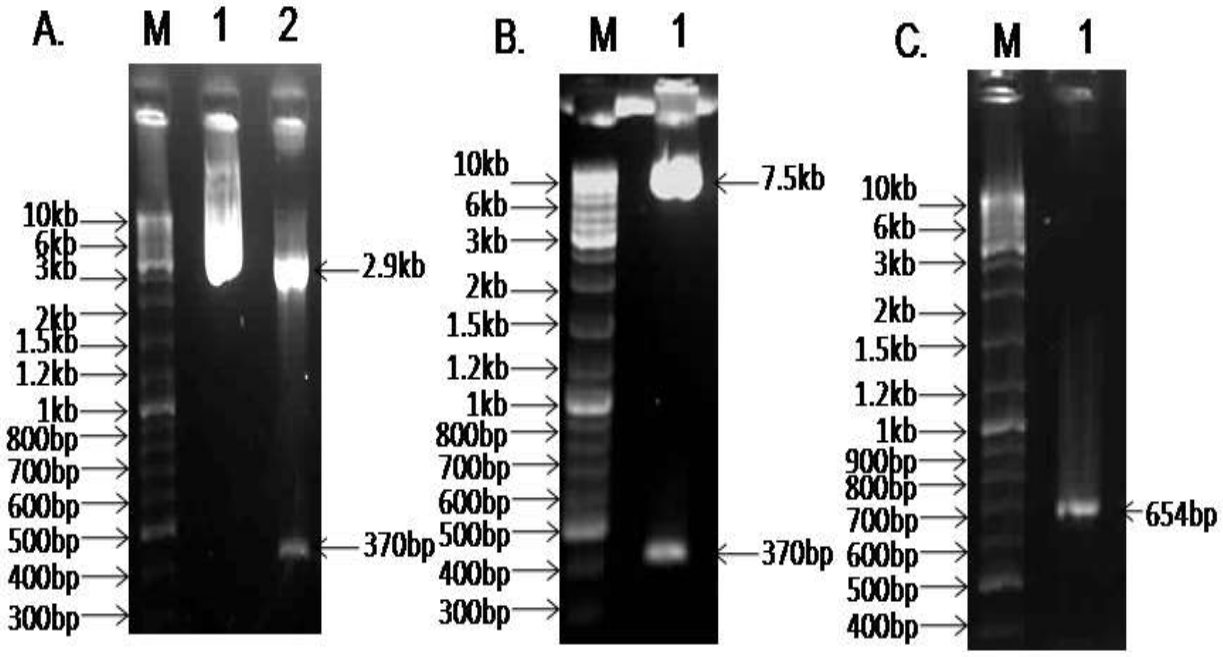

Figure A4: Cloning of 370 base pair of upstream activator region of *SIR2* in pCZ vector. Similar strategy of cloning 429 bp *UAS* of *SIR2* in pCZ vector was followed to clone 370 bp *UAS* of *SIR2* in pCZ vector. It was cloned first in pTZ vector and then sub cloned into pCZ vector in *BamHI* sites. (A) Screening of pTZ/370 bp *UAS* of *SIR2*: Lane 1 shows undigested plasmid and lane 2 shows *BamHI* digestion, which released the insert of 370 bp *UAS* of *SIR2* and the empty vector backbone of 2.9 Kb. (B) Screening of pCZ/370 bp *UAS* of *SIR2*: Lane 1 shows digestion by *BamHI* which released 370bp *UAS* of *SIR2* and 7.5 kb (pCZ). (C) Screening of pCZ/370 bp *UAS* of *SIR2* by PCR: Lane 1 shows 654 bp PCR amplified product which comprises 370 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp).

AI.5. Cloning of 307 base pair of upstream activator region of *SIR2* in pCZ vector [307_{UAS}]:

Genomic DNA isolated from wild type yeast strain was taken as template to amplify 307 base pair (bp) of upstream activator region (UAS) of *SIR2* using forward primer OSB194 and reverse primer OSB87 having *BamHI* overhang. 307 bp of $SIR2_{UAS}$ was then cloned into pTZ vector. The pTZ/307 $SIR2_{UAS}$ clone was confirmed by *BamHI* digestion which excised 307 bp of $SIR2_{UAS}$ and the 2.9 Kb pTZ vector (Figure A5.A.). This 307 bp of $SIR2_{UAS}$ was then gel eluted by digesting with *BamHI* and cloned into pCZ vector (7.5 kb) in *BamHI* sites. Confirmation of pCZ/307 $SIR2_{UAS}$ (or 307_{UAS}) was done by *BamHI* digestion which excised 307 bp of $SIR2_{UAS}$ (Figure A5.B.). Further the orientation of the insert was confirmed by PCR amplification of 307 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp) using forward primer OSB194 and reverse primer OSB41 (total size of the amplified product is 591bp) (Figure A5.C.).

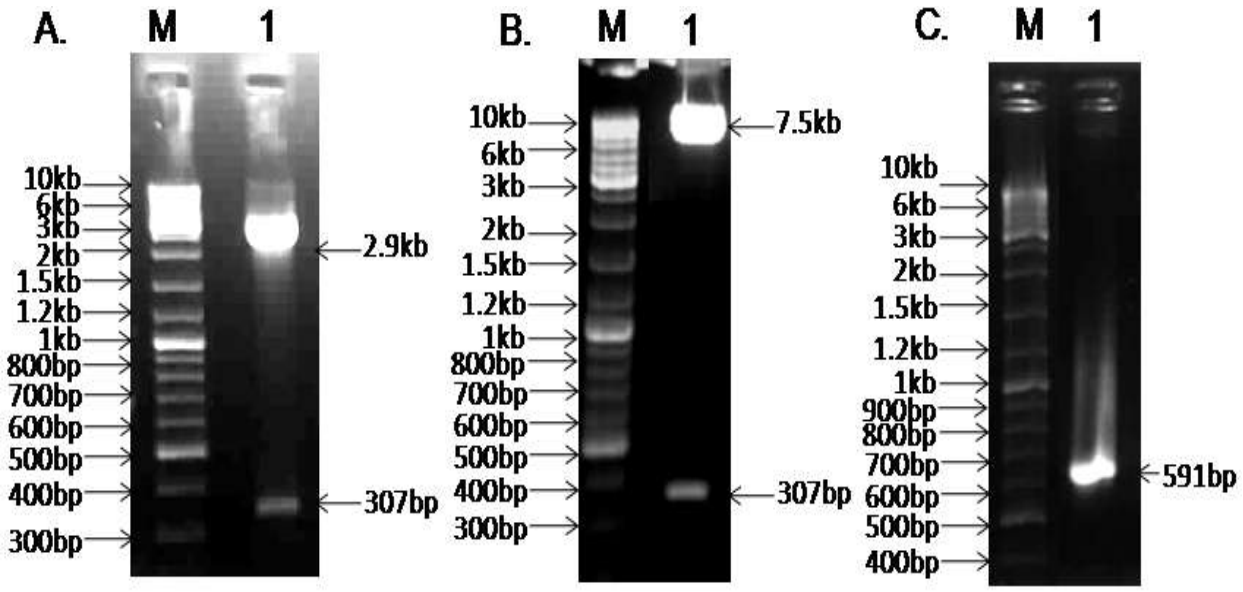

Figure A5: Cloning of 307 base pair of upstream activator region of *SIR2* in pCZ vector. Similar strategy of cloning 429 bp *UAS* of *SIR2* in pCZ vector was followed to clone 307 bp *UAS* of *SIR2* in pCZ vector. It was cloned first in pTZ vector and then sub cloned into pCZ vector in *BamHI* sites. (A) Screening of pTZ/307 bp *UAS* of *SIR2*: Lane 1 shows *BamHI* digestion, which released the insert of 307bp *UAS* of *SIR2* and the empty vector backbone of 2.9 Kb. (B) Screening of pCZ/307 bp *UAS* of *SIR2*: Lane 1 shows digestion by *BamHI* which released 307bp *UAS* of *SIR2* and 7.5 kb (pCZ). (C) Screening of pCZ/307 bp *UAS* of *SIR2* by PCR: Lane 1 shows 591bp PCR amplified product which comprises 307 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp).

AI.6. Cloning of 200 base pair of upstream activator region of *SIR2* in pCZ vector [200_{UAS}]:

Genomic DNA isolated from wild type yeast strain was taken as template to amplify 200 base pair (bp) of upstream activator region (UAS) of *SIR2* using forward primer OSB126 and reverse primer OSB87 having *BamHI* overhang. 200 bp of $SIR2_{UAS}$ was then cloned into pTZ vector. The pTZ/200 $SIR2_{UAS}$ clone was confirmed by *BamHI* digestion which excised 200 bp of $SIR2_{UAS}$ and the 2.9 Kb pTZ vector (Figure A6.A.). This 200 bp of $SIR2_{UAS}$ was then gel eluted by digesting with *BamHI* and cloned into pCZ vector (7.5 kb) in *BamHI* sites. Confirmation of pCZ/200 $SIR2_{UAS}$ (or 200_{UAS}) was done by *BamHI* digestion which excised 200 bp of $SIR2_{UAS}$ (Figure A6.B.). Further the orientation of the insert was confirmed by PCR amplification of 200 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp) using forward primer OSB126 and reverse primer OSB41 (total size of the amplified product is 484 bp) (Figure A6.C.).

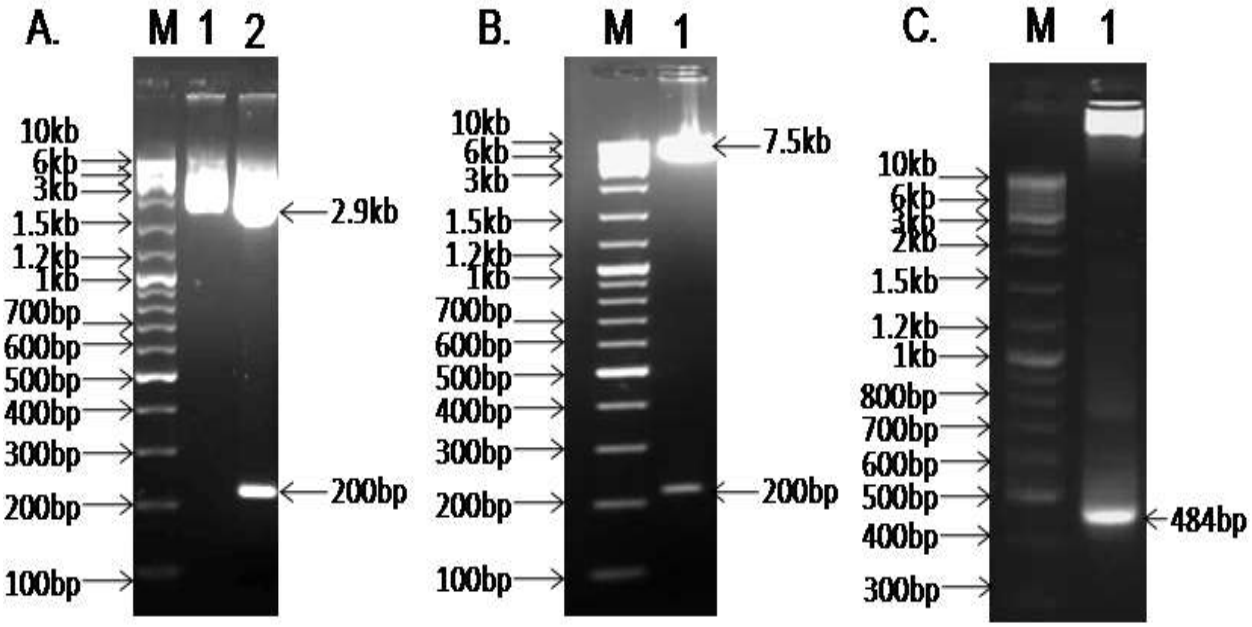

Figure A6: Cloning of 200 base pair of upstream activator region of *SIR2* in pCZ vector. Similar strategy of cloning 429 bp *UAS* of *SIR2* in pCZ vector was followed to clone 200 bp *UAS* of *SIR2* in pCZ vector. It was cloned first in pTZ vector and then sub cloned into pCZ vector in *BamHI* sites. (A) Screening of pTZ/200 bp *UAS* of *SIR2*: Lane 1 shows *BamHI* digestion, which released the insert of 200bp *UAS* of *SIR2* and the empty vector backbone of 2.9 Kb. (B) Screening of pCZ/200 bp *UAS* of *SIR2*: Lane 1 shows digestion by *BamHI* which released 200bp *UAS* of *SIR2* and 7.5 kb (pCZ). (C) Screening of pCZ/200 bp *UAS* of *SIR2* by PCR: Lane 1 shows 484bp PCR amplified product which comprises 200 bp of $SIR2_{UAS}$ with ORF of *LACZ* (284 bp).

AI.7. Cloning of *CUP9* in pESC-*HIS* vector [*pESC/ CUP9*]:

The strategy of the cloning is represented schematically in Figure A7A. Taking yeast genomic DNA as template, *CUP9* gene was amplified using forward primer, OSB203 having *BamHI* overhang and reverse primer, OSB204 having *SalI* overhang. The PCR product of size 921 base pair was cloned into pTZ (2.9 kb) vector. The pTZ/*CUP9* clone was confirmed by *BamHI* digestion, which released the insert of 921 base pair and the empty vector backbone of 2.9 Kb (Figure A7.B.). Then the *CUP9* gene was released by *BamHI* and *SalI* and sub cloned into the yeast expression vector, pESC (2μ, 6.7 kb) into *BamHI* and *SalI* sites under *GAL* promoter. pESC/*CUP9* clone was confirmed by *BamHI* and *SalI* digestion which released 921 base pair (*CUP9*) and 6.7 kb (pTZ) (Figure A7.C.).

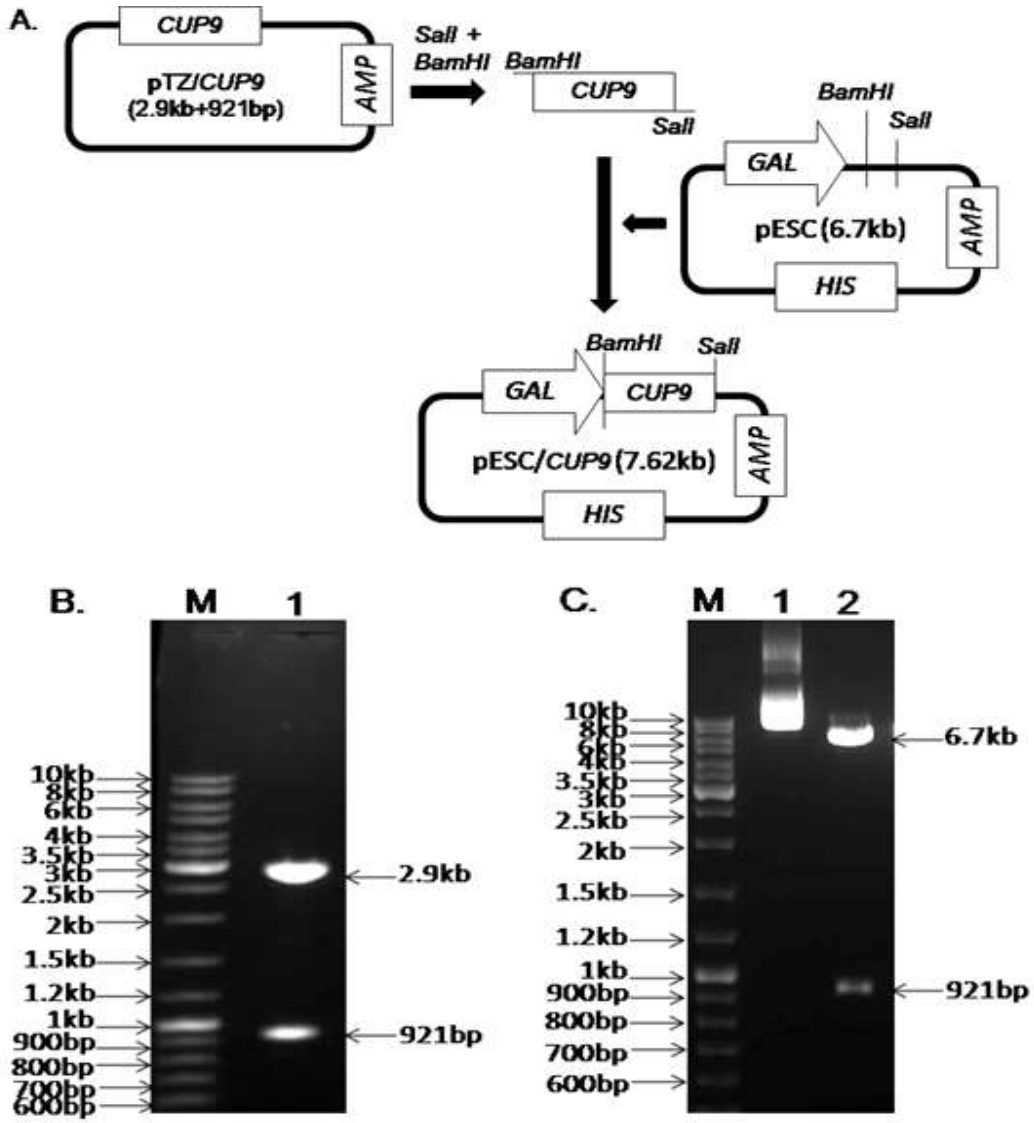

Figure A7: Cloning of *CUP9* in pESC-*HIS* vector. (A) Strategy of cloning *CUP9* in pESC-*HIS* vector. *CUP9* was cloned first in pTZ vector and then sub cloned into pESC vector in *BamHI* and *SalI* sites. (B) Screening of pTZ/*CUP9*: Lane 1 shows *BamHI* digestion, which released the insert of 921bp and the empty vector backbone of 2.9 Kb. (C) Screening of pESC/ *CUP9*: Lane 1 shows undigested plasmid and lane 2 shows digestion by *BamHI* and *SalI* which released 921 bp *CUP9* and 6.7 kb pESC vector.

SYNOPSIS:

The findings from last few years are helping researchers to understand a long-standing puzzle of heritable phenomena that regulate gene expression by modifying chromatin structure without involving changes in genome sequence. This phenomenon is defined as epigenetics [1]. Genes, packaged into chromatin, undergo a dynamic chromatin remodeling processes which are important for the initial step of gene expression. Decrease in nucleosome density is associated with relaxation of chromatin structure which causes transcriptional activation of genomic region [2]. This includes accessibility of gene promoters and regulatory regions to different transcription factors [3]. Epigenetic factors such as DNA methylation, histone modifications, action of small non coding RNAs are responsible for this regulatory process [4]. Epigenetic modification alters chromatin structure through several chromatin-associated proteins which includes linker histone 1, high mobility group proteins (HGP), histone modifying enzymes and components of chromatin remodeling complex [5, 6, 7].

Evidences suggest that environmental factors or stresses play a critical role in chromatin remodeling and stable alterations in gene expression and transmission of this non-DNA sequence information to next generation. Stresses can be either intrinsic, such as spontaneous gene mutation, or extrinsic which includes biotic and abiotic stress. Pathogen attack is an example of biotic stress whereas abiotic stress originates from unfavorable environmental conditions. Changes at the gene expression level are brought by different environmental factors such as diet, temperature changes, chemical pollutants and other external stresses. But the mechanistic links between environmental trigger and observed epigenetic modifications are yet to be understood. Histone tail modifications play an important role in response to abiotic stress in plants. For example, in tobacco and Arabidopsis, exposure to salinity, cold and abscisic acid tend to increase global enrichment level of H3 Ser-10

phosphorylation, H3 phospho-acetylation [17] and these histone modifications lead to up-regulation of many stress specific genes. Nutritional factors modulate gene expression in animal and human models. Experiment in mice shows methyl supplement causes diet induced phenotypic alterations at A^{vy} locus *via* increased C_pG methylation ensuing silencing of *agouti* gene expression [20]. Studies have explored that changes in the environmental temperature trigger epigenetic alterations in individuals. Exposure to heat shock and osmotic stress causes chromatin modification by phosphorylation of ATF2 resulting red eye color phenotype in drosophila that is stably inherited over successive generations [24]. Vernalization in plants is another striking example of epigenetic regulation of gene expression through which plants are instructed to flower early after having exposed to cold temperature [25].

Lower eukaryote *Saccharomyces cerevisiae* genome use histone acetylation-deacetylation as one of the measures of epigenetic mechanisms to control gene expression. Histone deacetylases (HDAC) are the transcriptional repressors which cause deacetylation of histones and thereby create localized regions of repressed chromatin. My overall research goal is to find out the link between environmental factors and the emerging phenotype occurring through the alteration in gene expression. To that end using *Saccharomyces cerevisiae* as a genetic model I have explored whether environmental cues like heat stress has any effect on the expression and activity of the type III NAD^+ dependent histone deacetylase Sir2. Sir2 is involved in transcriptional silencing of the silent mating type loci, *HML* and *HMR*, as well as genes near telomeres [6, 86].

Studies have shown that heat shock causes up regulation of a molecular chaperone heat shock protein 90. Hsp90 is responsible for the overall stability of cellular proteins at elevated temperature. It is a highly abundant eukaryotic protein, involved in maturation and folding of some special class of proteins collectively known as 'clients'. It dimerises in an ATP dependent manner with several co-

chaperones and provides the maturation of the target protein at a near native state [140]. In budding yeast there are two isoforms of Hsp90; Hsc82 (human ortholog of Hsp90β), which is constitutively expressed in the cell and Hsp82 (human ortholog of Hsp90α) which is induced whenever cells are exposed to any kind of stressed condition. Hsp90 level is significantly increased in the cell upon exposure to stress, including temperature, non physiological pH, nutrient deprivation and malignancy [140]. Recent studies have unraveled novel roles of Hsp90, where Hsp90 and its cochaperone p23 are involved in stabilization of different protein DNA complexes during RNA transcription, telomere maintenance, DNA replication *etc.* [215, 193, 194, 217]. Genome wide study revealed that yeast Hsp90 interacts with many telomeric proteins including Sir2 [168].

I have formulated three specific aims which have been investigated in this study.

Firstly, I wanted to investigate whether Hsp90 is required for functional maturation of Sir2. To that end, I have generated single knock out strains *⊿hsp82* and *⊿hsc82* as well as used a temperature sensitive strain and studied the endogenous level of Sir2p at restrictive temperature. Our result shows that deletion of either *HSP82* or *HSC82*, does not lead to any change in Sir2p stability. However if both the copies are inactivated (using a temperature sensitive strain at restrictive temperature) the endogenous level of Sir2p reduces significantly without any change in *SIR2* transcript. Also it is associated with drastic reduction in Sir2 activity in the cell. Our study shows that the conditional mutant shows complete loss of mating type silencing as well as telomere silencing activity. We have also established that Sir2 undergoes proteasomal degradation during Hsp82 inactivated condition, which is reversed in presence of proteasome inhibitor MG132. This is the general fate of major Hsp82 clients under Hsp82 inactivated condition. Thus our data collectively concludes that Sir2 is a direct client of Hsp82.

In the second specific aim, I wanted to find out whether heat shock or Hsp90 over-expression has any effect on the expression and activity of Sir2. Our result shows that a transient heat shock (exposure to 39^0C for 40 minutes) or over-expression of Hsp90 causes significant reduction in the endogenous level of Sir2 and results in the concomitant de-repression of the silent chromatin at telomeres. Most interestingly, the transient heat shock mediated de-repression of sub-telomeric chromatin is maintained over multiple generations before it gradually returns to its normal state. Our study shows that a transient heat shock mediated loss of Sir2p takes 7 days to revert back to its original level. As a result the subtelomeric genes remain de-repressed for about 90 generations. Also the *HMLa* transcript remains de-repressed upto 4days post heat shock in *MATa* cell indicating a defect in its mating behavior. In order to understand whether the reduction in Sir2p is due to regulation at the transcript level or at the protein level we estimated *SIR2* transcript at high temperature as well as under Hsp82 over expressed condition. Real time RTPCR shows about five fold reduction of *SIR2* transcript at above mentioned conditions compared to the wild type. We established that the upstream activator sequence of *SIR2* is regulated with Hsp82 over-expression.

In the third specific aim, I have identified the cis regulatory region as well as trans-regulatory elements (repressor) responsible for transcriptional down regulation of *SIR2* during heat shock. We have effectively mapped the cis regulatory element of *SIR2* gene and have identified a repressor binding site in the region spanning (-429 to -369) base pair of $SIR2_{UAS.}$ We have found that (-429 to -369) base pair of $SIR2_{UAS}$ is crucial for Hsp90 mediated transcriptional down regulation of *SIR2*. Using a series of genetic experiments we have identified a transcriptional repressor Cup9 which is responsible for transcriptional down regulation of *SIR2*. Our study shows that Hsp90 over expression (or heat shock) causes up regulation of *CUP9* both at the transcript as well as at the protein level and ChIP experiment confirms that Cup9 binds to the UAS_{SIR2} under Hsp82 over-

expressed condition. Deletion of *CUP9* abrogates Hsp90 over-expression mediated decrement of *SIR2* transcript. Conversely, Cup9 over-expression leads to the decrement of *SIR2* transcript ensuing de-repression of sub-telomeric genes.

Thus our study identified a cryptic pathway of Sir2 regulation under heat stressed condition. These findings thus highlight the mechanism by which environmental factors amend the epigenetic configuration of chromatin that is inherited in successive generations.

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HSP90 Controls *SIR2* Mediated Gene Silencing

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Abstract

In recent years, Hsp90 is found to interact with several telomeric proteins at various phases of cell cycle. The Hsp90 chaperone system controls assembly and disassembly of telomere structures and thus maintains the dynamic state of telomere. Here, for the first time we report that the activity of another telomeric protein Sir2p is modulated by Hsp82, the ortholog of Hsp90 from budding yeast (*Saccharomyces cerevisiae*). In a temperature sensitive Hsp90 deficient yeast strain (*iG170Dhsp82*), less abundant Sir2p is observed, resulting in de-repression of telomere silencing and a complete loss of mating type silencing. Intriguingly, over expression of Hsp90, either by exposing cells to heat shock or by introducing *HSP82* overexpression plasmid also yields reduced level of Sir2p, with a consequential loss of telomere silencing. Thus, Hsp90 homeostasis maintains the cellular pool of Sir2p and thereby controls the reversible nature of telomere silencing. Interestingly, such regulation is independent of one of its major co-chaperones Sba1 (human ortholog of p23).

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Introduction

Hsp90 is a highly abundant eukaryotic protein, which is involved in maturation and folding of some special class of proteins, which are primarily involved in signal transduction [1,2,3]. It dimerises in an ATP dependent manner with several cochaperones and provides the maturation of the target protein at a near native state [4]. In budding yeast there are two isoforms of Hsp90; Hsc82 (human ortholog of Hsp90β), which is constitutively expressed in the cell and Hsp82 (human ortholog of Hsp90α), which is induced whenever cells are exposed to any kind of stressed condition. It is known that expression of either one of the two isoforms of Hsp90 is required for yeast viability [5]. The two isoforms share 97% sequence identity and they comprise (1–2) % of the total cytosolic proteins. Hsp90 level is significantly increased in the cell upon exposure to stress, including temperature, nonphysiological pH, nutrient deprivation and malignancy [6]. Recent studies have unraveled novel roles of Hsp90, where Hsp90 and its cochaperone p23 are involved in stabilization of different protein DNA complexes during RNA transcription, telomere maintenance, DNA replication *etc.* [7,8,9,10]. Hsp90 and p23 also influence *in vitro* and *in vivo* telomerase activity [11]. It is also known that other cochaperones, for example, Hsp70, Hsp40 and Hop are also needed for proper maturation of telomerase [12]. However, unlike the steroid hormone receptor maturation, where Hsp90-p23 complex is only transiently attached to the target protein, in the case of telomerase activation, Hsp90 and p23 are found to be associated with functional telomerase complex even after the completion of telomere addition. In yeast, Hsp82 (the ortholog of Hsp90) helps in maintaining a dynamic equilibrium between the extendable and unextendable state of telomere through active interaction with Cdc13 [13]. In recent years, a plethora of proteomic, bioinformatics, and genetic approaches unravel many more telomeric proteins as potential clients of Hsp82. Examples include Cdc13, Stn1, [14,15], Sir2 [16], Mre11 [17], Ku80, Mec1 and Est1 [15]. Some of these gene products are involved in telomere position effect (TPE) [18], a phenomenon where transcription of gene is repressed by its close proximity to telomere.

In budding yeast, telomere silencing is initiated by recruitment of Sir2p-Sir4p complex at subtelomeric regions by telomere bound Rap1p and Ku70/Ku80 heterodimer. Sir2p mediated histone deacetylation helps Sir3p binding, which triggers the spreading of the Sir complex along the subtelomeric regions, causing heterochromatinisation. Sir2p, which is the key member of sirtuins, is conserved across the evolution [19]. It is a NAD^+ dependent histone deacetylase and is involved in transcriptional silencing of the silent mating type loci, *HML* and *HMR*, as well as genes near telomeres [20,21]. A high throughput study aims at identifying *S. cerevisiae* deletion mutants affecting telomere length reveal slightly short telomere phenotype in *hsc82* deletion mutant [22]. However contradictory results from other studies show no apparent change in telomere length in *hsp82* or *hsc82* deletion mutants [14]. It is well established that length of telomere positively regulates the efficiency of silencing, *i.e.*, the longer the telomere, greater is the silencing [23]. However, the effect of Hsp82 deficiency on TPE has not been explored. Moreover, why over-expression of Hsp82 leads to telomere length shortening is also poorly understood, except for the fact that *HSP82* and *HSC82* are the high copy suppressors for the *stn1-1* and *cdc13-1* mutants [14].

In this paper, we show that Hsp82 homeostasis plays a critical role in maintaining cellular pools of enzymatically active Sir2p. In *Δhsp82*, *Δhsc82* double knock out background, which is maintained

by *iG170Dhsp82* temperature sensitive allele, Sir2p level is found to be considerably diminished which is accompanied by complete loss of Sir2p function at restrictive temperature. Our result shows that at restrictive temperature, both the mating type silencing as well as telomere silencing function is completely lost, a phenotype similar to that observed in *Δsir2* cells. This conclusively proves that Sir2p function is dependent on Hsp82. On the other hand, over-expression of Hsp82 shows negative regulatory effect on telomere silencing but has no effect on mating type silencing. Our result shows that Hsp82 over expression diminishes the level of Sir2p. We hereby speculate that under normal physiological condition, Hsp82 stabilizes Sir2p and thereby maintains repressed state of subtelomeric chromatin. On the other hand, under stressed condition, which results during over-expression of *HSP82*, the level of Sir2p is down regulated that de-represses the silent chromatin state. Thus, it seems that the central role of Hsp82 is to maintain a dynamic equilibrium of the level of silencing proteins which may be responsible for the reversible nature of telomere silencing.

Materials and Methods

Yeast strains

Strains used in this study are listed in Table 1. The strain SLY20 used for TPE color assay is isogenic to W303a having *ADE2* marked telomere VIIL. The mating tester strains used for these studies are YDS31 and YDS32. The temperature sensitive strain *iG170Dhsp82* was kindly provided by Didier Picard [24]. This strain has the permissive temperature 25°C and restrictive temperature (35°C–37°C). Yeast knock out strains YSC1021-551935 (*HSP82::KAN*^{r}), YSC1021-552834 (*SBA1::KAN*^{r}), YSC1021-551520 (*HSC82::KAN*^{r}) were purchased from Thermo Scientific, Open Biosystems, Huntsville, AL, USA. All primers used in this study were purchased from Sigma Aldrich. Using the genomic DNA of YSC1021-551520 as a template and the pair of primers OSB1 (5′ACC AAG CGT TGG GTA ATG A3′) and OSB2 (5′ TGG TCA TTT GAC AGC TGA TG3′) KANMX cassette with *HSC82* flanking regions was amplified. The PCR product was then integrated into SLY20 and selected on G418 sulphate containing plates. The resultant *hsc82* null strain is referred as SLY4 in this paper. In a similar way, using genomic DNA of YSC1021-551935 (*HSP82::KAN*^{r}) as a template and the pair of primers OSB3 (5′ TGA CAC ACT AGA CGC GTC GG3′) and OSB4 (5′TAC CAA CCA GGT CCT TCC GC3′) KANMX cassette with *HSP82* flanking regions was amplified. It was then integrated to SLY20 to generate *hsp82* null strain SLY5. To knock out SBA1 gene, the genomic DNA of YSC1021-552834 was used as a template to amplify KANMX cassette with *sba1* flanking regions. Forward primer OSB5 (5′ TGC TAC CCG CCT TCC GAG TG3′) and the reverse primer OSB6 (5′ CAC ATA CAG TTC CAT TAC TTG AC3′) were used for this purpose. The PCR product was then integrated into SLY20 cells to generate *sba1* null strain SLY6.

The *sir2* null strain SLY12 (*sir2::KAN*^{r}) was made by integration of KANMX cassette with *SIR2* flanking regions in SLY20. All of the knockout genotypes were confirmed by PCR analysis.

The *HSP82* over expression plasmid pHCA/hsp82, which has *HIS3* marker, was transformed into SLY20, SLY5 and SLY6 strains and the transformed colonies were selected by growing them on SC-his medium to derive SLY13, SLY10 and SLY31 strains respectively. The blank vector pHCA was transformed into SLY20 and the transformed colonies were selected on SC-his medium to derive the strain SLY46. The 2 μ *HSP82* over expression plasmid pTA/hsp82 which has *TRP* marker was transformed into SLY20 strain; the transformed colonies were selected by growing them on SC-trp medium to derive SLY32 strain.

Plasmids

pHCA/*HSP82* plasmid was a gift from Didier Picard [25]. It is a CEN/ARS plasmid, derived from pRS313 vector, which over expresses *HSP82* under the control of GPD promoter.

Using genomic DNA of W303a as a template we amplified the *HSP82* gene using the forward primer OSB21 (5′ GAC GGA TCC ATG GCT AGT GAA ACT TTT GAA TTT C 3′) having *Bam*H1 site and the reverse primer OSB22 (5′ CGG GTC GAC CTA ATC TAC CTC TTC CAT TTC GG 3′) having *Sal*I sequence. The PCR amplified product was then cloned into *Bam*H1, *Sal*I double digested 2 μ expression vector pTA, which expresses *HSP82* under the control of GPD promoter. All the constructed plasmids were confirmed by sequencing.

TPE color assay

SLY20, SLY4, SLY5, SLY6, SLY13, SLY12, SLY31 and SLY46 cells were grown on appropriate medium and TPE was performed according to the protocol described [26].

Mating type frequency assessment

Wild type strain (*HSP82*), *Δhsp82*, *Δhsc82*, *YDS32 (MATα)* and *Δsir2* were grown to the mid exponential phase. About 400 cells from each culture were plated in YPD plate and incubated at 30°C. Also 400 cells of the wild type (*HSP82*), *Δhsp82*, *Δhsc82*, and *Δsir2* were mixed with same number of cells of *YDS32 (MATα)* and incubated at 30°C shaker for 10 minutes. Then each of them was spreaded on SD (synthetic dextrose) plate and incubated at 30°C incubator for 30 hours. The number of cells grown on each YPD and SD plates were counted. The mating frequency was calculated as = (No. of cells grown on SD plate/No. of cells on YPD plate)* 100. The value obtained in case of *Δsir2* strain was subtracted from each of the three strains. The experiment was repeated for 5 times and each bar shows the mean frequency ± SD.

Western blotting

The temperature sensitive strain *iG170Dhsp82* was grown at 25°C (permissive temperature) as well as 37°C (restrictive temperature) and protein was isolated from them by the procedure described below. Proteins were isolated from SLY4, SLY5, SLY6, SLY10, SLY13, SLY20 and SLY32 strains after growing them on proper medium at 30°C and equal amounts of proteins were loaded in SDS PAGE. For heat shock treatment, wild type cells (SLY20) were grown upto 0.3 O.D. at 595 nm. It was then divided into two batches, one batch of cells was subjected to heat shock at 39°C for 40 minutes, and other was grown at 30°C. Equal amount of cells were finally harvested and protein was isolated from them.

We isolated the protein using trichloroacetic acid (TCA) as described in [27] with little modifications. Each of the above strains was grown on 20 ml of appropriate medium until the $OD_{600\ nm}$ reaches 0.5. The cell was centrifuged and the pellet was washed first with distilled water and then with 500 μl 20% TCA. The pellet was finally resuspended in 200 μl 20% TCA and the cell lysis was done with glass beads keeping at cold temperature for 30 min. The TCA precipitated proteins were collected; washed with 5% TCA and dissolved in 60 μl 1× sample buffer (0.05 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol and 0.1% bromophenol blue), supplemented with 6.66 μl 1 M DTT, and 33 μl 1 M Tris HCl, [pH 9.0]. The protein samples were boiled for 3 minutes and appropriate volumes of samples were loaded on 10% SDS poly acrylamide gel. The gel

Table 1. Yeast strains used in this study.

Strains	Genotype
SLY20	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2*
YDS31	*MATa his1*$^{-}$
YDS32	*MAT*α *his1*$^{-}$
iG170Dhsp82	*MATa can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 HSP82::LEU2 HSC82::LEU2 HIS3::HSP82G170D*
YSC1021-551520	*MATa HSC82::KAN*^{r}
YSC1021-551935	*MATa HSP82::KAN*^{r}
YSC1021-552834	*MATa SBA1::KAN*^{r}
SLY4	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 HSC82::KAN*^{r}
SLY5	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 HSP82::KAN*^{r}
SLY6	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 SBA1::KAN*^{r}
SLY12	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 SIR2::KAN*^{r}
SLY13	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 pHCA/HSP82*
SLY10	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 HSP82::KAN*^{r} *pHCA/HSP82*
SLY31	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 SBA1::KAN*^{r} *pHCA/HSP82*
SLY32	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 pTA/HSP82*
SLY46	*MATa 15ade2-1, ura3-1, 112 his 3-11, trp1, leu2-3, VIIL::ADE2 pHCA*

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was then transferred to a polyvinylidene difluoride (PVDF) membrane and immuno blotted for Hsp82, Sir2 and Actin. The anti Hsp90 antibody (Calbiochem), the anti Sir2 (Santa Cruz Biotechnology Inc., CA) and anti Actin antibody (Abcam) were used at 1:5000 dilution. Horseradish peroxide-conjugated rabbit IgG (Santa Cruz Biotechnology Inc., CA) was used as a secondary antibody for Sir2 at 1:10000 dilution and HRP conjugated mouse IgG (Promega) was used as a secondary antibody for Hsp82 and Actin blot at the same dilution. The Western blots were developed using chemiluminescent detection system (Pierce). The bands on the gel were quantified using Gene Tools, Syngene and the relative densities, thus obtained, were plotted using GraphPad Prism 5 software. The mean value from four independent experiments was plotted with standard deviations ($\pm$SD). All blots were normalized against Actin.

RNA isolation

Total RNA was isolated from *iG170Dhsp82* after growing them at 25°C and 37°C, as well as from SLY20, SLY12 and SLY13 strains by using acid phenol method as described [28] with some modifications. 10 ml culture of cells were grown up to mid log phase ($OD_{600}\approx1$), centrifuged and the pellet was suspended in 400 µl TES buffer (10 mM Tris HCl, [pH = 7.5], 10 mM EDTA, 0.5% SDS). 400 µl phenol (pre equilibrated with water) was added and the mixture was incubated at 650C for 1 hour, with intermittent vortexing. The mixture was rapidly chilled on ice for 5 min, and centrifuged at 14000 rpm for 10 min at 40C. The aqueous layer was mixed with 400 µl chloroform, vortexed and centrifuged again for the same time at the same speed and temperature mentioned above. The extracted aqueous phase was precipitated by adding $1/10^{th}$ volume of 3 M sodium acetate [pH 5.2] and 2.2 volume of chilled ethanol. The precipitate containing RNA was washed with 70% ethanol and the pellet was dissolved in 30 µl DEPC treated water. Equal amount of RNA measured by spectroscopic analysis (JASCO spectrophotometer EMC-709) was then subjected to DNase I (Fermentas) digestion for 15 min at room temperature. DNase I was finally inactivated by incubating with 25 mM EDTA at 650C for 10 min. About 10 µg of total RNA from each sample was then reverse transcribed with oligo dT primer (Sigma Aldrich) using reverse transcriptase (Omniscript kit, Qiagen). Resulting cDNA was first quantified, diluted appropriately to normalize and then subjected to PCR amplification (27 cycles) using gene specific primers. The PCR products were run on 1.4% agarose gel and stained with ethidium bromide. We used OSB16 (5′TGA CCA AAC TAC TTA CAA CTC C3′) and OSB14 (5′TTA GAA ACA CTT GTG GTG AAC G3′) for amplifying 307 base pair at the 3′ end of *ACT1* transcript. OSB19 (5′ ATC ACG AGT AAG GAT CAA AG 3′) and OSB20 (5′ TTA TGG CTT TGT TAC GCT TG 3′) were used to amplify *YFR057w*, which is located in the subtelomeric region on chromosome VIR. We used OSB62 (5′ AAT CGG CGG ATG GGT TGG 3′) and OSB63 (5′ TCA TTC TTT CTT CTT TGC CAG 3′) for amplifying 308 base pair at the 3′end of the *HML*α*2* transcript.

Results

Sir2p function is dependent on Hsp82

Previously, yeast two hybrid screen, using Hsp82 as bait, has identified Sir2p interaction [16]. To explore whether Sir2p function is dependent on Hsp82, we examined both the mating type silencing as well as telomere position effect in a conditional mutant, where Hsp82 is functionally defective. We performed the assay using a temperature sensitive *iG170Dhsp82* strain. In this strain both *HSP82* and *HSC82* genes are deleted and the strain behaves as wild type when grown at permissive temperature 25°C, but as mutant when grown at restrictive temperature at 37°C [24]. This particular strain harbors mutant *G170Dhsp82* which is integrated into its chromosome. It tolerates little bit higher temperature than its counterpart where *G170Dhsp82* is maintained

episomaly in the cell [29]. Previous work by Nathan and Lindquist showed that the mutant *G170Dhsp82* when present as an episomal copy within the cell, its growth is significantly diminished above 34°C. However if it is integrated within the chromosome it can tolerate (2–3)°C higher temperature [24,30]. We allowed to mate *iG170Dhsp82* (*MATa*) with tester strain YDS32 (*MATα*) at 25°C and 37°C and compared its mating behavior with wild type (*HSP82*) and *Δsir2* strains. We found that loss of function of *HSP82* at 37°C impaired the mating ability of the strain and it behaved like *Δsir2* (Figure 1Ai). Conversely, at 25°C, the strain showed mating ability comparable to wild type cells. In order to rule out the possibility that the lack of silencing at restrictive temperature is not due to any lack of growth, we tested the viability of both temperature sensitive mutant and that of the tester strains at 37°C, and found that all the strains were viable at 37°C (Figure S1).

It was earlier reported that Hsp90 controls the pheromone signaling in yeast [25]. Therefore, it is important to understand whether the loss of mating type silencing of *G170Dhsp82* at restrictive temperature is due to the defect in pheromone signaling pathway alone, or also due to de-repression of *HMLα* locus. To address this question the semi quantitative RT-PCR was done to measure the *α2* transcript level from *G170Dhsp82 (MATa)* after growing them at 25°C as well as 37°C (Figure 1Aii). The quantification RT-PCR data showed negligible amounts of *α2* transcript at 25°C, whereas at 37°C significant amount of *α2* transcript was visible. This result was well corroborated with the sterility of *iG170Dhsp82* at 37°C, since in order for a *MATa* strain to behave as a mating compatible haploid strain, *α2* factor should remain repressed [31,32]. As a control, we had taken *Δsir2* strain where the RT-PCR result showed the presence of considerable amount of *α2* transcript, which made it sterile. Thus from this experiment it can be concluded that in *hsp82* deficient condition, there is a loss of silencing at HML, which is not only due to defect in pheromone signaling pathway [25] but also due to derepression of *MATα2* transcript in *iGD170hsp82 (MATa)*.

We also explored whether Hsp82 has any role in silencing of subtelomeric genes. For that purpose we monitored the mRNA level of *YFR057w* gene, which is located adjacent to chromosome

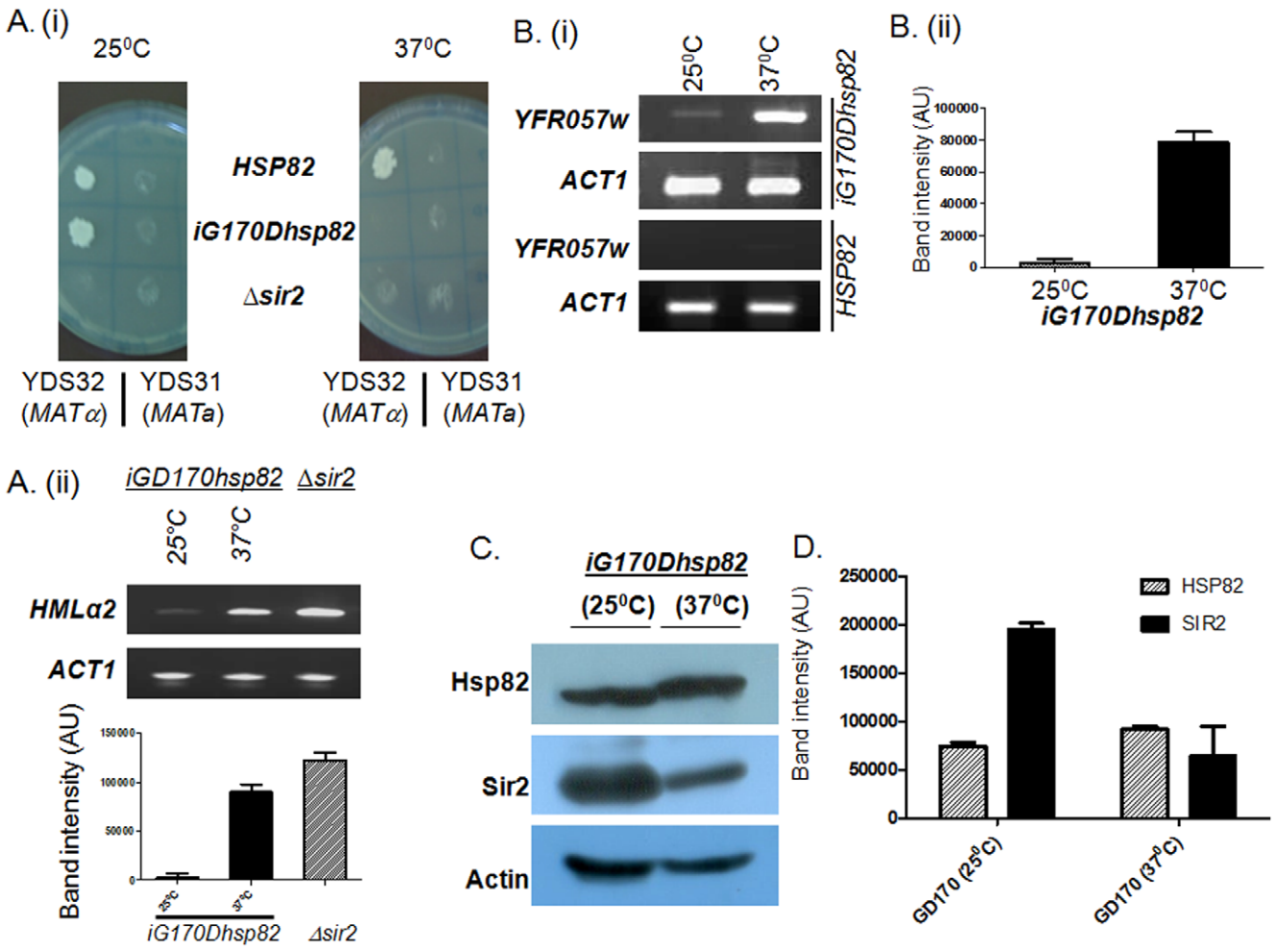

Figure 1. Sir2p function is dependent on Hsp82. (Ai) The temperature sensitive strain *iGD170hsp82* causes disruption of mating type silencing at restrictive temperature (37°C), where Hsp82 is non functional. (Aii) *iG170Dhsp82* at 37°C shows considerable amount of *HMLα2* transcript comparable to that present in *Δsir2* cells; whereas at 25°C negligible amount of *HMLα2* is seen. (Bi) Semi quantitative RT-PCR shows increase in *YFR057w* transcript in *iG170Dhsp82* at 37°C compared to 25°C, indicating that the strain has defect in telomere position effect, whereas wild type strain (*HSP82*) shows silencing at higher temperature (37°C). (Bii) Graphical representation of four independent experiments of (Bi) is done after normalization with *ACT1* control. (C) Western blot shows the relative abundance of Hsp82 and Sir2p at two temperatures. Actin is the loading control. (D) The quantification of Western blot (from 4 independent experiments) shows more than 50% reduction in steady state level of Sir2p at higher temperature. The data are normalized with respect to the loading control Actin. Each bar represents mean density ± SD.
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VIR telomere. *YFR057w* ORF codes for a protein of unknown function and is located within 1 kb from the end of chromosome. As Sir2p spreads as far as 3 kb length in the chromosome VIR [33], its ORF is repressed under normal conditions but de-repressed in *Δsir2* strain (Figure S2). We quantified the steady state level of *YFR057w* transcript in *iG170Dhsp82* at both 25°C and 37°C and compared with *ACT1* transcript. The semi quantitative RT-PCR showed significant increase in *YFR057w* transcript at 37°C compared to 25°C, whereas the level of *ACT1* transcript remained the same at both temperatures (Figure 1B). In order to rule out the possibility that the loss of silencing is not a mere effect due to shift in temperature we measured the *YFR057w* transcript level of wild type strain (SLY20) at both 25°C and 37° (Figure 1B). No loss of silencing was observed in wild type strain at higher temperature. This result is consistent with the previous finding that higher temperature causes more silencing [34]. The above two experiments conclusively prove that Hsp82 has a role in regulating telomere silencing as well as mating type silencing.

Since, Sir2p is the key protein involved in both of these silencing mechanisms; we wanted to see whether there is any change in the level of Sir2p in *iGD170hsp82* strain at different temperatures by Western blot analysis. When monitored at non permissive temperature (37°C) the level of Sir2p was found to be substantially diminished (Figure 1C), whereas the level of Hsp82 was comparable at both the temperatures, Act1p being a loading control. The experiment was repeated four times and the mean value of the quantification of the band intensity on Western blot showed that there was at least 50% reduction in the level of Sir2p at 37°C compared to 25°C (Figure 1D). We hereby speculate that Sir2p may be a putative client of Hsp82, whose activity inside the cell is directly or indirectly regulated by Hsp82. At restrictive temperature when Hsp82 is nonfunctional, Sir2p level is reduced by 50% and thus at lower levels it is unable to provide mating type silencing and telomere silencing activity. Thus for transcriptional silencing at telomere and at mating type loci, the budding yeast requires native Hsp82 protein.

Effect of Hsp82 over-expression on mating type silencing and transcriptional silencing at telomere

Next, we wanted to determine whether single knockout mutant allele *Δhsp82* or *Δhsc82* has any effect on Sir2p function. We generated two deletion mutants, *hsp82* and *hsc82* in SLY20 strain, which had *ADE2* gene next to telomere at chromosome VIIL. Under normal condition, when SLY20 was allowed to grow on low adenine medium at 30°C, they developed mostly as red colored colonies due to the lack of *ADE2* expression, whereas *Δsir2* null strain SLY12 developed as white colored colonies owing to the expression of *ADE2*. Our result showed that deletion mutants SLY4 (*Δhsc82*), SLY5 (*Δhsp82*) exhibited wild type phenotype, *i.e.* they all produced pink colored colony (Figure 2Ai) due to transcriptional silencing of *ADE2* gene, suggesting that Hsp82 and Hsc82 are redundant to each other. Similarly when we checked *YFR057w* transcript level of the single deletion mutants, we found *YFR057w* was silenced in all of them as expected from earlier experiment (Figure 2Aii). Thus single knockout alleles do not show any change in transcriptional silencing at subtelomeric locus and are redundant to each other.

As Hsp82 level is increased within the cells whenever cells are exposed to any kind of stressed conditions, we aimed to determine the effect of elevated amount of Hsp82 on Sir2p level and examined whether the silencing function of Sir2p was altered in Hsp82 over-expressed condition. We mimicked the Hsp82 over-expressed condition within the cell by transforming a centromeric plasmid pHCA/hsp82 in SLY20 to generate SLY13 strain, where Hsp82 was expressed under a strong promoter GPD. Interestingly, we found SLY13 cells resulted mostly in white colored colonies indicating a decrease in TPE (Figure 2Ai), however SLY46 strain containing the blank vector showed pink color colonies. In order to test whether the loss of TPE, due to over-expression of *HSP82*, is restricted to chromosome VII alone or other subtelomeric genes are also de-repressed, we monitored the mRNA level of *YFR057w* gene. We quantified the steady state level of *YFR057w* transcript in Hsp82 over-expressed cell (SLY13) and compared with wild type cell (SLY20). The semi quantitative RT-PCR showed significant increase in *YFR057w* transcript in Hsp82 over-expressed cells compared to the wild type, whereas *ACT1* transcript remained same in both the genotype (Figure 2B). This leads us to speculate that over-expression of Hsp82 acts as a negative regulator of transcriptional silencing at telomeres.

In order to find out the effect of single knock out (*Δhsp82* or *Δhsc82*) strains or Hsp82 over-expression on mating type silencing, we measured the mating ability of SLY4, SLY5, SLY10, SLY13, and SLY32 and compared with wild type and *Δsir2* strains. It was observed that all of them exhibited wild type mating phenotype when mated with the tester strain YDS32 (*MATα*) (Figure 3A). The mating type frequency for each of the deletion mutants (*Δhsp82* and *Δhsc82*) were plotted (Figure S3) and it was found to be comparable with that of the wild type. We also determined the level of *MATα2* transcript in *HSP82* overexpressing cell (SLY13) and compared its level with wild type (SLY20) and *Δsir2* (SLY12) strains. While *Δsir2* strain showed abundant levels of *MATα2* as expected, SLY13 showed negligible amount of *MATα2* transcript comparable to that of the wild type (Figure 3B). We, therefore, conclude that neither the single knockout mutants nor the over-expression of Hsp82 affect mating type silencing.

We were also interested to explore whether Sba1 (human ortholog of p23), which is a cochaperone of Hsp82 has any role in Sir2p maturation. To this end we created *Δsba1* deletion strain SLY6. We had also over-expressed Hsp82 in *sba1* null background (SLY31). While doing TPE assay for both SLY6 and SLY31, we found that SLY6 mostly developed as pink colored colonies whereas SLY31 showed white colored colonies (Figure 2Ai). The *Δsba1* deletion strain could also silence the subtelomeric gene *YFR057w* (Figure 2Aii) like wild type. The mating type silencing function of both SLY6 and SLY31 remained unperturbed (Figure 3A). These results indicate that Hsp82 mediated homeostasis of Sir2p is independent of Sba1.

Over-expression of Hsp82 reduces the cellular pool of Sir2p in a dose dependent manner

As over-expression of Hsp82 leads to down regulation of transcriptional silencing at telomeres, we monitored whether there was any change in Sir2p level as a function of Hsp82 expression. Equal amount of proteins isolated from different strains was loaded and they were probed with anti-Hsp90 antibody as well as anti-Sir2 antibody. Our result showed that in Hsp82 over-expressing cells (SLY10 and SLY13 strains) the level of Sir2p was considerable diminished (Figure 4A). The quantification of the bands from at least three independent experiments showed that in SLY13 strain while Hsp82 amount was increased by 50% compared to the wild type, the level of Sir2 was reduced by more than 50% in some cases (Figure 4B). In all other strain background such as SLY4 (*Δhsc82*), SLY5 (*Δhsp82*), SLY6 (*Δsba1*) the level of Sir2p remained unaltered. These results also suggest that Hsc82 (which is poorly detected by the antibody) compensates for the lack of Hsp82 (in SLY5) and thus the level of Sir2p is not affected.

Since cellular level of Hsp82 changes in response to different stressed condition, it is important to know whether Sir2p level is

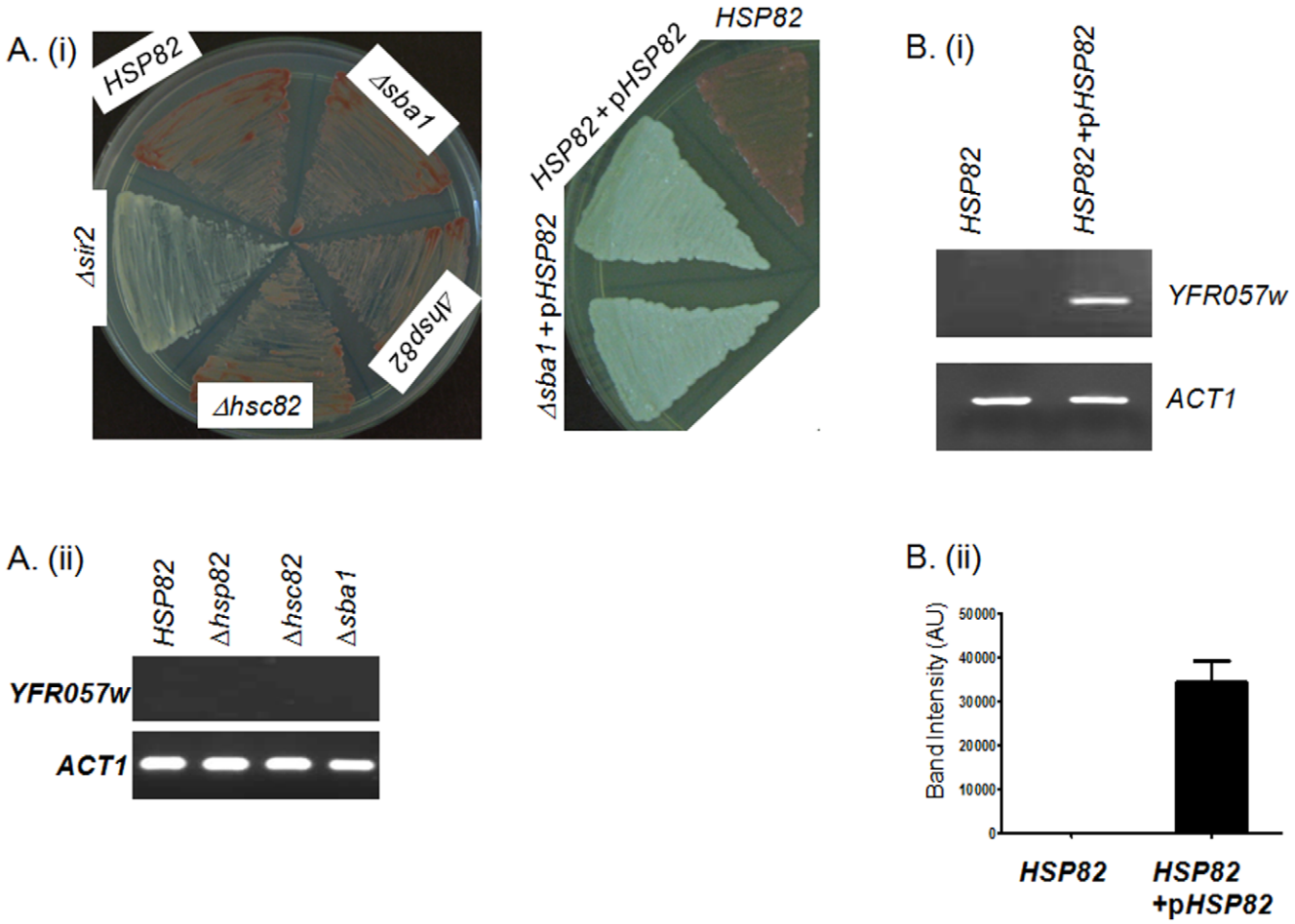

Figure 2. Over-expression of *HSP82* negatively regulates TPE but not mating type silencing. (Ai) ADE2 color phenotype of various strains *i.e.*, *HSP82* (wild type), *Δhsp82*, *Δhsc82*, *Δsba1*, *Δsir2*, over-expressing *HSP82* (*HSP82+pHSP82*) and over-expressing *HSP82* in *Δsba1* background. Pink colored colony represents telomere silencing and white colored colony represents de-repression of telomere silencing. (Aii) *YFR057w* is silenced in all single mutants as well as in wild type strain. (Bi) Over-expression of *HSP82* de-represses the expression of *YFR057w*. *ACT1* serves as loading control. (Bii) Quantification of *YFR057w* transcript is done from 4 independent experiments in *HSP82* overexpressed cells. The band intensity values are normalized against *ACT1*. Each bar represents mean density ± SD.
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dependent on Hsp82 in a dose dependent manner or not. In order to address this question, we generated another strain SLY32, which over-expresses Hsp82 from a multicopy 2 μ plasmid (pTA/hsp82). We compared the level of Hsp82 and Sir2 among SLY20 (endogenous *HSP82*), SLY13 (endogenous plus one copy from a CEN plasmid) and SLY32 (multiple copies of *HSP82*) strains by Western blot analysis. Our result showed a gradual increment in Hsp82 protein levels from SLY20 to SLY13 to SLY32, which was in accordance with the copy number. However, the level of Sir2p showed a reverse trend. The most dramatic reduction in Sir2p level was observed in SLY32 cells, while SLY13 exhibited a moderate decrement compared to the wild type cells (SLY20) (Figure 4C). The quantification of the bands, taking the average from independent three sets of experiment, showed that while the increase in Hsp82 level is 4 times in SLY32 strain compared to the wild type, the decrease in Sir2 level was at least by 75% compared to the wild type and by 50% compared to the SLY13 strain (Figure 4D). Thus, it appears that under stressed condition the increased level of Hsp82 results in concomitant reduction of the total cellular pool of Sir2p, which begs us to speculate that such adaptation in Sir2p abundance in response to Hsp82 homeostasis could be responsible for the reversible nature of transcriptional silencing at telomere.

Heat shock induces derepression of telomere silencing in yeast

Our findings propose that the Sir2p level and/or their function is controlled by Hsp82 homeostasis, which may be responsible for reversible nature of telomere silencing in yeast. This observation led us to think whether heat shock induced elevated level of Hsp82p is sufficient to alter the Sir2p level and consequently its function. To achieve this we exposed wild type cells (SLY20) to 39°C heat shock for 40 minutes. This is the optimum condition for induction of heat shock gene *HSP82* [35]. After heat shock we harvested the cell and isolated protein from them. The Western blot analysis showed (Figure 5Ai) similar kind of reduction in Sir2p level in heat stressed condition compared to cells growing at 30°C. The experimental data were plotted (Figure 5Aii) which showed heat shock induced the expression of Hsp82p which in turn reduced about 50% of the total cellular pool of Sir2p.

In order to find out the effect of heat shock on telomere silencing we aimed to monitor the transcript level of the

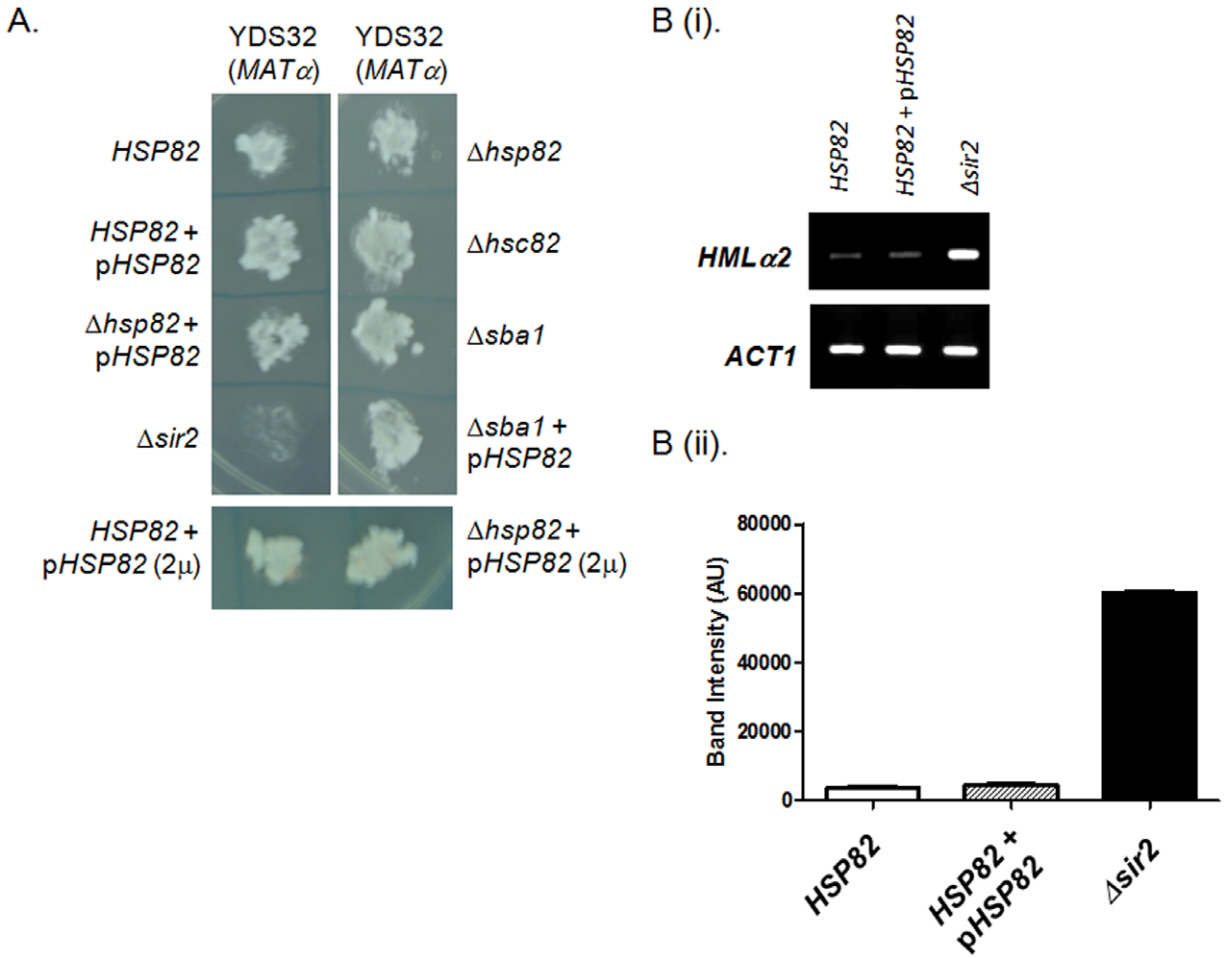

Figure 3. Single mutants as well as overexpression of *HSP82* do not alter mating type silencing. (A) Mating type silencing phenotype of different strains (as indicated) when mated with the tester strain YDS32 (*MAT*α). *Δsir2* acts as a negative control. (Bi) RT-PCR analysis of *HMLα2* transcripts in different strain backgrounds (as indicated) shows that *HMLα2* transcript levels are similar in *HSP82* overexpressing cells and wild type cells, *ACT1* serves as loading control. (Bii) Quantification of *HMLα2* transcript from three independent experiments are normalized with *ACT1* and plotted. Each bar represents mean density ± SD.
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subtelomeric gene *YFR057w*. For that we isolated the total RNA from the wild type cells grown under normal (30°C) as well as stressed condition (exposing at 39°C for 40 min). The semi-quantitative RTPCR showed the abundance of *YFR057w* transcript as a result of heat shock, indicating loss of telomere silencing (Figure 5B). These data strengthen our model that Hsp82 homeostasis is indeed responsible for the reversible nature of transcriptional silencing at telomere. Though the mechanism of this is not clear at present, but it is evident that Hsp82 is directly or indirectly controlling the cellular abundance of Sir2p.

Discussion

Several important findings came out through this study. Firstly, our study shows for the first time that Sir2p function is directly or indirectly dependent on Hsp82, and its steady state level in the cell is also controlled by Hsp82. We observe that in *iGD170hsp82* temperature sensitive mutant, where Hsp82 is nonfunctional at restrictive temperature, there is significant reduction of Sir2p which is associated with loss of mating type silencing as well transcriptional silencing at telomere. As Hsp82 controls pheromone signaling pathway in yeast, we wanted to decipher whether the loss of mating type silencing at restrictive temperature is also due to derepression at the *HMLα* locus in *MATa* strain. Our experimental result shows significant amount of *MATα2* transcript at 37°C, which is well correlated with the sterile nature of *iG170Dhsp82 (MATa)*. The Western blot shows that considerable amount of Sir2p is still present at the restrictive temperature (albeit at 50% reduced level); however functional studies at hidden mating locus demonstrated that these Sir2 proteins are inadequate for its functional activity. It has been reported earlier that silencing in yeast is dependent on temperature [34]. Increase in the temperature is associated with stronger TPE and mating type silencing in yeast. As an explanation of this effect it has been shown that at higher temperature Sir2-Net1 complex is destabilized whereas Sir2-Sir4 complex is having no effect on temperature [36]. Thus at higher temperature more Sir2 is available to bind with Sir4 leading to an increase in telomere and mating type silencing at the expense of ribosomal silencing in yeast. In our system the derepression of transcriptional silencing both at telomere and *MAT* locus at higher temperature is directly linked with the lack of Hsp82/Hsc82 activity.

Secondly, over-expression of Hsp82 in the cell causes reduction of Sir2p level in a dose dependent manner. This has important

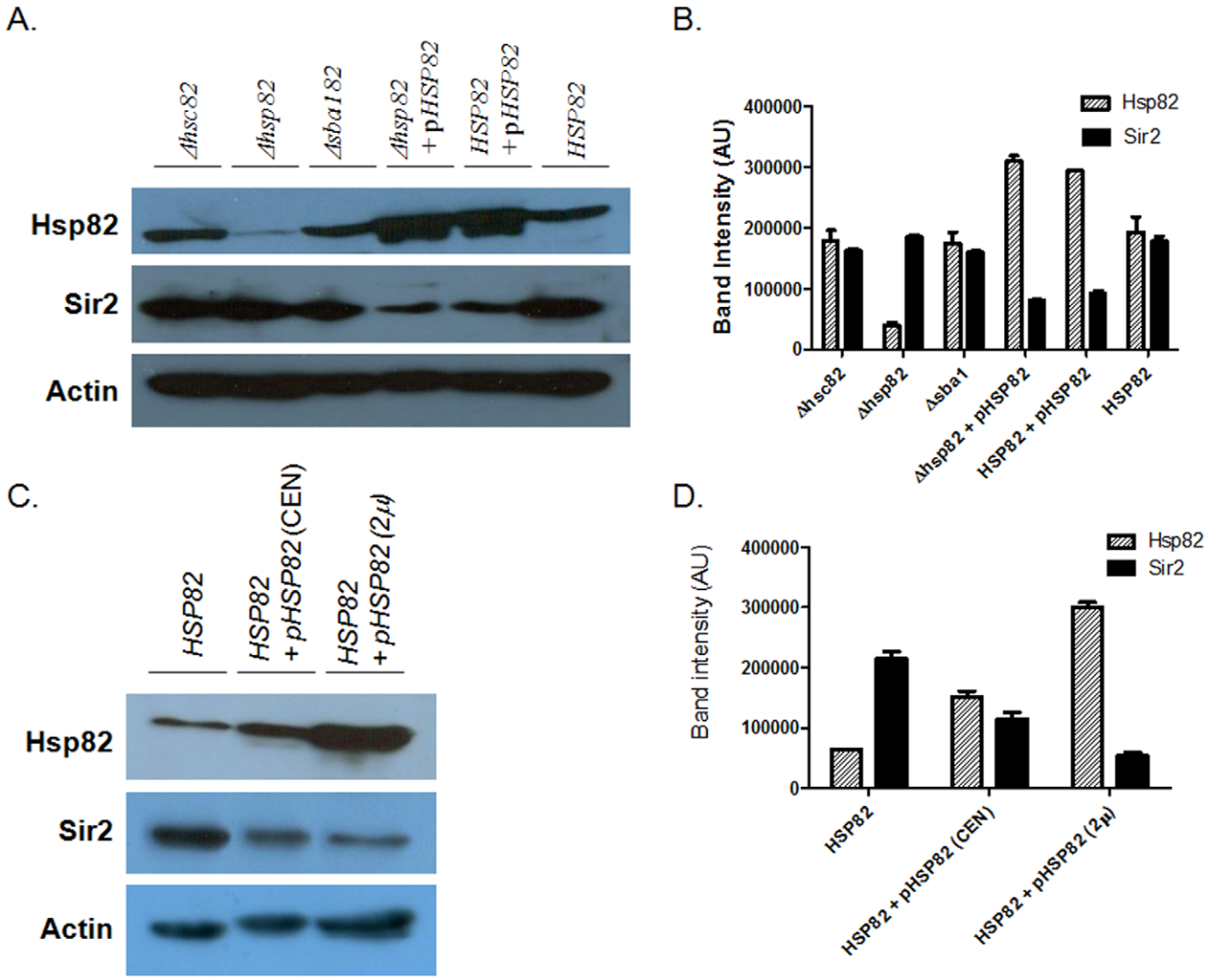

Figure 4. Western blot analysis shows over-expression of *HSP82* down regulates Sir2p in a dose dependent manner. (A) Different lanes are marked with the respective genotypes. p*HSP82* implies over-expression of *HSP82* from a CEN plasmid. Total proteins from each lysates are probed with anti Hsp82 antibody, anti-Sir2 antibody and with anti-Actin antibody (as loading control). (B) Graphical representation of (A), where densitometric measurements of the bands from four experiments are plotted, after normalizing with Actin band intensities. Each bar represents mean density ± SD. (C) Multiple levels of Hsp82 over-expression is shown using high (2 μ) and low copy plasmids (CEN), to demonstrate dose response between Hsp82 and Sir2p levels. (D) The graphical representation of Western blot as in figure (C) shows over expression of Hsp82 from a high copy plasmid confers more reduction of Sir2p level. Each bar represents the mean density ± SD from 4 independent experiments. The data are normalized with respect to Actin.
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consequence as occasionally the amount of Hsp82 increases within the cell when they are exposed to heat shock or subjected to other stressed condition. Our study demonstrates, in a condition where Hsp82 is overexpressed, there occurs a gradual decrement of Sir2p which impairs its ability in transcriptional silencing at telomere, although silencing at *HML*/*HMR* locus remains unperturbed. This data suggests that under stressed condition, though Sir2p level is diminished, there are enough Sir2p still present to perform other cellular activities. We speculate that although Sir2p is the key protein to control both mating type silencing and telomere silencing, the threshold cellular concentration of Sir2p for these two different functions may be different. In case of silencing at the mating type loci, the reduced cellular level of Sir2p, which is the consequence of Hsp82 over-expression, may be sufficient to maintain the silence state, however, it may be limiting for silencing the stretch of subtelomeric regions spread over 32 chromosomal ends. The question remains that why at low concentration, Sir2p comes out of the subtelomeric region, but not from the mating type loci. Considering that telomere silencing is a reversible phenomenon, meaning that the silencing complex is in a dynamic equilibrium between DNA bound and unbound states; and mating type silencing is not reversible under normal conditions, provoke us to speculate that the binding affinity of silencing complex towards the silencer sequences at the cryptic mating type loci is much stronger than that for the subtelomeric regions. Thus, binding of Sir proteins at the silent mating loci may be achieved at much less cellular concentration.

It is important to understand how deficiencies of Hsp82 as well as overexpression of Hsp82 both lead to derepression of TPE. Our results suggest that under overexpression condition the loss of TPE

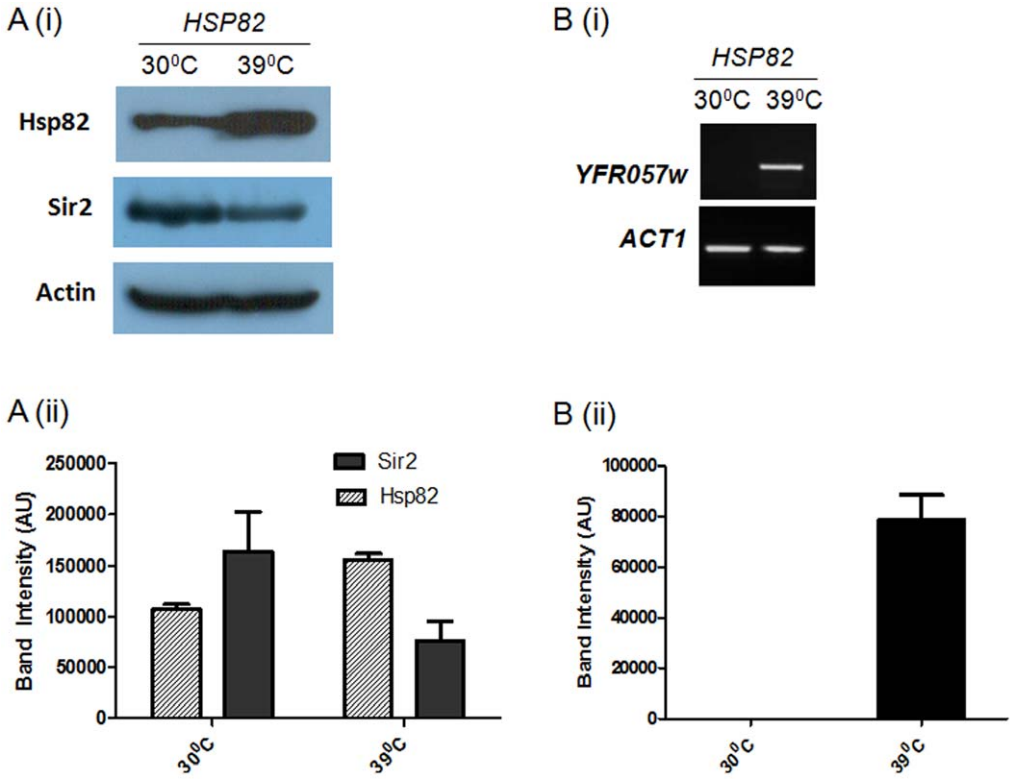

Figure 5. Heat shock induces derepression of transcriptional silencing at telomere. (Ai) The Western blot shows the relative levels of Hsp82p, Sir2 and Act1 in wild type strain at normal *vs*. heat shock condition. (Aii) Three independent experimental data are normalized with respect to Actin and plotted. Each bar represents mean density ± SD. (Bi) Heat shock induces the derepression of subtelomeric gene *YFR057w*, *ACT1* being the loading control. (Bii) Three independent experiments are performed and the data are plotted after normalizing with *ACT1*. Each bar represents mean density ± SD.
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activity is due to the reduced cellular pool of Sir2p; on the other hand in *Δhsp82Δhsc82* double mutant cell (*iG170Dhsp82*) the derepression of silencing is not only due to reduced level of Sir2p but could also be due to the presence of functionally inactive Sir2p. It appears that even the low abundance of active Sir2p (as low as $1/4^{th}$ of the usual amount) is good enough to ensure mating type silencing (as observed in SLY32 cells). The absence of *MATα2* transcript in *HSP82* overexpressing cells also confirms their mating ability. We reason that these cells are capable of mating type silencing, because they still possess wild type *HSC82* and *HSP82* genes due to which Sir2p maturation and functional activation are perhaps unaltered. Taking all these together, it is very tempting to propose that loss of TPE under overexpression condition and knockout condition could be mechanistically distinct. At present we do not know whether Hsp82 is directly controlling the cellular pool of Sir2p or it does so through a mediator.

Thirdly, our result suggests that Hsp82 over-expression leads to euchromatinisation of chromosome ends. The transitions between the two states of chromatin are very well studied in TPE model in budding yeast *S. cerevisiae* [18]. However, the reversible nature of such heterochromatin-euchromatin transition and *vice versa* is poorly understood. Although, the epigenetic marks (especially H3 and H4 modifications) associated with such changes are well established [37], the regulation at the DNA-protein interface is ill understood. It is known to a greater extent that how do the silencing proteins nucleate and then spread along the heterochromatin [38]. However, the molecular events occurring during the exit of the silencing complex from the heterochromatin giving rise to euchromatin formation is largely unknown. Findings from this work leads us to speculate that may be under *in vivo* condition Hsp82 is required to maintain a dynamic equilibrium of total amount of silencing proteins. This idea has been proven under stressed conditions such as heat shock that alter Hsp82 levels in the cells. We have exposed the cells to heat shock and under that conditions observed that total Sir2p abundance is also reduced by more than 50% which is associated with complete lose of silencing at telomere. Thus the adaptation in Sir2p abundance in response to the Hsp82 homeostasis could be responsible for the reversible nature of transcriptional silencing at telomere. We speculate that Hsp82 modulates interaction of silencing complex (Sir2-Sir3-Sir4) with DNA in such a way that it facilitates the removal (or degradation) of the major silencing proteins, once the chromatin does not need to be maintained at the heterochromatin state. Similar effect is also observed at telomere end, where Hsp82 acts as a releasing factor for Cdc13 to control the telomere protein dynamics, and maintains the extendable and unextentable states of telomere [13].

Finally, our genetic analysis shows that Hsp82 mediated maturation of Sir2 is independent of its cochaperone Sba1. This observation does not fit with classical steroid hormone receptor chaperone model, where Sba1 is found to be required at the last stages during client release. Thus, apart from traditional chaperonic role, newer roles of Hsp82 are emerging, where it acts as a regulator of various DNA protein interactions occurring inside the cell. Our report also provides evidence along the same line. Future studies need to be done to understand how Hsp82 actually regulates Sir2 level.

Supporting Information

Figure S1 Temperature sensitivity of *iG170Dhsp82*. (A) Growth kinetics of *iG170Dhsp82* at indicated temperatures. This strain shows slow growth phenotype at 37°C. (B) *iG170Dhsp82* cells, wild type cells (*HSP82*), tester strains YDS32 (*MAT*α) and YDS31 (*MATa*) all show comparable growth at 37°C on YPD plate.
(TIF)

Figure S2 *Δsir2* strain shows de-repression of telomere silencing at *YFR057w* locus. Semi quantitative RT-PCR shows increase in *YFR057w* transcript in *Δsir2* strain compared to the wild type strain (*SIR2*). *ACT1* transcript level remains comparable in both the strains.
(TIF)

Figure S3 Mating type frequency of wild type, *Δhsp82* and *Δhsc82*. Wild type (*HSP82*), *Δhsp82* and *Δhsc82* mating type frequencies show comparable values.
(TIF)

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Author Contributions

Conceived and designed the experiments: SB MKB. Performed the experiments: SL MKB RS. Analyzed the data: SB MKB. Contributed reagents/materials/analysis tools: SB MKB. Wrote the paper: SB.

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Heat Stress-Induced Cup9-Dependent Transcriptional Regulation of *SIR2*

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The epigenetic writer Sir2 maintains the heterochromatin state of chromosome in three chromosomal regions, namely, the silent mating type loci, telomeres, and the ribosomal DNA (rDNA). In this study, we demonstrated the mechanism by which Sir2 is regulated under heat stress. Our study reveals that a transient heat shock causes a drastic reduction in the *SIR2* transcript which results in sustained failure to initiate silencing for as long as 90 generations. Hsp82 overexpression, which is the usual outcome of heat shock treatment, leads to a similar downregulation of *SIR2* transcription. Using a series of genetic experiments, we have established that heat shock or Hsp82 overexpression causes upregulation of *CUP9* that, in turn, represses *SIR2* transcription by binding to its upstream activator sequence. We have mapped the *cis* regulatory element of *SIR2*. Our study shows that the deletion of *cup9* causes reversal of the Hsp82 overexpression phenotype and upregulation of *SIR2* expression in heat-induced Hsp82-overexpressing cells. On the other hand, we found that Cup9 overexpression represses *SIR2* transcription and leads to a failure in the establishment of heterochromatin. The results of our study highlight the mechanism by which environmental factors amend the epigenetic configuration of chromatin.

Increasing amounts of evidence suggest that environmental factors lead to stable alteration in gene expression by modifying chromatin structure. The genome of the lower eukaryote *Saccharomyces cerevisiae* uses histone acetylation-deacetylation as one of the epigenetic mechanisms to control gene expression. Histone deacetylases (HDAC) are the transcriptional repressors which cause deacetylation of histones, thereby creating localized regions of repressed chromatin. They are categorized into three groups based on their homology to yeast proteins: RPD3 (class I), HDA1 (class II), and Sir2 (class III) (1, 2). Sir2 deacetylates histone H3 (at K9, K14, and K56) and H4 (particularly K16) to regulate telomeric heterochromatin structure in yeast (3, 4). Our previous studies have demonstrated that heat stress and concomitant overexpression of Hsp90 result in euchromatinization of silent subtelomeric chromatin by reducing the steady-state level of Sir2 (5).

Sir2 protein mediates silencing at the silent mating-type loci HML and HMR, telomeres, and ribosomal DNA locus through a series of protein-protein interactions. During telomere silencing, Rap1 and a Ku70/80 heterodimer, which are telomere binding proteins, recruit the Sir2/Sir4 complex (6). Sir2p deacetylates neighboring nucleosomes and facilitates the binding of Sir4 and Sir3 to hypoacetylated H3 and H4 (7, 8). Sir3 and Sir4 recruit additional Sir2, and thus, the renewal of this cycle causes the spread of the Sir complex along the chromosome (9). The propagation of silencing complex along the chromosome requires the NAD^+-dependent histone deacetylase activity of Sir2. Mutation at the NAD^+ binding pocket of Sir2 makes it severely defective in telomere silencing (10). Nicotinamide (NAM), which is generated as the by-product of the enzymatic reaction, acts as a noncompetitive inhibitor of Sir2 (11). It has been demonstrated that *PNC1*, which codes for nicotinamidase, acts as a positive regulator of Sir2 activity by causing deamidation of NAM and thus increasing the replicative life span of yeast (12). Surprisingly, how *SIR2* gene is regulated at the transcription level under normal conditions or in response to different environmental cues is not understood at all.

Cup9 was originally identified as the gene that permits the cell to tolerate very high doses of copper, which are otherwise toxic. However, the mechanism of such an effect was not known (13). Published microarray experiments revealed that its transcription increases severalfold when cells are exposed to hypoxia and osmotic stress and when grown in the presence of alternate carbon sources (14, 15). Cup9 is a homeodomain transcriptional repressor having a high degree of identity with human PBX proteins (pre-B cell leukemia transcription factor) that are crucial for embryonic development (16). It also shows identity with the *S. cerevisiae MATα2* locus (17). About 36 targets of Cup9 have been documented so far, among which the best characterized is the master peptide transporter (dipeptide and tripeptide) *PTR2*. It is known that Cup9, along with the corepressors Tup1 and Ssn6 (18), reduces peptide import in cells by repressing *PTR2* transcription (19, 20).

Hsp90 is an evolutionarily conserved molecular chaperone found in organisms ranging from *Escherichia coli* (HtpG) to yeast (Hsc82 and Hsp82) to humans (Hsp90α and Hsp90β). It regulates diverse cellular functions by providing maturation to a specific group of proteins known as clients. A high-throughput evaluation by LUMIER assay (21) revealed that 60% of the Hsp90 clientele belongs to the kinase family, 30% belongs to the ubiqui-

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tin ligases, and about 7% are the transcription factors (22). Steroid hormone receptors are the most extensively characterized transcription factors that are chaperoned by Hsp90 (23). Unlike the other chaperones, such as Hsp70 and Hsp40, which act early in the folding process, the Hsp90 family interacts with the substrates at the later stages of protein folding (24, 25). It forms a multichaperone complex with Hop, Hip, Aha1, p23, CyP40, or FKBP and binds to the target proteins (26). Heat shock (HS) and other proteotoxic stresses trigger overexpression of Hsp90 due to the activation of the transcription factor Hsf1,which homotrimerizes and translocates to the nucleus from the cytoplasm and causes the transcriptional activation of Hsp90 (27, 28).

Previous experiments in our laboratory showed that Hsp82 (the yeast ortholog of Hsp90) homeostasis controls the abundance as well as activity of Sir2. The Hsp82 null condition not only leads to the reduced abundance of Sir2 but also results in the inactivation of Sir2 proteins. On the other hand, Hsp82 overexpression leads to the reduction of the steady-state level of Sir2 protein, though it does not affect the mating type silencing activity of Sir2 (5). In this report, we provide mechanistic insights into Hsp82 overexpression phenotype which occurs while cells are exposed to heat shock. Our work demonstrates that heat stress (or overexpression of Hsp82) leads to transcriptional downregulation of *SIR2*, which is inherited through successive generations before it returns to the normal level. Our work identified the transcriptional repressor Cup9, which negatively regulates *SIR2* gene expression. We demonstrate that under heat shock as well as Hsp82 overexpression, Cup9 is upregulated and is recruited at the $SIR2_{UAS}$ region. Thus, our work explains the mechanism behind the alteration of the epigenetic state of chromatin in response to environmental cues such as heat stress.

MATERIALS AND METHODS

Plasmids. The sequences of all the primers used in this study are given in Table 1. The reporter plasmid pCZ is a high-copy-number yeast expression vector having the *LACZ* reporter gene under the control of the *CYC1* promoter. Using SLY20 genomic DNA as a template, we amplified 429 bp, 370 bp, 307 bp, and 200 bp of *SIR2* upstream activator sequence ($SIR2_{UAS}$) and cloned them individually in a *LACZ* reporter plasmid, replacing its original *CYC1* promoter. The cloned vectors are referred to as 429_{UAS}, 370_{UAS}, 307_{UAS}, and 200_{UAS}, respectively, in this paper. We also made a vector by removing a built-in *CYC1* promoter to obtain a promoterless control; it is referred to as pCZdelcyc1.

Using SLY20 genomic DNA as a template, we amplified *CUP9* and cloned it in a 2μ C-terminally Myc-tagged vector, pESC-HIS (Agilent Technologies). This generated pESC-CUP9-MYC, which overexpresses *CUP9* under the control of the *GAL* promoter.

Yeast strains. Strains used in this study are listed in Table 2. 429_{UAS}, 370_{UAS}, 307_{UAS}, and 200_{UAS} deletion constructs along with the reporter plasmid without the *CYC1* promoter were transformed into strain SLY20 to generate isogenic strains SLY57, SLY84, SLY83, SLY56, and SLY64, respectively. The *HSP82* overexpression plasmid pRS313/HSP82 (5) was transformed into strains SLY20, SLY57, SLY84, SLY83, and SLY56, and colonies were selected on SC-his medium to generate isogenic strains SLY13, SLY61, SLY86, SLY85, and SLY60, respectively. The empty vector *pHCA* was also transformed into SLY20, SLY57, SLY84, SLY83, and SLY56, and colonies were selected on SC-his medium to generate isogenic strains SLY13C, SLY61C, SLY86C, SLY85C, and SLY60C, respectively.

Using plasmid pFA6a-TRP1 as a template (29), we amplified the *TRP1* cassette with *CUP9* flanking regions. The product was then integrated into SLY20 and selected on a medium lacking tryptophan to generate a Δ*cup9* strain, which is referred to as SLY71 in this paper. *HSP82* overexpression plasmid pRS313/HSP82 and the empty vector were transformed into SLY71 to generate SLY77 and SLY77C, respectively. Using the same strategy, we created Δ*sum1*, Δ*rim101*, and Δ*sok2* strains, which are referred to as SLY75, SLY73, and SLY74, respectively, in this paper. We transformed *HSP82* overexpression plasmid and the empty vector into SLY75, SLY73, and SLY74 to generate SLY81 and SLY81C, SLY79 and SLY79C, and SLY80 and SLY80C, respectively.

The *CUP9* overexpression plasmid pESC-CUP9-MYC and the empty vector pESC-MYC were transformed into SLY20 to create SLY90 and SLY91, respectively.

In order to Myc tag the C-terminal end of the Cup9 protein expressed from the chromosomal locus, a *13MYC-KanMX6* cassette (29) was amplified with regions flanking this portion of *CUP9*. It was then integrated into SLY20 to generate *CUP9 MYC*-tagged strain SLY87. *HSP82* overexpression plasmid pHCA/HSP82 was transformed into SLY87 to generate SLY88.

TPE color assay. The SLY20 strain used for the TPE color assay is isogenic to W303a, having *ADE2* marked at telomere VIIL. SLY13C, SLY12, SLY13, SLY77, SLY77C, SLY81, SLY79, SLY80, SLY90, and SLY91 cells were grown on appropriate medium, and the telomere position effect (TPE) assay was performed according to the protocol described previously (5).

Antibodies. The anti-Hsp90 antibody (Calbiochem) and antiactin antibody (Abcam) were used at a 1:5,000 dilution. The anti-Sir2 antibody (Santa Cruz Biotechnology Inc., CA) and anti-Myc antibody (Abcam) were used at 1:200 and 1:8,000 dilutions, respectively. Horseradish peroxidase (HRP)-conjugated rabbit IgG (Santa Cruz Biotechnology Inc.) was used as a secondary antibody for Sir2 and Myc at a 1:10,000 dilution, and HRP-conjugated mouse IgG (Promega) was used as a secondary antibody for Hsp82 and actin at a 1:10,000 dilution. A chemiluminescence detection system (Pierce) was used to develop Western blots.

RNA isolation and real-time RT-PCR. Total RNA was isolated by the acid-phenol method as described in our earlier paper (5). For real-time PCR, cDNA was diluted (1:50) and used for PCR using a reverse transcription-PCR (RT-PCR) kit (Roche). The real-time analysis was done using the Applied Biosystems 7500 fast real-time PCR system. Primers used for the amplification of 200- to 300-bp stretches at the portions of *ACT1*, *SIR2*, *LACZ*, *CUP9*, *YFR067w*, and *HML*α corresponding to the C terminus are listed in Table 1. The threshold cycle (C_T) value of the *ACT1* transcript of each sample was used to normalize the corresponding C_T values of *SIR2*, *LACZ*, *CUP9*, *YFR057w*, and *HML*α transcripts. The normalized C_T values of *SIR2*, *LACZ*, *CUP9*, *YFR057w*, and *HML*α from different samples were compared to obtain ΔC_T values. The relative levels of mRNA were estimated as $2^{-\Delta\Delta CT}$. The mean values (±standard deviations [SD]) from three independent experiments were plotted using GraphPad Prism 6 software.

ChIP assay. The chromatin immunoprecipitation (ChIP) assay was performed as described previously (30), with some modifications. A 50-ml quantity of cells was grown to an optical density at 600 nm (OD_{600}) of 1.2 and cross-linked with 1% formaldehyde at 30°C for 15 min. Glycine at 2.5 M was added, and the cells were shaken for 5 min before being spun down and washed with PBS buffer (10 mM KH_2PO_4, 40 mM K_2HPO_4, 150 mM NaCl) containing dithiothreitol (DTT). The cells were then suspended in 2 ml of spheroplast buffer (18.2% sorbitol, 1% glucose, 0.2% yeast nitrogen base, 0.2% Casamino Acids, 25 mM HEPES [pH 7.4], 50 mM Tris, 1 mM dithiothreitol) along with 0.8 mg of lyticase and incubated at 30°C for 30 min to generate spheroplasts. The spheroplasts were first washed in 500 μl of ice-cold PBS buffer containing phenylmethylsulfonyl fluoride (PMSF). Then they were resuspended in HEPES–Triton X-100 buffer (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5]) containing 0.5 mM PMSF and protease inhibitor cocktail (Roche) and spun down at 7,000 rpm for 7 min. Ultimately, the spheroplasts were resuspended in HEPES–NaCl buffer (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5]) containing 0.5 mM PMSF and protease inhibitor cocktail and again centrifuged at 7,000 rpm

TABLE 1 Primers used in this study

Primer name	Sequence	Purpose
OSB 125	5′ ATC CTC GAG CTG CAA CTC CTC AAT GTG TC 3′	Forward primer used to amplify 429-bp $SIR2_{UAS}$
OSB 193	5′ ATC CTC GAG GTA TAT GCT TAT ATG CAT GCG 3′	Forward primer used to amplify 370-bp $SIR2_{UAS}$
OSB 194	5′ATC CTC GAG CCA AGC TAC ATC TAG CAC TC 3′	Forward primer used to amplify 307-bp $SIR2_{UAS}$
OSB 126	5′ ATC CTC GAG CTT TGG CCG CCA GTT GCG 3′	Forward primer used to amplify 200-bp $SIR2_{UAS}$
OSB 87	5′ ATC GGA TCC GGT CAT CCA GCT TTA ATG TGC CG 3′	Common reverse primer used to amplify all above deletion constructs of $SIR2_{UAS}$
OSB 203	5′ GAC GGA TCC ATG AAT TAT AAC TGC GAA ATA C 3′	Forward primer used to amplify *CUP9* for cloning in pESC-MYC-tagged vector
OSB 204	5′ CGA GTC GAC ATT CAT ATC AGG GTT GGA TAG 3′	Reverse primer used to amplify *CUP9* for cloning in pESC-MYC-tagged vector
OSB 164	5′ CTT TTA TGC TAA CAA CCT TCG AGA ATA GTT ACA TTC GAA GCG GAT CCC CGG GTT AAT TAA 3′	Forward primer used for *CUP9* knockout
OSB 165	5′ TAT AAT TAT ATG AAT ATT TAA GTA ATG CAT TGA TAA GTG AGA ATT CGA GCT CGT TTA AAC 3′	Reverse primer used for *CUP9* knockout
OSB 170	5′ AAG TTT CAT ACA TAA TTA ACA AAA TTC GTT TGT TGC GGG GCG GAT CCC CGG GTT AAT TAA 3′	Forward primer used for *SUM1* knockout
OSB 171	5′ TTT TAT CTA TTC TCG AAA CTG CCC CAA CGT ACG GAC CAG CGA ATT CGA GCT CGT TTA AAC 3′	Reverse primer used for *SUM1* knockout
OSB 173	5′ ACT GAA AAC GGT AAA GTA GGT TTG TTT AAA TTG ACT TAA GCG GAT CCC CGG GTT AAT TAA 3′	Forward primer used for *RIM101* knockout
OSB 174	5′ GCA AAG AAA CAA CTA AGA ATA AAA TAT CCG ACA ATC CAT AGA ATT CGA GCT CGT TTA AAC 3′	Reverse primer used for *RIM101* knockout
OSB 161	5′ CAA AAT CAT CCT TAT ATA ACC CTG GTA AGG TCC TTT TGT CCG GAT CCC CGG GTT AAT TAA 3′	Forward primer used for *SOK2* knockout
OSB 162	5′ GAT TAA AGT AAC ATA ATT ATC CAA GGA ATT CAT AGT TGT TGA ATT CGA GCT CGT TTA AAC 3′	Reverse primer used for *SOK2* knockout
OSB 196	5′ GCT GGA AGA ATT GAA AAA GCT ATC CAA CCC TGA TAT GAA TCG GAT CCC CGG GTT AAT TAA 3′	Forward primer used to generate *MYC* tag at the portion of *CUP9* corresponding to the C terminus at the chromosomal locus
OSB 197	5′ TAT AAT TAT ATG AAT ATT TAA GTA ATG CAT TGA TAA GTG AGA ATT CGA GCT CGT TTA AAC 3′	Reverse primer used to generate *MYC* tag at the portion of *CUP9* corresponding to the C terminus at the chromosomal locus
OSB 16	5′ TGA CCA AAC TAC TTA CAA CTC C 3′	Forward primer used to amplify *ACT1* for real-time RT-PCR
OSB 14	5′ TTA GAA ACA CTT GTG GTG AAC G 3′	Reverse primer used to amplify *ACT1* for real-time RT-PCR
OSB 131	5′ CTG ATT AAT CGT GAT CCC GTC 3′	Forward primer used to amplify *SIR2* for real-time RT-PCR
OSB 132	5′ CTT AGA GGG TTT TGG GAT GTT C 3′	Reverse primer used to amplify *SIR2* for real-time RT-PCR
OSB 121	5′ CAA CTG ATG GAA ACC AGC C 3′	Forward primer used to amplify *LACZ* for real-time RT-PCR
OSB 122	5′ TTA CGC GAA ATA CGG GCA G 3′	Reverse primer used to amplify *LACZ* for real-time RT-PCR
OSB 189	5′ CTA ATG ACA ACG CGA ATA ATA C 3′	Forward primer used to amplify *CUP9* for real-time RT-PCR
OSB 190	5′ CAA TTC ATA TCA GGG TTG GAT AG 3′	Reverse primer used to amplify *CUP9* for real-time RT-PCR
OSB 19	5′ ATC ACG AGT AAG GAT CAA AG 3′	Forward primer used to amplify *YFR057w* for real-time RT-PCR
OSB 20	5′ TTA TGG CTT TGT TAC GCT TG 3′	Reverse primer used to amplify *YFR057w* for real-time RT-PCR
OSB 62	5′ AAT CGG CGG ATG GGT TGG 3′	Forward primer used to amplify *HMLα* for real-time RT-PCR
OSB 63	5′ TCA TTC TTT CTT CTT TGC CAG 3′	Reverse primer used to amplify *HMLα* for real-time RT-PCR

for 7 min. Finally, the spheroplasts were resuspended in 100 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris [pH 8.1]) containing 0.5 mM PMSF and protease cocktail inhibitor and sonicated (Elma; model-S-60H) to generate an average DNA fragment size of 0.5 to 1 kb. After centrifugation, approximately 1.1 ml of supernatant was added to 1 ml of IP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris [pH 8.1], 167 mM NaCl, 0.5 mM PMSF, and protease cocktail inhibitor) and left on ice for 15 min to form the chromatin fraction. Immunoprecipitation was performed with 1 μg of anti-Myc antibody to precipitate Cup9. $SIR2_{UAS}$ was amplified using the primers OSB 125 and OSB 87 in a reaction volume of 50 μl using 1/75 of immunoprecipitates and 1/50 of input DNA. Samples were subjected to electrophoresis on 1.5% agarose. Cup9 binding was also measured at the *ACT1* locus using the primers OSB 16 and OSB 14. The control antibody for ChIP was rabbit IgG.

Southern hybridization. Telomere Southern blotting was carried out according to the protocol published earlier (31). Briefly, yeast cells were grown in yeast extract-peptone-dextrose (YPD) medium to a density of 1.5×10^7 cells per milliliter. Heat shock was carried out by exposing the cells to 39°C for 40 min and subsequently returning them to 30°C and then growing them for 7 days. The genomic DNA was isolated from the control as well as from the post-HS samples collected after 2 h and those collected on the 4th and 6th days. Equal amounts of genomic DNA from each sample were subjected to XhoI digestion. Subsequently, all the digested samples were electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane. For probe preparation, a 625-bp poly(G·T)/poly(C·A) fragment (kindly provided by Arthur Lustig) was labeled with [α-^{32}P]dCTP using a Deca label DNA labeling kit (Fermentas). The Southern blot was finally exposed to X-ray film and developed.

RESULTS

HS or Hsp82 overexpression induces transcriptional downregulation of *SIR2*. In an earlier study (5), we had established that when cells are exposed to heat shock (HS) (at 39°C for 40 min), the

TABLE 2 Yeast strains used in this study

Strain	Genotype	Reference or source
SLY20	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2*	5
SLY12	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sir2::KAN*^{r}	5
SLY13C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pHCA	This study
SLY13	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pHCA/HSP82	5
SLY56	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/200$_{UAS}$	This study
SLY57	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/429$_{UAS}$	This study
SLY60C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/200$_{UAS}$, pHCA	This study
SLY60	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/200$_{UAS}$, pHCA/HSP82	This study
SLY61C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/429$_{UAS}$, pHCA	This study
SLY61	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/429$_{UAS}$, pHCA/HSP82	This study
SLY64	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZdelcyc1	This study
SLY71	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 cup9::TRP1*	This study
SLY74	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sok2::TRP1*	This study
SLY73	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 rim101::TRP1*	This study
SLY75	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sum1::TRP1*	This study
SLY77C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 cup9::TRP1* pHCA	This study
SLY77	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 cup9::TRP1* pHCA/HSP82	This study
SLY80C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sok2::TRP1* pHCA	This study
SLY80	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sok2::TRP1* pHCA/HSP82	This study
SLY79C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 rim101::TRP1* pRS313	This study
SLY79	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 rim101::TRP1* pRS313/HSP82	This study
SLY81C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sum1::TRP1* pRS313	This study
SLY81	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 sum1::TRP1* pRS313/HSP82	This study
SLY83	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/307$_{UAS}$	This study
SLY84	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/370$_{UAS}$	This study
SLY85C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/307$_{UAS}$, pHCA	This study
SLY85	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/307$_{UAS}$, pHCA/HSP82	This study
SLY86C	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/370$_{UAS}$, pHCA	This study
SLY86	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pCZ/370$_{UAS}$, pHCA/HSP82	This study
SLY87	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 CUP9-13MYC-KANMX6*	This study
SLY88	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2 CUP9-13MYC-KANMX6* pHCA/HSP82	This study
SLY90	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pESC/CUP9MYC	This study
SLY91	*MAT***a** *leu2-3,112 trp1 ura3-1 ade2-1 his3-11,15 VIIL::ADE2* pESC(MYC)	This study

total cellular pool of Sir2 was reduced considerably. Similarly, Hsp82 overexpression, a general phenomenon associated with heat shock response, also caused drastic reduction in Sir2p in a dose-dependent manner. To investigate whether the reduction of Sir2p is merely at the protein level or extends to the transcript level as well, we analyzed *SIR2* mRNA under heat stress. To this end, we exposed SLY20 cells to 39°C for 40 min, a condition that yields overexpression of Hsp82, and compared the level of *SIR2* mRNA with that of the wild-type (WT) cells. We also transformed the cells with an Hsp82 overexpression plasmid (centromeric expression vector) (SLY13) and compared the level of *SIR2* mRNA with that of the cells containing the empty expression vector (SLY13C). Under both conditions, we measured the levels of Hsp82 protein, which were higher than that of the control cells (Fig. 1A, bottom). The semiquantitative RT-PCR showed that Hsp82 overexpression (artificially or under heat stress) downregulated the level of *SIR2* (Fig. 1A). Quantitative analysis by real-time RT-PCR revealed about a 5-fold reduction in the *SIR2* transcript under the heat shock/Hsp82 overexpression condition compared to that in the control cells (Fig. 1B).

Transient heat shock leads to transgenerational transmission of derepressed subtelomeric chromatin. We focused on the effects of HS over multiple generations. To this end, we exposed cells of the SLY20 strain to heat stress at 39°C for 40 min and

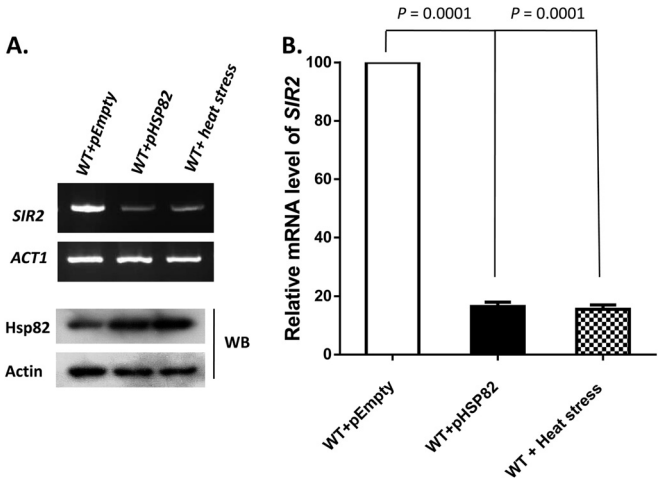

FIG 1 Heat shock or Hsp82 overexpression induces transcriptional downregulation of *SIR2*. (A) (Top) Semiquantitative RT-PCR shows the *SIR2* transcript in cells exposed to heat shock (39°C for 40 min) and in cells harboring an Hsp82 overexpression vector compared to that present in wild-type cells. (Bottom) Western blotting (WB) was done with anti-Hsp82 and antiactin antibodies. (B) The relative mRNA levels of *SIR2* under the above-mentioned conditions (indicated on the *x* axis) were plotted after normalization with *ACT1* mRNA. In each case, the mean value (±SD) from three independent experiments with three independent harvests of cells was calculated and was plotted using GraphPad Prism6 software. *P* values were calculated using the two-tailed Student *t* test.

subsequently returned them to 30°C. These heat-stressed cells were maintained for several generations (up to 10 days), with regular medium changes every 24 h. In parallel, we maintained a wild-type culture which was not subjected to heat shock. We collected the total RNA before HS (0 h), 2 h post-HS, and thereafter at an interval of every 24 h. We repeated the semiquantitative RT-PCR three times with independent harvests of cells; the results of one of the representative experiments are presented in Fig. 2A. Our results showed that HS-mediated reduction in the *SIR2* transcript continued through successive generations. The *SIR2* transcript was barely observed in the 4th- and 6th-day HS samples. However, it started to return to a level comparable to that of the unstressed cells on the 7th day post-HS. The real-time RT-PCR result showed that the relative level of *SIR2* mRNA had been reduced to nearly 25% and 15% in the 4th-day and 6th-day HS cultures, respectively. However, in the 7th-day HS culture, the *SIR2* transcript level was comparable to that of the control (Fig. 2B). Our observation was supported by Western blotting with anti-Hsp82 and anti-Sir2 antibodies. Our results indicated that Hsp82 overexpression under heat stress led to a significant reduction in Sir2p in the 2 h and the 4th day post-HS. It is important to note that in the 7th-day HS sample, the Sir2p level went back to that of the unstressed cells, and there was a significant reduction of Hsp82 (Fig. 2C). The quantification of the relative band intensities from three independent experiments showed that the Hsp82p level increased 2.2 times in the 2 h post-HS, remained 2.1 times higher in the 4 days post-HS than the level of control, and returned to the level of the control after 7 days (Fig. 2D). Similarly, the relative band intensity of Sir2 was reduced by half in the 2 h post-HS and remained at that level up to 4 days and then returned to the level of the control cells on the 7th day (Fig. 2E). Our previous work showed that Hsp82 overexpression leads to derepression of telomere silencing, without any change in mating type silencing in yeast (5). We wanted to investigate whether a transient heat shock leads to any transgenerational mating type silencing defect in yeast. To that end, we monitored the *HML*α transcript at various time intervals in the post-HS sample and compared it to that of the control cells, which were never exposed to heat shock. The real-time RT-PCR showed that the relative levels of *HML*α in the 2-h post-HS sample were slightly higher than those in the control cells; however, they increased significantly (5 times) on the 4th day post-HS and ultimately returned to the normal level on the 7th day (Fig. 2F). To test the telomeric silencing activity of Sir2 in HS samples, we measured the transcription of subtelomeric gene *YFR057w* by real-time RT-PCR analysis. Under normal conditions, Sir2 represses the transcription of *YFR057w* by spreading through the subtelomeric ends of chromosome. Our results showed that there was no significant change in the relative mRNA level of *YFR057w* in the 2-h post-HS sample compared to that of the control. However, in the 4th-, 6th-, and 7th-day post-HS samples, the *YFR057w* transcript had increased 4.5-fold, 3.6-fold, and 3-fold, respectively, and it was repressed again from the 8th day onwards (Fig. 2G). We measured the growth of control and HS culture for 7 days, and the kinetics showed that they were dividing at the same rate (Fig. 2H).

It was previously reported that prolonged heat shock (at 37°C) causes telomere shortening in yeast (32, 33). To understand whether telomere shortening is responsible for the derepression of subtelomeric chromatin, we wanted to find out whether transient heat shock also leads to such changes in the telomere structure. For that purpose, we monitored the length of the telomere for 7 days after a transient heat shock. We performed three independent experiments, and our results showed that transient heat shock leads to shortening of telomere length (Fig. 3). Telomere length remained short up to the 4th day and then returned to the wild-type length.

Mapping the *cis* regulatory region of Sir2 affected by Hsp82 overexpression. To further characterize the transcriptional repression of *SIR2*, we cloned the upstream regulatory element of *SIR2* (−429 to −1) into a reporter plasmid carrying *LACZ* and named it 429_{UAS} (Fig. 4A). Similarly, three more constructs (370_{UAS}, 307_{UAS}, and 200_{UAS}) which spanned across the $SIR2_{UAS}$ (−370 to −1, −307 to −1, and −200 to −1) were generated, and they were individually cloned as the exclusive promoter region of the *LACZ* expression cassette. To provide a negative control, we generated a reporter plasmid without any promoter. Eventually all the constructs were transformed into SLY20 to generate isogenic strains SLY57 (429_{UAS}), SLY84 (370_{UAS}), SLY83 (307_{UAS}), SLY56 (200_{UAS}), and SLY64 (negative control), respectively. Expression of the *LACZ* gene from these constructs directly correlated with the transcriptional activity of different regions of the *SIR2* upstream regulatory element.

To test whether $SIR2_{UAS}$ is affected by Hsp82 overexpression, we compared *LACZ* transcription of 429_{UAS} in the presence and the absence of Hsp82 overexpression. Semiquantitative RT-PCR revealed that overexpression of Hsp82 resulted in a significant reduction of the *LACZ* transcript in the strain carrying both 429_{UAS} and pHSP82 (SLY61) compared to that in the strain carrying 429_{UAS} and pEmpty (SLY61C) (Fig. 4B). Western blotting (Fig. 4B, bottom) confirmed overexpression of Hsp82 in SLY61 compared to SLY61C. Quantitative analysis by real-time RT-PCR revealed that the *LACZ* transcript was reduced 2.4-fold under the Hsp82 overexpression condition compared to the wild type (Fig. 4C). In order to find the repressor binding site in $SIR2_{UAS}$, we quantified the relative levels of *LACZ* mRNA in cells harboring various deletion constructs. The transcription of *LACZ* in SLY64 was considered the baseline. We found about an 80-fold increase in *LACZ* in the cells carrying full-length (429_{UAS}) upstream activator sequence of *SIR2* compared to that of the negative control (no promoter). However, *LACZ* transcription further increased (7-fold) in 370_{UAS} and in 307_{UAS} (Fig. 4D). This provides evidence of a repressor binding site in the region spanning bp −429 to −369 of $SIR2_{UAS}$. 200_{UAS}, however, displayed about a 4-fold reduction in *LACZ* transcription compared to that of 429_{UAS}. Next, we aimed to narrow down the *cis* regulatory element of *SIR2* that is regulated via heat stress. For this, we mimicked the heat shock condition by transforming an Hsp82 overexpression plasmid in the cells carrying individual constructs. As a control, we transformed the empty plasmid in cells carrying different *LACZ* fusion constructs. Real-time RT-PCR data showed about a 2-fold reduction in *LACZ* transcription in cells carrying 429_{UAS} along with Hsp82 overexpression (Fig. 4E). However, Hsp82 overexpression in 370_{UAS}, 307_{UAS}, and 200_{UAS} did not affect the relative mRNA level of *LACZ* compared to that of cells having the empty plasmid. Western blotting in each of the four fusion constructs showed overexpression of Hsp82 (Fig. 4E, bottom). Together, these data suggest that the region spanning bp −429 to −369 of *SIR2* is crucial for its regulation during Hsp82 overexpression.

Bioinformatics prediction of transcription factor binding to $SIR2_{UAS}$. We analyzed the 429 bp upstream of *SIR2* regulatory

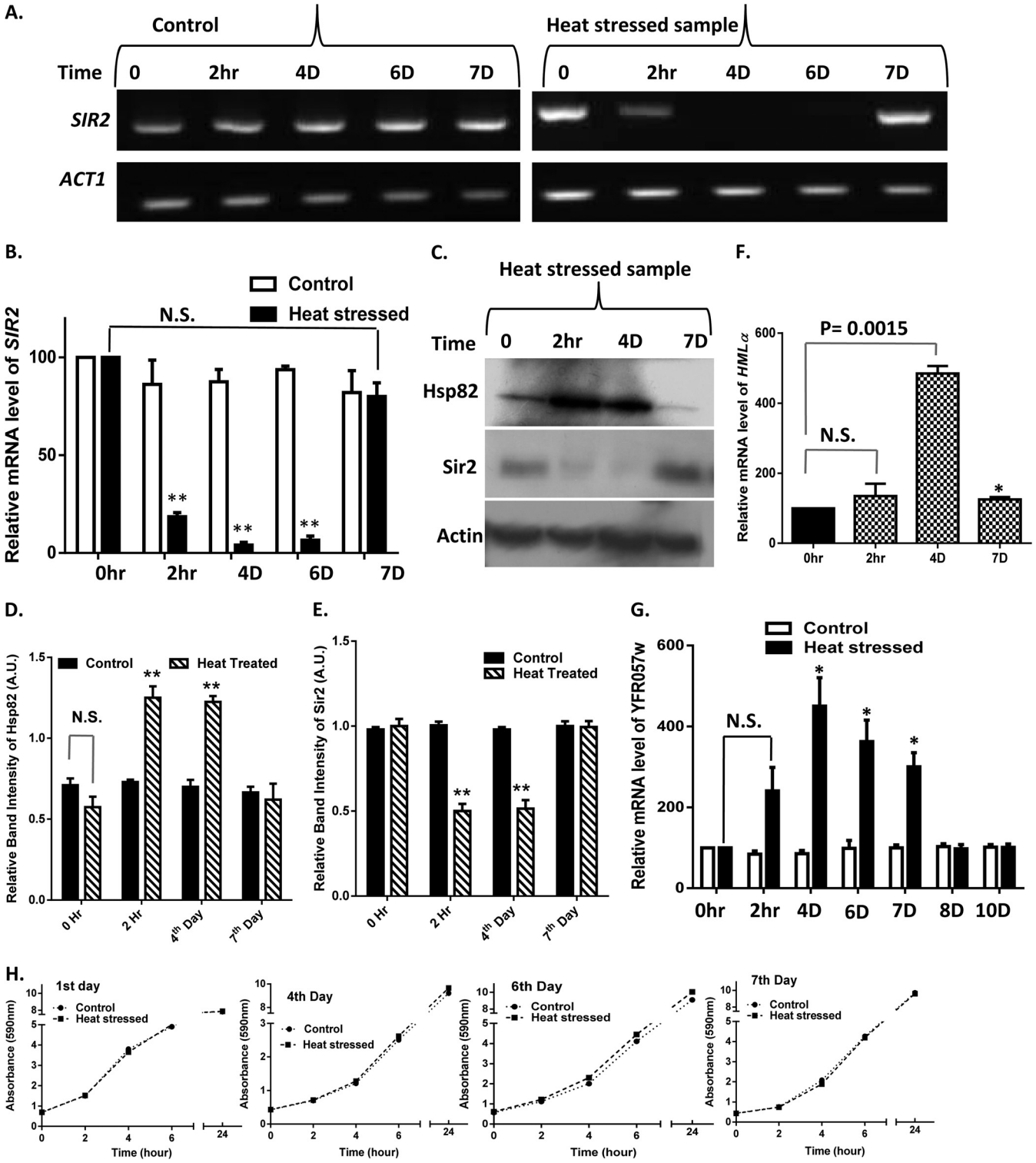

FIG 2 Transient heat shock leads to transgenerational transmission of derepressed subtelomeric chromatin. (A) Wild-type cells were exposed to heat shock (39°C) for a period of 40 min and then returned to 30°C. They were allowed to grow for 10 days as described in Results. The *SIR2* transcript profile was monitored after 2 h and on the 4th, 6th, and 7th days and compared with that of the control cells, which were not exposed to heat shock. The experiment was repeated three times; the results from one representative semiquantitative RT-PCR are presented. (B) Relative mRNA levels of *SIR2* in normal and heat-stressed cells at different time points (as indicated on the *x* axis) were plotted. Error bars indicate SD ($n = 3$ experiments); asterisks indicate values significantly different from the control, as follows: **, $P < 0.01$, and *, $P < 0.05$. N.S., not significant. *ACT1* was used as the normalization control. (C) A Western blot was developed with control and heat-stressed samples at different time intervals using anti-Sir2, anti-Hsp82, and antiactin antibodies. (D) Densitometric measurements of Hsp82 from three independent Western blots were plotted for control and heat-treated samples at the indicated time points. Error bars indicate SD. (E) Densitometric measurements of Sir2 from three independent Western blots were plotted with control (before heat shock) and heat-treated samples at the indicated time points. Error bars indicate SD. (F) Relative mRNA levels of *HMLα* in *MAT***a** haploids before and after heat shock at the time points given in the *x* axis are plotted. Error bars indicate SD ($n = 3$). *, $P < 0.05$. (G) Relative mRNA levels of *YFR057w* in wild-type cells and cells exposed to heat shock (39°C for 40 min) at different time points are shown. Error bars indicate SD ($n = 3$). *, $P < 0.05$. (H) Growth kinetics of wild-type and heat-stressed cells were monitored for 7 days. The graph represents a comparison between their growths on the 1st, 4th, 6th, and 7th days.

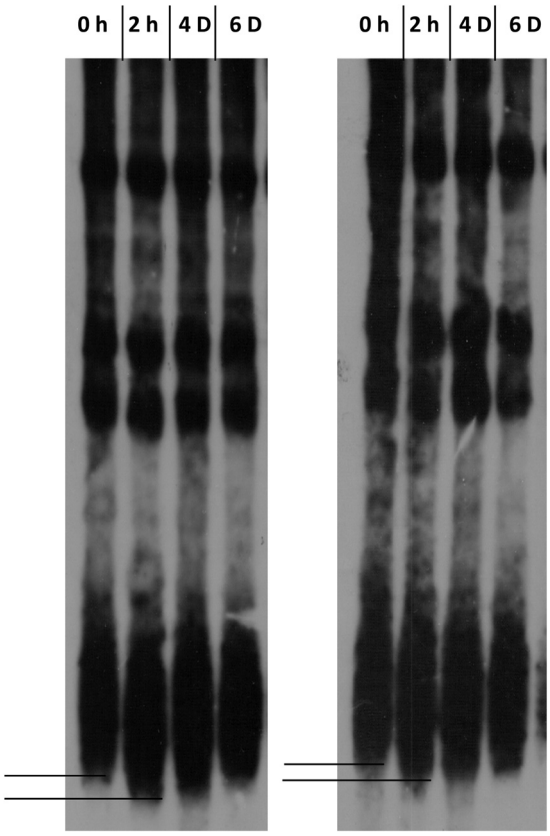

FIG 3 Transient heat shock leads to telomere shortening in wild-type cells. Strain SLY20 was subjected to heat shock at 39°C for 40 min and then was grown at 30°C for 6 days. Genomic DNA was isolated at different time intervals (as marked at the top) and subjected to XhoI digestion, and telomere length was measured using Southern blot hybridization. The experiment was repeated with three independent colonies; two telomere blots are represented. The difference between the lengths of telomeres grown at 30°C and the 2-h post-HS sample is represented by solid lines.

region for transcription factor binding sites of *Saccharomyces cerevisiae*. The analysis for finding transcription factors was performed by the statistical method (34) employed in the widely used TRAP (transcription factor affinity prediction) web tool (35). TRAP was developed to predict transcription factor binding affinities to DNA. Based on the analysis with TRAP, eight transcription factors were found to have high binding affinity, namely, Cup9, Rim101, Sok2, Tod6, Phd1, Tec1, Dot6, and Sum1, out of which four are transcriptional repressors. The transcription factors from both the databases TRANSFAC (36) and JASPAR (37), with their ranks, are shown in Table 3. From the data, it is evident that Cup9 shows highest binding affinity (*P* value, 0.007). Its binding sequence belongs to the region from −411 to −402.

Reversal of the heat shock/Hsp82 overexpression phenotype in *cup9* deletion strain. Based on the bioinformatics analysis, we characterized all four repressors, namely, Cup9, Sum1, Rim101, and Sok2, to identify the putative repressor of *SIR2* transcription. We constructed four deletion strains, namely, Δ*cup9*, Δ*rim101*, Δ*sok2*, and Δ*sum1* mutants, and screened each of them using various genetic experiments in an Hsp82 overexpression background. Semiquantitative RT-PCR showed no significant reduction in *SIR2* transcript in the Δ*cup9* strain (SLY77C) carrying the empty vector or the Hsp82 overexpression plasmid (SLY77) (Fig. 5A). On the other hand, the Δ*sok2*, Δ*sum1*, and Δ*rim101* strains displayed significant reductions in *SIR2* transcription upon Hsp82 overexpression. Real-time RT-PCR also displayed no significant reduction in relative mRNA levels of *SIR2* in the Δ*cup9* strain with and without the *HSP82* overexpression plasmid (Fig. 5B). However, other deletion strains exhibited considerable reductions in the *SIR2* transcript with *HSP82* overexpression similar to that in the wild type. By Western blotting, we observed the presence of a comparable amount of Sir2p in the *cup9* deletion strain harboring the Hsp82 overexpression plasmid and that having an empty vector (Fig. 5C). However, Sir2p was considerably reduced under Hsp82 overexpression in Δ*sum1*, Δ*rim101*, and Δ*sok2* cells compared to those carrying the empty plasmid. Relative band intensity (with respect to actin) revealed about a 50% reduction of Sir2p in the Δ*sum1*, Δ*rim101*, and Δ*sok2* strains carrying the Hsp82 overexpression plasmid, but it remained unaltered in the Δ*cup9* strain with Hsp82 overexpression (Fig. 5D). These data are indicative of Cup9 being the mediator through which Hsp82 regulates *SIR2* transcription. We wanted to monitor which of the deletion strains abrogate Hsp82 overexpression-mediated derepression of subtelomeric genes. With this in mind, we performed two independent functional assays of Sir2 using two different subtelomeric genes. First, we monitored the Sir2 function by a color assay scoring subtelomeric *ADE2* expression. The Δ*cup9*, Δ*sum1*, Δ*rim101*, and Δ*sok2* strains were generated in an isogenic background of SLY20 in which the telomere region of chromosome VIIL was marked with *ADE2*. The wild-type and Δ*cup9* strains both showed a pink color phenotype due to the silencing of the *ADE2* gene, whereas the Δ*sir2* strain exhibited a white color phenotype correlating with derepression of *ADE2*. However, Hsp82 overexpression in the Δ*cup9* strain retained the pink color phenotype, as opposed to the white color phenotype observed during Hsp82 overexpression in the WT as well as in the Δ*sum1*, Δ*rim101*, and Δ*sok2* strains (Fig. 5E). In order to understand whether the maintenance of heterochromatinization is locus specific or not, we compared the levels of the *YFR057w* transcript, which is located near the chromosome VIR telomere. Our data showed that although Hsp82 overexpression caused derepression of *YFR057w* in wild-type cells, the Δ*cup9* strain did not display any silencing defect, as *YFR057w* remained silent even in the presence of Hsp82 overexpression (Fig. 5F). To score the silencing activity of Sir2p in a more quantitative manner, we performed real-time RT-PCR analysis, which showed no significant alteration in the *YFR057w* transcript in Δ*cup9* cells with and without Hsp82 overexpression (Fig. 5G). Our results imply that out of the four transcriptional repressors, deletion of only *CUP9* restores wild-type-like Sir2 function under the Hsp82 overexpression condition. In other words, we observed increased expression of Sir2p specifically in the *cup9* knockout strain during Hsp82 overexpression, which correlated well with the maintenance of Sir2 silencing function.

Cup9 expression and its binding to $SIR2_{UAS}$ are enhanced by heat shock and Hsp82 overexpression. From the previous experiment, it was apparent that Hsp82-mediated transcriptional downregulation of Sir2 is dependent on Cup9. That led us to estimate the steady-state level of Cup9 under heat stress. We grew the cells at three different temperatures, 30°C, 37°C, and 39°C, and observed that the level of the *CUP9* transcript was upregulated at 39°C (Fig. 6A). Real-time RT-PCR data showed that there was no change in the *CUP9* transcript with an increase in temperature

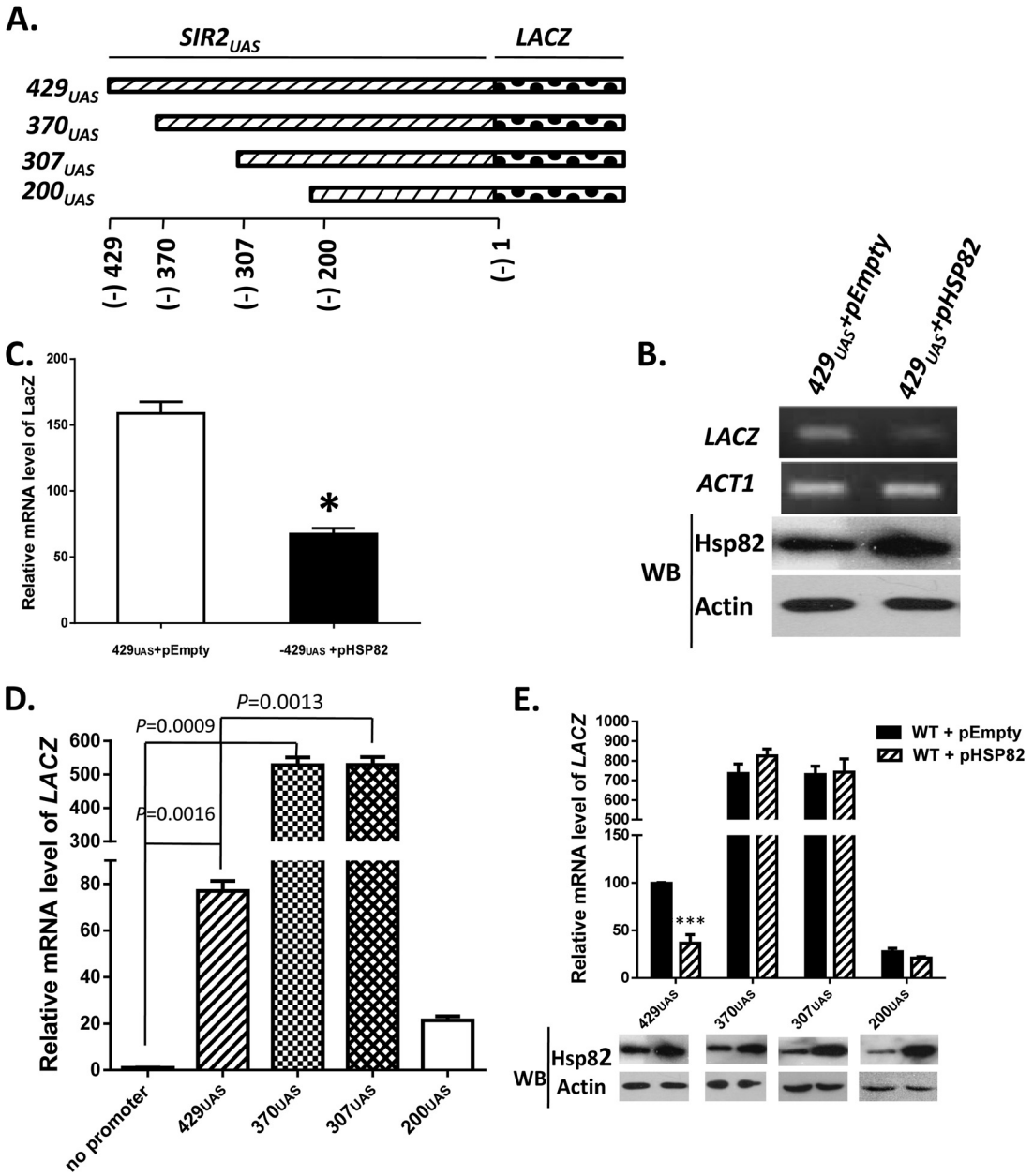

FIG 4 Mapping of the *cis* regulatory region of Sir2 that is affected by Hsp82 overexpression. (A) Upstream activator sequences of *SIR2*, 429, 370, 307, and 200 bp, were cloned in the upstream region of *LACZ* to generate four reporter plasmids, namely, 429_{UAS}, 370_{UAS}, 307_{UAS}, and 200_{UAS}. (B) (Top) 429_{UAS} reporter plasmid was transformed into wild-type cells and cells harboring overexpressing Hsp82. Semiquantitative RT-PCR shows the relative levels of the *LACZ* transcript between wild-type cells and cell harboring Hsp82 overexpression plasmid. (Bottom) Western blotting was done with anti-Hsp82 and antiactin antibodies. (C) Real-time RT-PCR shows relative levels of *LACZ* mRNA between wild-type cells and cells bearing the Hsp82 overexpression plasmid. Error bars indicate SD ($n = 3$ experiments); the asterisk indicates a value significantly different from the control ($P < 0.05$). (D) A *LACZ* reporter plasmid without any promoter was transformed into wild-type cells and included in the experiment. Real-time RT-PCR was used to compare the relative mRNA levels of *LACZ* in cells harboring four different reporter plasmids along with cells having no promoter. *P* values were calculated using the two-tailed Student *t* test. (E) (Top) In cells with plasmids bearing each of the four reporter constructs, Hsp82 overexpression plasmid was transformed. Real-time RT-PCR shows how the relative abundance of *LACZ* was affected in the presence of the *HSP82* overexpression plasmid. The relative abundances of mRNA from these constructs were plotted after normalization against *ACT1* mRNA. Each bar represents mean mRNA level (±SD) from three independent experiments. ***, $P < 0.001$. (Bottom) Western blotting was done using anti-Hsp82 and antiactin antibodies.

from 30°C to 37°C. However, at 39°C, *CUP9* was upregulated 2.5-fold (Fig. 6B). We also monitored the level of the *CUP9* transcript under the Hsp82 overexpression condition and observed that it had significantly increased compared to that of the wild type (Fig. 6C). Real-time RT-PCR analysis showed more than 2-fold upregulation of the *CUP9* transcript under the Hsp82 overexpression condition (Fig. 6D). Our observation was further corroborated by the endogenous level of Cup9 protein under the Hsp82 overexpression condition. We tagged *CUP9* with *MYC* at the chromosomal locus. Western blot analysis showed very low levels

TABLE 3 Transcription factors from both of the databases JASPAR and TRANSFAC

Serial no.	Rank	Matrix no.	Name of position-specific matrix in databases[a]	*P* value[b]	Sequence[c]
1	1	M01549	F$CUP9_01	0.00742	TCCTCAATGTGTCAATTAAC
	2	MA0288.1	CUP9	0.00791	AATGTGTCA
2	3	M01030	F$RIM101_01	0.0176	CCAAGCTA
3	4	M01621	F$SOK2_01	0.0278	GCCTGCAACT
	5	MA0385.1	SOK2	0.0329	TATATGCATGCG
4	6	MA0350.1	TOD6	0.0341	ATTTTTCCCTCATCGGCACAT
5	7	M01523	F$PHD1_01	0.0343	ATGCTTATATGCATGCGCATA
6	8	M01534	F$TEC1_01	0.0345	TTGCCAAAATTCTTGCTTTC
7	9	M01537	F$DOT6_01	0.0355	ATTTTTCCCTCATCGGCACAT
	10	MA0351.1	DOT6	0.0363	ATTTTTCCCTCATCGGCACAT
8	11	MA0398.1	SUM1	0.0424	TTAATTTAT

[a] Names beginning with "F" are from the TRANSFAC database; all other names are from the JASPAR database.
[b] Probability of observing a certain or higher affinity in a given sequence. An accurate *P* value computation for the TRAP tool scores allows determination of which factors are the most likely to regulate a given target gene. It is set to normalize an observed affinity for a random-sequence model and to give a statistical meaning to the statement that one factor binds stronger than another.
[c] Sequence of the transcription factor binding site.

of Cup9-Myc in normal cells. However, an increased expression of Cup9-Myc was associated with overexpression of Hsp82 (Fig. 6E) as well as found under heat stress (Fig. 6F). Next, we used chromatin immunoprecipitation (ChIP) to analyze Cup9 recruitment at the upstream regulatory region of *SIR2* in the presence of Hsp82 overexpression. We used Hsp82-overexpressing cells in which Cup9 (Cup9-Myc) was abundantly present and used anti-Myc antibody to immunoprecipitate chromatin-bound Cup9. Under the Hsp82 overexpression condition, we observed a bright signal of Cup9 specifically at the upstream regulatory element of *SIR2* but not on the control *ACT1* locus (Fig. 6G).

Cup9 overproduction reduces the endogenous level as well as the function of Sir2. In order to explore whether the endogenous level and activity of Sir2 are directly regulated by Cup9, we analyzed them under the Cup9 overexpression condition. To that end, Cup9 overexpression plasmid pESC-CUP9 and the empty vector pESC were transformed into wild-type strain SLY20 to generate SLY90 and SLY91, respectively. This vector overexpresses Myc-tagged Cup9 under the control of a galactose-inducible promoter. The *SIR2* transcript was quantified in those backgrounds by semiquantitative RT-PCR. The results showed that Cup9 overexpression entirely diminished the *SIR2* transcript in SLY90 compared to that in the SLY91 strain (Fig. 7A). Real-time RT-PCR analysis showed a nearly 10-fold reduction of the *SIR2* transcript in a Cup9 overexpression background (Fig. 7B). Our results were further confirmed after estimating Sir2p in a Cup9 overexpression background. Western blot analysis showed that Cup9 overexpression caused a modest reduction in the Sir2p level specifically, without any alteration to the Hsp82 or Act1 protein level (Fig. 7C). We subsequently investigated the silencing function of Sir2 under the Cup9 overexpression condition using three independent assays. We observed that SLY90 cells having a reduced level of Sir2 developed into white colonies, indicating derepression of the subtelomeric *ADE2* gene, whereas SLY91 containing the empty vector developed as pink colonies due to the silencing of *ADE2* (Fig. 7D). Also, quantification of the *YFR057w* transcript by real-time RT-PCR showed a nearly 8-fold increase in transcripts in cells harboring the Cup9 overexpression plasmid compared to those in the WT (Fig. 7E). We also compared the *HML*α transcript by real-time RT-PCR in a Cup9 overexpression background but observed no significant change. These results indicate that Cup9 overexpression brings down the Sir2 level moderately, as a result of which the silencing activity of Sir2 at a hidden mating locus is maintained, though subtelomeric silencing activity is diminished.

DISCUSSION

In this article, we provide a mechanistic understanding of how Hsp90 homeostasis regulates Sir2 function in the cell. We provide compelling evidence that Hsp90 regulates the transcription of *SIR2* under heat stress and thereby controls the cellular abundance of Sir2 protein. Previous work in our laboratory had demonstrated that heat shock treatment as well as Hsp90 overexpression caused a drastic reduction in the endogenous level of Sir2 (5). The reduced pool of Sir2 was functionally active, but its limiting quantity was insufficient to establish silencing across all 32 telomeres. However, it was adequate to silence hidden mating type loci. Findings from this work help in understanding how Hsp90 overexpression results in a reduced pool of Sir2 protein.

Molecular players involved in transcriptional regulation of *SIR2* gene have remained elusive. Here, we report for the first time the identification of a transcriptional repressor that regulates *SIR2* gene transcription. We provide several lines of evidence that unequivocally establish Cup9 as the transcriptional repressor of *SIR2* gene expression.

First, in the Δ*cup9* background, neither heat shock nor overexpression of Hsp82 had any effect on *SIR2* transcription. Second, Cup9 overexpression caused a drastic reduction in *SIR2* transcription, resulting in the derepression of subtelomeric genes. Such an effect was independent of heat stress. Third, bioinformatics analysis predicted a Cup9 binding sequence within (−419 to −399) upstream regions of *SIR2*. Finally, a chromatin immunoprecipitation assay further demonstrated that endogenously expressed Cup9 was recruited at the 5′ end on the yeast *SIR2* promoter under the Hsp82 overexpression condition.

Our current data show that under heat stress, Cup9 expression

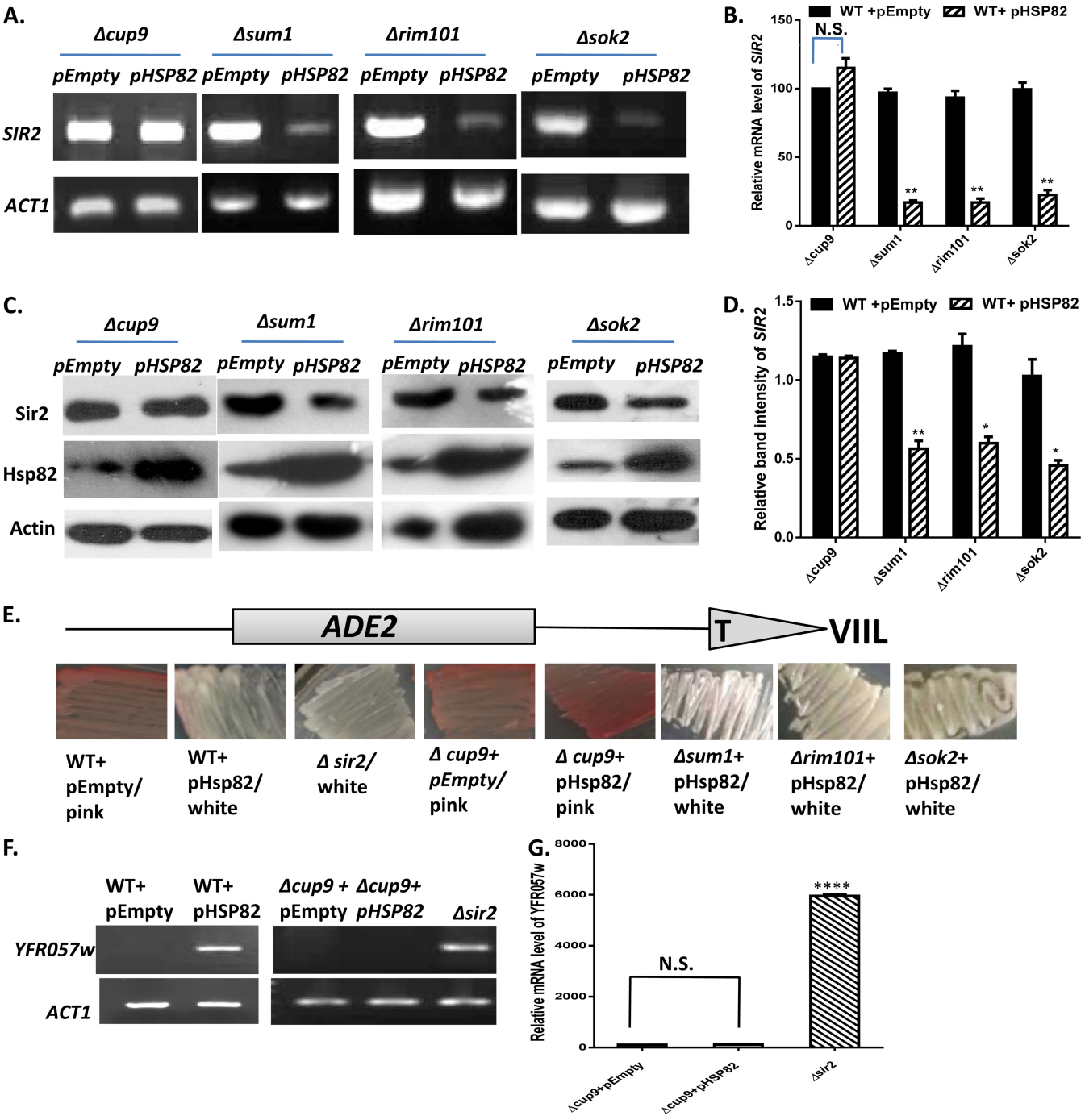

FIG 5 Reversal of heat shock/Hsp82 overexpression phenotype in the *cup9* deletion strain. (A) Δ*cup9*, Δ*sum1*, Δ*rim101*, and Δ*sok2* strains were generated as described in Materials and Methods. An Hsp82 overexpression plasmid was transformed into each of the four deletion strains, and semiquantitative RT-PCR displays no alteration in the *SIR2* transcript in the Δ*cup9* strain having an *HSP82* overexpression background; *ACT1* acted as the normalization control. (B) Real-time RT-PCR data for the relative quantity of *SIR2* mRNA between the above-mentioned strains (presented on the *x* axis). Each bar represents the mean mRNA level (±SD) from three independent experiments. *P* values were calculated using the two-tailed Student *t* test. **, $P < 0.01$. (C) Western blot analysis was done with the protein extracted from the above-mentioned strains using antiactin, anti-Hsp82, and anti-Sir2 antibodies. (D) Densitometric measurements of Sir2 (after normalization with actin) from three independent experiments were plotted for the strains indicated on the *x* axis. Error bars indicate SD. **, $P < 0.01$; *, $P < 0.05$. (E) *ADE2* reporter gene located at chromosome VIIL was used for the telomere silencing assay. Wild-type cells, wild-type cells carrying an Hsp82 overexpression plasmid, Δ*sir2* cells, Δ*cup9* cells, and Δ*cup9*, Δ*sum1*, Δ*rim101*, and Δ*sok2* cells each carrying the Hsp82 overexpression plasmid were grown and plated as described in Materials and Methods. It should be noted that Δ*sum1*, Δ*rim101*, and Δ*sok2* strains carrying Hsp82 overexpression plasmid behave like wild-type cells carrying the Hsp82 plasmid. They are different from Δ*cup9* cells carrying the Hsp82 overexpression plasmid, which show intense pink coloration. (F) A telomere silencing assay was done with *YFR057w* localized adjacent to telomere VI-R. Semiquantitative RT-PCR was done to study the expression of *YFR057w* in wild-type cells with and without the Hsp82 overexpression plasmid and in the Δ*cup9* strain in the presence and absence of the Hsp82 overexpression plasmid. The Δ*sir2* strain was used as a control. The *ACT1* transcript was measured as a loading control. (G) Real-time RT-PCR was done to quantify the relative abundance of *YFR057w* in the Δ*cup9* strain with or without Hsp82 overexpression plasmid and compare it with the same in the Δ*sir2* strain. ****, $P < 0.0001$.

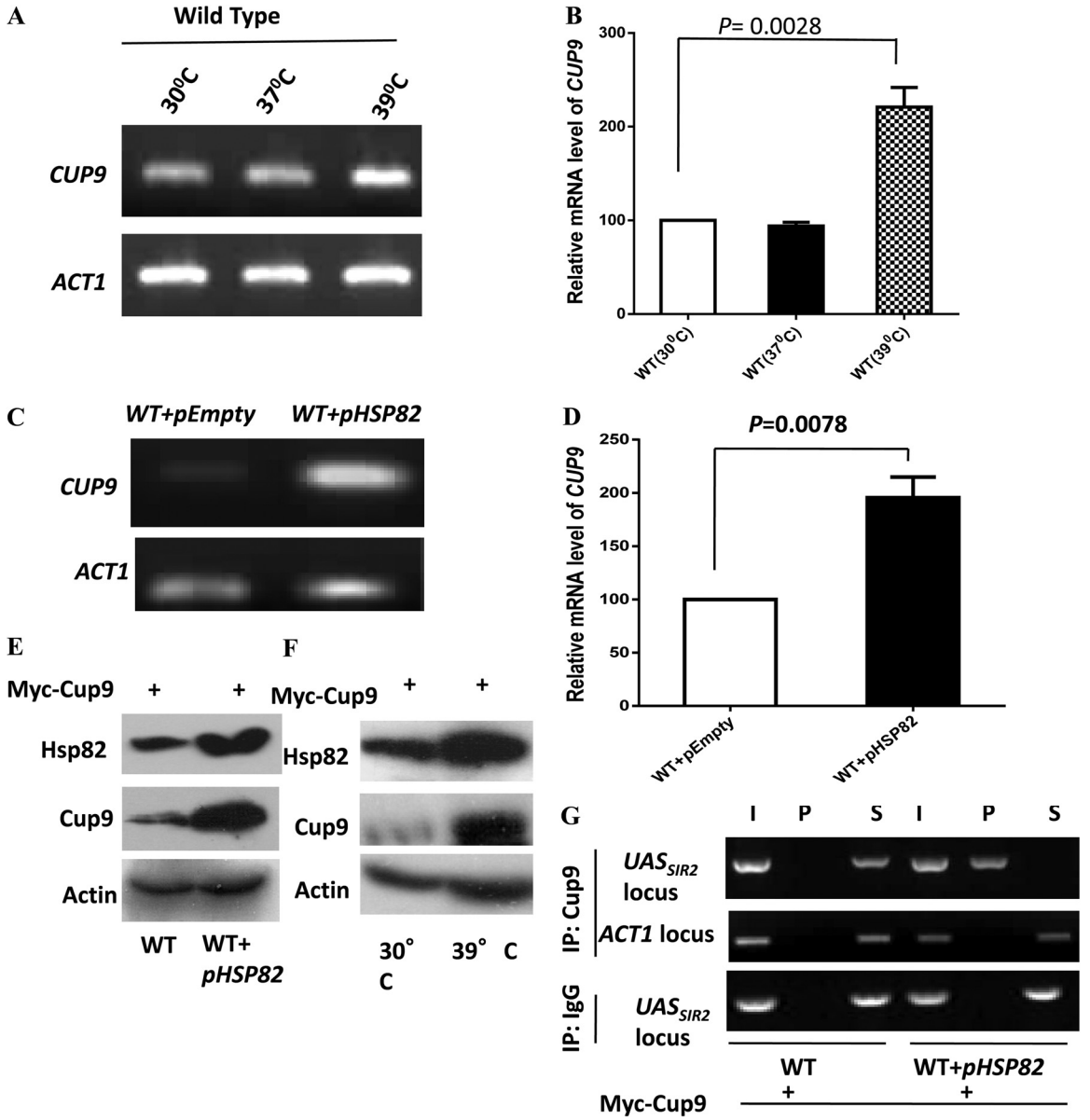

FIG 6 Heat shock and Hsp82 overexpression induce Cup9 expression and lead to its association with $SIR2_{UAS}$. (A) Wild-type cells were divided into three groups: one grown at 30°C, another exposed to 37°C for 2 h, and the last exposed to 39°C for 40 min. Semiquantitative RT-PCR results for all groups show the *CUP9* transcript profile at the different temperatures. *ACT1* acted as the normalization control. (B) Real-time RT-PCR shows the quantitative abundance of the *CUP9* transcript at 39°C compared to that at 30°C. Each bar represents mean mRNA level (±SD) from three independent experiments. *P* values were calculated using the two-tailed Student *t* test. (C) Wild-type cells and cells bearing the Hsp82 overexpression plasmid were used to assess the level of the *CUP9* transcript by employing semiquantitative RT-PCR. The experiment was repeated three times; data from one representative experiment are presented here. (D) Real-time RT-PCR reveals the quantitative abundance of the *CUP9* transcript in cells bearing *HSP82* overexpression plasmid compared to that in the wild type. (E) In wild-type cells, *CUP9* was *MYC* tagged at the chromosomal locus as described in Materials and Methods. Proteins isolated from wild-type cells and cells having the Hsp82 overexpression plasmid were subjected to Western blot analysis using anti-Myc (Cup9), anti-Hsp82, and antiactin antibodies. (F) The same cells were subjected to 39°C for 40 min, and heat-treated and untreated cells both were subjected to immunoblotting using antiactin, anti-Hsp82, and anti-Myc antibodies. (G) ChIP assays were performed using *CUP9 MYC*-tagged cells in the absence and presence of Hsp82 overexpression plasmid. Anti-Myc antibodies were used with control IgG (immunoglobulin G). Input (I), immunoprecipitated DNA (P), and supernatant (S) were amplified by semiquantitative RT-PCR with primers that covered $SIR2_{UAS}$. The experiment was repeated twice; data from one representative experiment are presented. I, P, and S DNA were also amplified using primers that cover *ACT1*, which acted as a negative control.

is induced. Under such conditions, Cup9 binds to the $SIR2_{UAS}$, leading to the transcriptional downregulation of *SIR2* and thereby causing derepression of subtelomeric genes. This was further supported by a reporter gene analysis using various deletion constructs of $SIR2_{UAS}$. The effect of Hsp82 on transcriptional downregulation of the reporter gene was abolished when the Cup9 binding region was deleted from $SIR2_{UAS}$. Currently, it is not known how Hsp82 regulates Cup9 expression. Previously, it was observed that copper stress causes transcriptional upregulation of *CUP9* when cells are grown on lactose medium (13). Our study

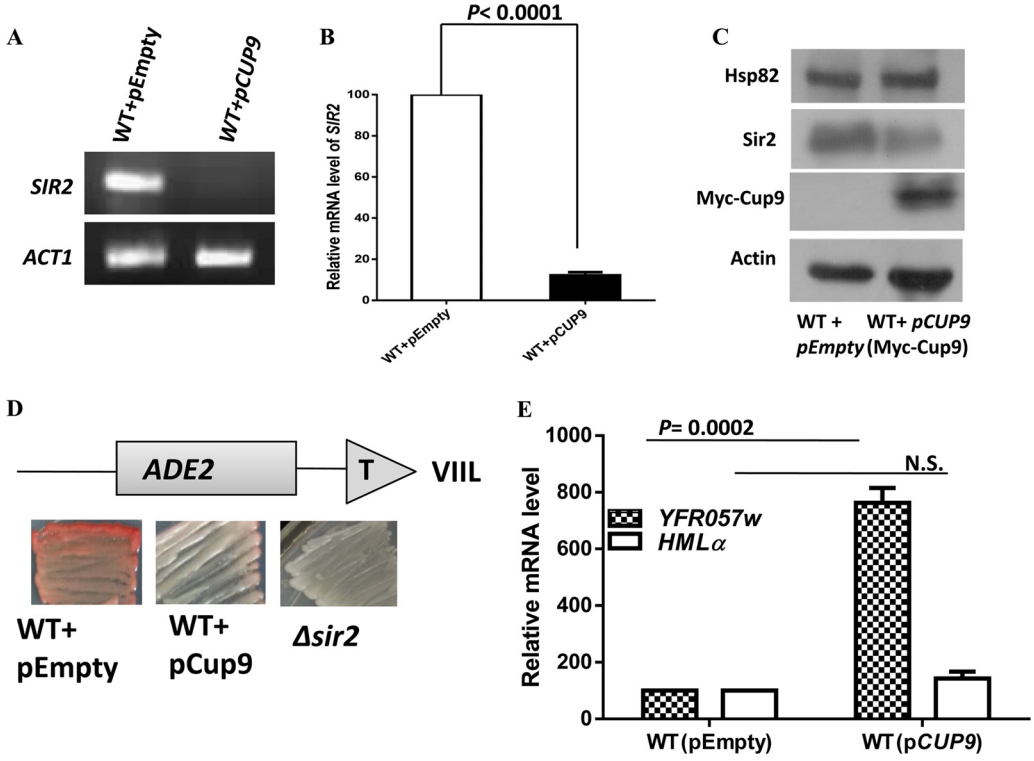

FIG 7 Cup9 overproduction reduces the endogenous level as well as the function of Sir2. (A) Cup9 was induced by adding galactose to a final concentration of 2%. The overnight culture grown in galactose medium was used as a secondary inoculum in galactose-containing medium and was grown for an additional 5 h (until the mid-log phase) before the isolation of RNA. The effect of Cup9 overexpression on *SIR2* transcription was measured by semiquantitative RT-PCR. (B) Real-time RT-PCR quantification of *SIR2* mRNA levels in Cup9-overexpressing cells relative to those of the wild-type cells is shown as the average of three experiments. Error bars indicate SD. *P* values were calculated using the two-tailed Student *t* test. *ACT1* mRNA was used as the normalization control. (C) The overnight culture grown in galactose medium was used for Western blot analysis. The immunoblot shows the reduction of endogenous Sir2 protein upon Cup9 overexpression. The Cup9 overexpression vector harbors C-terminally Myc-tagged Cup9. (D) The *ADE2* reporter gene at telomere VIIL was used for the telomere silencing assay. Wild-type, Cup9-overexpressing, and Δ*sir2* cells were grown and plated as described in Materials and Methods. Pink colonies indicate a silenced *ADE2* gene, and white colonies indicate transcriptionally active *ADE2*. (E) Cup9 overexpression induces the derepression of another subtelomeric gene, *YFR057w*. However, silencing at the hidden mating type loci is maintained, as seen by the comparable level of the *HML*α transcript. *ACT1* was used as the normalization control. Error bars indicate SD ($n = 3$).

demonstrates that expression of *CUP9* is also upregulated during heat shock treatment. It was previously reported that high temperature (37°C) strengthens mating and telomere silencing (38). Our results corroborate this finding, since *CUP9* transcription remained unaltered between 30°C and 37°C. However, at 39°C, there was a nearly 2.5-fold increase in the *CUP9* transcript. It has been observed that expression of *HSP90* increases severalfold not only during heat shock but also in response to several other environmental stresses (39–41). Thus, it will be interesting to investigate whether upregulation of Cup9 is a specific or general stress response phenomenon.

Transcriptional regulation of Sir2 in yeast in response to environmental stimuli has never been identified before. However, in a human cell line, it has been demonstrated that SIRT1p associates with HIC1 (hypermethylated in cancer 1) and the complex binds to the *SIRT1* promoter to repress its own transcription (42). Epigenetic silencing of *HIC1* through hypermethylation of the *HIC1* promoter has been associated with aging. Reduction in HIC1 expression results in *SIRT1* upregulation, which results in excessive deacetylation and deactivation of p53 function and thus increases the cancer risk in mammals. Any such feedback inhibition of *SIR2* expression by Sir2 itself in yeast is not known. To the best of our knowledge, Cup9 is the only repressor identified so far that regulates *SIR2* gene expression in yeast.

Dietary restriction is proven to be an environmental factor that increases longevity from yeast to mammals (43, 44). NAD^+-induced histone deacetylase activity of Sir2 is required for increased longevity during starvation, and this effect was not observed in a *sir2* mutant strain (45). It had been demonstrated previously that under normal conditions, Cup9 is a short-lived protein, having approximately 5 min as its half-life (46). The presence of imported di- or tripeptides causes the activation of E3 ubiquitin ligase Ubr1 and accelerates the Ubr1-dependent ubiquitylation of Cup9 (46). Thus, under normal conditions, due to the unstable nature of Cup9, Sir2 can be transcribed optimally during glucose starvation. However, under heat shock conditions (39°C), an elevated level of Cup9 leads to repression of *SIR2* transcription, thereby mimicking *sir2* knockdown in yeast, which is inherited for many generations. Thus, it is tempting to predict that Sir2 may not influence longevity under heat stress conditions. This hypothesis is supported by the following lines of evidence. First of all, the Δ*sir2* mutant strain does not show life span extension in yeast, and upon heat treatment, the steady-state level of Sir2 decreased drastically and remained almost undetectable for up to 6 days in our Western

blot analysis. Second, deletion of *cup9*, a condition that increases Sir2 abundance, yielded an extended life span under cold conditions (47). Another study performed with *Caenorhabditis elegans* (48) also documented that decreasing the temperature progressively lengthened the life span of worms. It will be interesting to explore whether such Hsp90-induced regulation of mammalian sirtuins also results in their suboptimal activity.

Our work reveals that transient heat shock results in heritability of derepressed subtelomeric chromatin. Previously, it had been reported that telomere structure regulates the heritability of silenced subtelomeres (49). An elongated telomere track leads to the increased inheritance of the silenced subtelomeric state and is independent of yeast chromatin assembly factor 1 (yCAF-1). In another study, it was reported that prolonged exposure to heat stress (37°C) as well as Hsp82 overexpression led to telomere shortening in wild-type cells (32, 33). Thus, it is important to understand whether transient heat shock causes any change in telomere structure and can thereby influence telomere silencing. Our study shows that transient heat shock leads to shortening of the telomere length, which is gradually restored to the wild-type length (at the end of the 6th day). The restoration of telomere length might be one of the factors for the reappearance of the telomere position effect, since TPE is not reestablished before the telomere length returns to normal. On the other hand, the reappearance of Sir2p is also likely to be the reason behind restoration of wild-type-like silencing at the telomere, as the timing of Sir2 reappearance and that of the reestablishment of TPE coincide very closely. Thus, it is possible that transient heat shock-mediated derepression as well as reestablishment of TPE is multifactorial and a period of 7 to 8 days is required for the full reestablishment of subtelomeric silencing.

Although the mechanism behind heritable repression of *SIR2* is not clear at present, this work has unraveled a cryptic pathway of *SIR2* regulation that is induced under heat stress (39°C) or under a condition in which Hsp90 is overexpressed in cells. Our work showed that a short period of heat shock rendered the cells *sir2* knockdown cells for more than 90 generations. Our finding on derepression of the *HML*α transcript upon transient heat shock implies a likely defect in yeast mating behavior which has tremendous implications for yeast physiology. This observation is also important in a broader context, as Sir2 is one of the epigenetic factors that establishes silencing at subtelomeric regions in many eukaryotes. Sir2-mediated telomere silencing plays a major role in mutually exclusive expression of virulent multigene family in protozoan parasites such as *Plasmodium* and trypanosomes (50, 51). Such a mechanism controls antigenic variation and thereby causes evasion of the host immune system (52). In light of our findings, it will be interesting to explore whether exposure to febrile temperature (around 39°C) as a natural consequence of *Plasmodium* infection has any correlation with derepression of subtelomeric virulent genes as a consequence of poorer Sir2 activity.

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We declare that we have no conflicts of interest.

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