Molecular Studies of Longevity-Associated Genes in Yeast and Mammalian Cells

by

Gregory Liszt

B.S. Molecular, Cellular, and Developmental Biology
Yale University

Submitted to the Department of Biology
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY IN BIOLOGY

at the
Massachusetts Institute of Technology
February, 2006

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Signature of Author

Department of Biology

Certified by

Leonard Guarente
Novartis Professor of Biology
Thesis Supervisor

Accepted by

Stephen Bell
Chairman, Biology Graduate Committee
ABSTRACT

Aging is a complex process affecting diverse organisms from bacteria to humans. Despite strong evolutionary arguments against the conservation of a single mechanism of aging, a variety of conserved single gene mutations have been found to extend life span and stave off aging in several different species. The study of these mutations yields important insights into the biology of aging.

In *Saccharomyces cerevisiae*, aging can be studied by mutations that extend the replicative potential of mother cells. With successive cell divisions, instability at the rDNA locus and extrachromosomal rDNA circle accumulation exponentially increase the likelihood of senescence and mortality. Aging can be forestalled by caloric restriction, a regimen that increases the activity of Sir2p, an NAD-dependent protein deacetylase and important regulator of aging in yeast and some metazoans. Caloric restriction activates respiration, reducing cellular NADH levels and relieving the competitive inhibition of Sir2p by this metabolite.

*SSD1* promotes longevity by a Sir2p-independent mechanism that affects neither ERC formation nor rDNA silencing. Ssd1p directly represses the translation of the mitochondrial and cell wall glycoprotein Uth1. This repression, which requires a physical interaction between Ssd1p and the 5’-UTR of the *UTH1* mRNA, is necessary and sufficient to account for diverse effects of Ssd1p on cell integrity, stress resistance, and life span. Future studies should determine whether Ssd1 plays a role in the maintenance of longevity in higher organisms.

Mammalian genomes contain seven Sir2 homologs (SIRT1-7) involved in diverse processes including fat and muscle cell differentiation, p53- and FOXO-dependent apoptosis, stress resistance, and DNA-damage repair. Mouse SIRT6, a nuclear protein, is
broadly expressed throughout the body and displays a robust auto-ADP-ribosyltransferase activity unique among Sir2 family members. SIRT7, a nucleolar homolog of Sir2, physically interacts with RNA polymerase I (Pol I), colocalizing with the Pol I complex at the transcribed regions of the rDNA. SIRT7 activates rDNA transcription by an enzymatic mechanism, suggesting a novel model coordinating cellular energy status with ribosome biogenesis via changes in SIRT7 activity.

Thesis Supervisor: Leonard Guarente
Title: Novartis Professor of Biology, MIT
DEDICATION

This thesis is dedicated to my parents, Miki and Harvey Liszt.
ACKNOWLEDGEMENTS

First, I thank my thesis advisor Leonard Guarente for his guidance, wisdom, patience and understanding, all of which were indispensable to my success in graduate school. I am very lucky to have had you as a teacher.

I would also like to thank my thesis committee: Rick Young, Peter Sorger, Paul Garrity, and David Sinclair.

I thank Matt Kaeberlein for scientific insight, project ideas, collaboration, and general inspiration.

Profound thanks go to the members of Crooked Still (Rushad Eggleston, Corey DiMario, and Aoife O’Donovan) for inspiring me musically and making these years in grad school so unbelievable and adventurous.

Special thanks go to Ethan Ford for constantly sharing his insights, teaching me so many lab techniques, collaborating with me scientifically, and keeping me company for so many years. We had a good run of it.

Special thanks also go to Nick Bishop, whose passion for the science of aging and beatboxing skills have always kept me inspired. Sorry to have abandoned you so close to the end. And thanks for the help with the Xmas Rap.

Thanks to Andy Tolonen, my roommate, classmate, running partner, and good friend.

Thanks to Kayvan Zainabadi for scientific insights and personal advice.

Thanks to Martin Kurtev for always bringing life to the lab when no one else was around.

I would also like to thank the entire Guarente lab for scientific and social camaraderie over the last six years. I have been lucky to share the company of so many brilliant and helpful people. Especially my collaborators, Matt Kaeberlein, Su-Ju Lin, Ethan Ford, and Martin Kurtev, without whom I would never have finished this thesis.

Thanks to Sarah Buckley for being a great UROP.

Thanks to all the musicians who have enriched my life here in Boston, especially Jake Armerding, Casey Driessen, the Wayfaring Strangers, and everyone from the Cantab.

And most importantly, thanks to my family: Miki and Harvey Liszt, for their profound love and support; Jeff, for all that plus being a best friend; Danielle, for being such an inspiring future sister-in-law; Nonni for being such an amazing person and loving grandmother; and Aunt Mimi and Uncle Bev for always being there for me.
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Chapter 1

Introduction -

Genetic Pathways of Aging from Yeast to Mammals
WHY STUDY AGING IN MODEL ORGANISMS?

Although we all possess an intuitive understanding of what it means to age, the scientific study of aging has proven notoriously complex. Aging, after all, is characterized by the modification or breakdown of nearly every system within an organism, and distinguishing the important changes from the unimportant ones poses a daunting problem. Indeed, aging differs drastically from most other basic biological processes in that aging is primarily a nonadaptive trait, conferring no benefit to the individual. As such traits arise precisely because of the lack of natural selection, there is no reason to believe that natural selection would conserve a particular mechanism of aging throughout evolution.

For this reason, the study of aging in model organisms, which began in earnest in the 1990’s, was met with skepticism (and even some ridicule). In the wild, very few organisms even survive to reach old age (reviewed in (Kirkwood, 2005), instead dying of causes such as predation, starvation, cold, accident, etc. Therefore, throughout evolution very little pressure has selected for alleles that confer benefits late in life. Evolutionary theory thus holds that aging should consist of the incidental failure of several systems rather than the onset of a specific program controlled by particular genes.

The discovery of numerous conserved single gene mutations slowing aging and extending life span thus opened a rift between evolutionary theory and experimental reality. As a result, the control of aging has been rethought in evolutionary terms, and is now commonly regarded as possessing significant adaptive benefit. Which is not to say that aging results from the execution of a specific genetic program, merely that the generalized decay of aging can be prevented by means that have evolved over time.
Consider the case of dietary restriction, discussed in detail later in this chapter: This intervention, which entails reducing excess food consumption, has been shown to extend the life span of a variety of organisms (Guarente and Picard, 2005; Merry, 2002), likely involving by activating a conserved genetic mechanism. One hypothesis explaining this finding is that organisms with the ability to stave off aging and postpone reproduction during times of nutrient scarcity are at a tremendous selective advantage over those that maintain a constant rate of aging under such conditions. Consistent with this hypothesis, calorie-restricted rodents show signs of increased physiologic maintenance function and reduced reproduction (Weindruch et al., 2002), both of which would prime them for Darwinian success should food conditions improve and their physiology return to normal.

This chapter details several of the main advances in model systems aging research, also addressing the extent to which genetic control of aging is conserved between species. Genomic stability, metabolism, and stress resistance appear as common themes in aging from yeast to mammals. In metazoans, control of these processes is superimposed against hormonal signaling pathways, with endocrine regulation emerging as a key means of regulating the rate of aging.

In the chapters that follow, the goal of the research is two-fold: first, to arrive at a more complete understanding of aging in the unicellular model organism *Saccharomyces cerevisiae*, and second, to begin to apply the knowledge of yeast aging to the more complex mammalian systems.
AGING IN YEAST

Early studies

Background:

Over 45 years ago, Mortimer and Johnston made the observation that was to become the basis for the application of genetic analysis to the study of aging. Observing the repeated divisions of individual yeast mother cells, these scientists noted that in a genetically identical population of yeast, each cell ceased to divide after giving birth to a finite number of daughter cells (Mortimer and Johnston, 1959). For a given strain of yeast, the likelihood of mortality increased exponentially with increasing numbers of cell divisions. When Mortimer and Johnston plotted their data, they made a striking observation: that the mortality curve for a population of yeast closely resembled the mortality curves for many other organisms, including humans (Figure 1). The study of yeast aging was born.

In the decades that followed, radical advances characterized the study of yeast biology. On the forefront of genetic research, yeast provided an invaluable model system for the investigation of such basic biological processes as the cell division cycle, the secretory pathway, and the DNA damage response, among many others. However, it was not until the 1990’s that the powerful tools of molecular biology, combined with the resources of the fully sequenced yeast genome, were applied to the study of yeast aging (Sinclair et al., 1998).

Budding yeast reproduce by asymmetric cell division, with a pre-existing mother cell giving birth to a smaller daughter cell consisting predominantly of newly-synthesized materials (Guthrie and Fink, 1991). Practically, this asymmetry of cell division enables
the microscopic separation of mother and daughter cells upon completion of cytokinesis, forming the experimental basis of the yeast life span assay. Mean and maximum lifespan are relatively constant for a given strain, although different strains of yeast vary drastically over a mean life span range of less than 15 generations to greater than 30 (Sinclair et al, 1998). This initial finding demonstrated a strong genetic influence on longevity, and encouraged the search for single gene mutations affecting the rate of aging.

Age-related changes:

Aging in yeast is accompanied by a battery of phenotypic changes, ranging from obvious changes in cell size (Mortimer and Johnston, 1959) to more subtle differences in transcription and protein localization (Kennedy et al., 1997; Mortimer and Johnston, 1959; Smeal et al., 1996). Mortimer and Johnston hypothesized, a priori, that the onset of senescence is caused when cell volume reaches some upper limit (Mortimer and Johnston, 1959). Three lines of evidence now refute that hypothesis. First, inducing an increase in size by temporary G1 arrest fails to shorten life span (Kennedy et al., 1994). Second, populations of old senescent cells span a wide range of sizes, defying a critical prediction of Mortimer and Johnston’s hypothesis (G.L., unpublished observation). Finally, of the multitude of single gene changes known to extend life span, very few affect cell size (Bitterman et al., 2003).

Age-dependent changes in cell surface characteristics also accompany the journey into yeast old age. Notably, each round of cell division leads to the deposition of a chitin bud scar on the surface of the mother (but not the daughter) cell. These bud scars can be stained with calcofluor white, permitting visualization under a microscope and enabling
the determination of cell age. Early hypotheses attributing bud scar accumulation as the cause of aging have now been generally refuted (Sinclair et al., 1998), mostly on the grounds that induced chitin accumulation on the cell surface does not curtail life span (Egilmez and Jazwinski, 1989). Also, theoretical models of the yeast cell surface estimate that the cell wall can accommodate more than three times the number of bud scars than usually accumulate over the course of normal aging (Sinclair et al., 1998).

The first molecular phenotype associated with aging in *S. cerevisiae* was a loss of transcriptional silencing (Smeal et al., 1996), and it was the characterization of this phenotype that ultimately led to the discovery of *SIR2* as a central regulator of yeast aging (Kaeberlein et al., 1999). Haploid yeast cells typically exist as one of two distinct mating types, known as *MATα* and *MATα*, determined by the sequence of the gene expressed at the *MAT* locus. Although mating type information is only expressed from the single allele present at the *MAT* locus, haploid yeast harbor silenced copies of both mating type alleles, present at *HM* loci (Guthrie and Fink, 1991). Under normal conditions, silencing of these loci is accomplished by the SIR (Silent Information Regulator) protein complex, consisting of Sir2p, Sir3p, and Sir4p (Gottschling et al., 1990; Ivy et al., 1986; Rine and Herskowitz, 1987). The SIR complex also directs silencing at telomeres (Gottschling et al., 1990), where gene expression is abolished over telomeric repeats and nearby subtelomeric regions. Sir2p, the catalytic component of this complex, is an NAD+ dependent protein deacetylase (Imai et al., 2000; Landry et al., 2000b; Smith et al., 2000), and is discussed at length later on in this chapter.

Progressive sterility has been shown to affect aging yeast mother cells (Muller, 1985), and this phenotype is now known to be attributable to changes in the localization
of the SIR complex (Smeal et al., 1996). This molecular phenotype of aging is most likely an effect of the aging process rather than a cause, as deletion of the HM loci cures age-related sterility without extending cellular life span (Kaeberlein et al., 1999; Smeal et al., 1996). Interestingly, telomeric silencing is lost with age (Kim et al., 1996), and this loss of silencing correlates with the absence of the SIR complex. The current explanation for these findings is that the SIR complex relocalizes to the nucleolus to counteract the nucleolar instability that eventually limits mother cell division (Kennedy et al., 1997; Sinclair et al., 1998; Sinclair and Guarente, 1997). Supporting this model, the long-lived SIR4-42 mutant constitutively relocalizes the entire SIR complex to the nucleolus, even in young cells, thereby forestalling nucleolar fragmentation and prolonging life span (Kennedy et al., 1997).

**Extrachromosomal rDNA Circles: a Cause of Aging**

The first molecular cause of aging to be determined for yeast synthesized these findings and provided a rationale for the importance of the nucleolus in cellular senescence (Sinclair and Guarente, 1997). The yeast nucleolus houses the rDNA, a tandem array of 150-200 repeated copies of the genes encoding ribosomal RNA (rRNA). Each 9.1 kb repeat encodes a 35S rRNA and a 5S rRS and 5S rRNAs are transcribed in opposing directions, after which the 35S RNA is processed into mature 25S, 18S, and 5.8S rRNA molecules (reviewed in (Nomura, 2001). According to current evidence, the stalling of the replication fork within the rDNA triggers the induction of homologous recombination, as DNA repair enzymes recognize this feature and process it into a double stranded break (Kim and Wang, 1989; Park et al., 1999). Homologous recombination
between neighboring rDNA repeats has been shown to result in excision of circular molecules containing one or more complete copies of the rDNA unit (Park et al., 1999), each one of which directs its own replication through the presence of three ARS consensus sequences. Of these three potential origins of replication, it is estimated that an average of one fires each S phase (Miller and Kowalski, 1993). Importantly, the same extrachromosomal rDNA circles (ERCs) that multiply exponentially with each successive round of cell division predominantly fail to segregate to daughter cells, instead accumulating in mother cells at levels approaching 1000 copies per cell (Sinclair and Guarente, 1997). This number of ERCs contains a quantity of DNA roughly equivalent to all the rest of the yeast genome.

Several lines of experimental analysis have supported an ERC model of aging, indicating that ERC accumulation is not just a hallmark of yeast aging but actually a cause. First, the ectopic release of an ERC in young cells accelerates the onset of senescence, demonstrating that formation of an ERC is limiting for life span (Defossez et al., 1999; Sinclair and Guarente, 1997). Second, mutation of the rDNA unidirectional replication fork block component Fob1p eliminates ERC formation and extends life span (Defossez et al., 1999). Indeed, most mutations extending the yeast life span in a strain-independent manner correlate with reduced ERC levels and can be rationalized by their effects on ERC formation and accumulation (Kaeberlein et al., 2005).
Sir2p, a central regulator of aging

Genetic analysis:

Multiple studies from the last six years have established an important role for Sir2p as a central regulator of aging (Anderson et al., 2003a; Kaeberlein et al., 1999; Lin et al., 2000; Lin et al., 2002). While the other members of the SIR complex indirectly affect aging through modulation of silencing at the HM loci and telomeres (Kaeberlein et al., 1999), Sir2 plays an independent role in establishing rDNA heterochromatin (Bryk et al., 1997) and repressing the unequal crossing-over responsible for ERC formation (Kobayashi et al., 2004) and, hence, normal aging. At the nucleolus, Sir2p acts within the RENT (regulator of nucleolar silencing and telophase exit) complex, whose other members Net1p, Cdc14p, and Nan1 (Ghidelli et al., 2001; Shou et al., 2001) have not been implicated in the aging process. The initial observation that SIR2/sir2 heterozygous diploids displayed an intermediate life span between those of the two homozygotes suggested that Sir2p dosage might be limiting for life span (Kaeberlein et al., 1999). Indeed, addition of a single extra copy of SIR2 extends life span of multiple strains by up to 40%, while the deletion of SIR2 shortens life span by approximately 50% in several strain backgrounds (Kaeberlein et al., 2005; Kaeberlein et al., 1999). Consistent with the ERC model of aging, SIR2 levels correlate not only with life span but also with ERC levels and rate of marker gene loss by rDNA recombination. Interestingly, a recent study indicates that Sir2p influences not the overall rate of rDNA recombination, but rather the relative frequency of unequal sister chromatid crossing over between different rDNA repeats, hence creating the appearance of increased total recombination in rDNA marker loss and ERC accumulation assays (Kobayashi et al., 2004).
Providing further support for the role of Sir2p in ERC-mediated aging, genetic analysis has shown that *fob1* deletion and *SIR2* overexpression fail to additively increase life span, and are therefore likely to affect aging through the same pathway (Kaeberlein et al., 1999). By a similar token, *fob1* deletion restores wild type life span to a *sir2* mutant, most likely by rescuing the increased ERC formation caused by *sir2* mutation.

**Biochemistry:**

Due to its ability to promote silencing, Sir2p was long suspected of possessing histone deacetylase activity. Despite the fact that Sir2p levels were known to correlate with histone deacetylation at targeted areas of the yeast genome (Braunstein et al., 1993; Grunstein, 1997; Jenuwein and Allis, 2001) attempts to demonstrate an *in vitro* deacetylation activity for Sir2p met with years of failure. In 1999, however, major strides were made in the enzymology of Sir2p and its metazoan homologs. Initially, Sir2p and several homologs were found to possess an ADP-ribosyltransferase activity, catalyzing the breakdown of a nicotinamide adenine dinucleotide (NAD) substrate and the addition of an ADP-ribose moiety to target proteins (Frye, 1999; Tanny et al., 1999). However, this reaction was soon overshadowed by the discovery of a more robust NAD-dependent histone deacetylase activity present in yeast Sir2p as well as its closest mouse homolog, SIRT1 (Imai et al., 2000; Landry et al., 2000b; Smith et al., 2000). In yeast, this activity is essential for the maintenance of silencing and life span, as mutations abolishing deacetylation generally eliminate silencing in vivo and shorten life span (Imai et al., 2000). Interestingly, some mutations in conserved residues of Sir2p affect *in vitro* histone deacetylase activity and *in vivo* silencing differently (Armstrong et al., 2002),
suggesting that Sir2p might influence silencing by deacetylating non-histone substrates within the cell. Despite this inconsistency, Sir2p has been shown to display a substrate preference for lysine 16 of the histone H4 (Imai et al., 2000; Landry et al., 2000b; Smith et al., 2000), a tail residue critical for silencing.

Several unique features characterize the Sir2p enzymatic reaction. Unlike other histone deacetylases, the Sir2, or Class III, deacetylases are not inhibited by trichostatin A (Imai et al., 2000). Histone deacetylation by Sir2p also cannot proceed without NAD, nor can other nicotinamide adenine dinucleotides such as NADH, NADP, or NADPH substitute for NAD (Imai et al., 2000). In fact, NADH and nicotinamide both function as effective inhibitors of Sir2p catalytic activity, providing an effective mechanism for regulating Sir2p function in vivo (Anderson et al., 2003a; Bitterman et al., 2002; Lin et al., 2004). The enzymology of NADH inhibition is discussed in detail in Appendix 2, along with the in vivo consequences of this inhibition in calorie-restricted cells.

The requirement of NAD for the Sir2 enzymatic reaction is also unexpected for thermodynamic reasons. The deacetylation reaction catalyzed by trichostatin A-sensitive HDACs, a simple hydrolysis reaction, is energetically favorable, begging the question of why Sir2 would couple such a reaction to the breakdown of NAD, a metabolically important compound (Bitterman et al., 2003). Curiously, NAD is not a cofactor in the Sir2-catalyzed reaction but is actually consumed by it, with hydrolysis of a glycosidic bond occurring between the nicotinamide and ADP-ribose moieties. The complete reaction catalyzed by Sir2 encompasses two hydrolysis steps that are likely coupled (Landry et al., 2000a; Min et al., 2001; Tanner et al., 2000). In the first of these, the aforementioned glycosidic bond within NAD is hydrolyzed, releasing a predicted 8.2kcal
of free energy per mol (Moazed, 2001). Subsequently, the C-N bond between the acetyl group and lysine is hydrolyzed in a deacetylation reaction that occurs with 1:1 stoichiometry. The final peculiarity of this reaction is that Sir2 catalyzes the transfer of the acetyl group to the ADP-ribose cleaved from NAD, resulting in the formation of O-acetyl ADP-ribose (AAR) (Jackson and Denu, 2002; Liou et al., 2005; Tanner et al., 2000).

Thus, the Sir2 reaction produces two major products in addition to deacetylated lysine: nicotinamide and AAR. While Sir2p is generally believed to influence silencing through the deacetylation of histone H4, it is formally possible that any of these three products of the Sir2 reaction could be required for creation and maintenance of the silenced state. Nicotinamide, a precursor of nicotinic acid in the cell, has been shown to strongly inhibit Sir2 enzymatic activity (Bitterman et al., 2002; Jackson et al., 2003) even doing so in several in vivo contexts, discussed below. The function of O-acetyl ADP ribose, however, has been somewhat elusive. This molecule was originally proposed to trigger some sort of signal transduction cascade, as metabolic instability of the type it displays is a hallmark of many signal transduction initiators (Jackson and Denu, 2002). Experimentally, injection of AAR has been shown to block cell cycle progression of Xenopus oocytes (Borra et al., 2002), consistent with this model. More recently, however, AAR has been shown to play an integral role in the assembly of the SIR complex in yeast, promoting the association of multiple copies of Sir3p with Sir2p/Sir4p and inducing important structural changes in this complex as a result (Liou et al., 2005).
**Caloric Restriction and the Regulation of Sir2p**

*Requirement of SIR2 and NAD+ in CR*

The question of how Sir2 is regulated emerged at the forefront of aging research with the observation that this protein is required for life span extension induced by caloric restriction (Lin et al., 2000). For almost 70 years, it has been known that decreasing the amount of food consumed by laboratory rodents significantly lengthens their life span and promotes youthful vigor (McCay et al., 1989). Studies have since shown that variation of calorie content was the critical factor retarding the aging process (Masoro, 1984a; Masoro, 1984b; Weindruch et al., 1988), and calorie restriction (CR), as it has come to be known, is now known to extend life span of diverse species ranging from yeast (Lin et al., 2000) to possibly even primates (Ingram et al., 2004). In yeast, CR can be accomplished by two different methods: reducing the glucose content of the growth media or inducing genetic mutations that decrease cellular glucose uptake or utilization. Both methods have succeeded in extending replicative life span, likely by the same mechanism (Kaeberlein et al., 2004b; Lin et al., 2000), as both methods combined do not synergize to give an even longer life span.

Of the dozens of genes ever reported to extend the yeast life span, those that mimic CR have shown most consistent effect on life span across multiple strain backgrounds (Kaeberlein et al., 2005). Yeast utilize glucose as a preferred source of carbon, tightly regulating the expression of a variety of cell surface glucose transporters of different substrate affinity depending on the concentration of glucose present in the growth media (Ozcan and Johnston, 1999; Rolland et al., 2001). Once inside the cell, glucose is phosphorylated by hexokinase to yield glucose-6-phosphate, a critical substrate
in glycolysis. Deletion of HXK2, one of the three genes encoding hexokinase enzymes, extends life span by a mechanism that does not synergize with caloric restriction (Kaeberlein et al., 2004b; Lin et al., 2000). This result has been interpreted to mean that \textit{hxk2A} mimics low glucose, a very plausible conclusion given that other genetic mutations predicted to reduce carbon flow through glycolysis also fail to synergize with \textit{hxk2A} (Lin et al., 2000).

The cyclic AMP (cAMP)-dependent protein kinase plays a critical role in the cellular response to glucose, increasing in activity when glucose is prevalent and decreasing when glucose in the extracellular medium is depleted (reviewed in (Thevelein and de Winde, 1999)). Several mutations reducing PKA signaling activity significantly increase life span, and at least one mutation causing constitutive activation of PKA shortens life span by 40% (Lin et al., 2000). Mutation of the adenylate cyclase (Cdc35p) and the GTP-GDP exchange factor Cdc25p both fall into the former category, and the temperature sensitive \textit{cdc25-10} allele has been used as a genetic model for CR in several studies (Lin et al., 2000; Lin et al., 2002).

In order to address the question of whether CR extends life span by a regulated mechanism, Lin and coworkers investigated a possible role for Sir2p in this process. Several lines of evidence now point to a key role for Sir2p in coordinating CR with an extension of life span. First, CR fails to extend the life span of both \textit{sir2A} and \textit{sir2A fob1Δ} mutants, indicating that Sir2p activity is necessary in order for CR to extend life span (Lin et al., 2000). Second, Npt1p, a component of the predominant cellular pathway of NAD biosynthesis, is also required for CR, presumably to make NAD available as a co-substrate for Sir2p under food-restricted conditions (Lin et al., 2000). Third, CR
increases Sir2 activity at the rDNA, increasing silencing, decreasing recombination, and reducing ERC accumulation, all three of which are tightly associated with life span extension by Sir2p (Lin et al., 2000; Lin et al., 2002).

Two models of Sir2p activation

Very interestingly, CR causes an increase in respiration necessary and sufficient to extend life span in a Sir2p-dependent fashion (Lin et al., 2002). In the presence of sufficient glucose concentrations, yeast cells rely on anaerobic fermentation for energy, even though this process is far less energy-efficient than respiration. Under conditions of CR, however, cells undergo a shift from fermentation to respiration, with the Hap4p transcription factor directing the expression of key genes required for this shift (Forsburg and Guarente, 1989). Overexpression of Hap4p in cells grown on glucose mimics CR by the criteria established for other genetic CR models (Lin et al., 2002). Like these other models, Hap4p overexpression requires Sir2p to extend life span. The exact mechanism by which increased respiration is translated into an increase in Sir2p activity is still controversial, although electron transport per se is definitely required upstream of Sir2p in the pathway. Yeast cells lacking cytochrome 1 (Cyt1p), an essential component of the mitochondrial electron transport chain, fail to exhibit an extension of life span or an increase in Sir2p activity when subjected to conditions of CR (Lin et al., 2002).

The metabolic shift to respiration induced by CR has been shown to cause an increase in the NAD/NADH ratio, although by decreasing NADH levels (Lin et al., 2004), not increasing NAD levels as originally predicted (Anderson et al., 2003b). As presented in detail in Appendix B, NADH, a key electron donor in respiration,
competitively inhibits Sir2p activity, such that a decrement in cellular NADH accounts for a significant increase in Sir2p activity during CR. In agreement with this model, overexpressing NADH dehydrogenase is sufficient to increase Sir2p activity and extend life span (Lin et al., 2004).

The major competing model explaining Sir2p activation by CR relies on a similar principle of relief-of-inhibition, although in this case the proposed inhibitor reduced by CR is nicotinamide, not NADH (Anderson et al., 2003a; Anderson et al., 2003b). Mechanistically, this model invokes changes in a key NAD salvage pathway enzyme to account for a reduction in nicotinamide when extracellular glucose is reduced (Anderson et al., 2002). This regimen results in upregulation of the nicotinamidase Pnc1p, which converts nicotinamide into nicotinic acid, a compound that does not inhibit Sir2p (Bitterman et al., 2002). Consistent with this model, CR largely fails to extend life span when PAIC1 is deleted. Also consistent with this model, overexpression of PAIC1 dramatically extends yeast life span in a Sir2p-dependent manner, and PAIC1-overexpressors do not live any longer when calorie restricted (Anderson et al., 2003a). Interestingly, Pnc1p is induced by several other life-extending conditions, including high osmolarity, mild heat stress, and low amino acid concentrations (Anderson et al., 2003a), although of these, only high osmolarity has been experimentally proven to mimic calorie restriction (Kaeberlein et al., 2002).

Sir2-independent effects of CR:

A recent report indicates that one particularly long-lived yeast strain, BY4742, demonstrates a Sir2-independent life span extension under CR (Kaeberlein et al., 2004b).
The authors found that CR elicited a greatly extended life span from sir2Δ fob1Δ mutants, and that CR synergized with fob1 deletion or SIR2-overexpression to create the longest-lived yeast strains ever reported. This interesting finding suggests that CR may activate more than one pathway affecting life span, raising the question of whether this new pathway bears any mechanistic similarity to the one already described. Notably, a study by another group investigating the Sir2-independent effects of CR has implicated Hst2p, the closest yeast Sir2 homolog, as a key player in the process (Lamming et al., 2005). Overexpression of HST2 suppresses rDNA recombination, promotes rDNA silencing, and extends life span. Deletion of this gene largely abrogates the effect of CR in a sir2Δ fob1Δ background, thus likely accounting for the Sir2p-independent effects of CR observed in the original study. Interestingly, Hst1p, another Sir2p homolog, plays a residual role in CR in the absence of Sir2p and Hst2p (Lamming et al., 2005), suggesting that Sir2 family members display a certain degree of flexibility in their roles within the cell. These results have raised the possibility that in other organisms multiple members of the Sir2 family might cooperate to promote longevity and survival in response to moderate food shortages. The Sir2p response to yeast CR is diagrammed in Figure 2.

Natural polymorphisms affecting yeast aging

As strain-dependent genetic differences account for much of the controversy surrounding the Sir2-independent effects of CR, it now becomes relevant to discuss the two examples of naturally polymorphic loci known to affect the aging process. These two loci, MPT5 (also known in some studies as UTH4 or PUF5) and SSD1, bear several striking similarities to one another. Both MPT5 and SSD1 are large genes characterized
by numerous polymorphisms in common laboratory strains (Kennedy et al., 1997; Sutton et al., 1991; Uesono et al., 1997). Both encode RNA-binding proteins that act in parallel to affect cell wall integrity, high temperature growth, and life span, as well as other global processes (Kaeberlein and Guarente, 2002). Both interact genetically with a wide array of mutations in diverse genes, and both function as post-transcriptional regulators (Gerber et al., 2004; Kaeberlein et al., 2004a; Tadauchi et al., 2001).

Mpt5p, a polymorphic repressor of translation

The first genetic screen for long-lived mutants in yeast identified mutations in four complementation groups that rescued the stress sensitivity and short life span of the starting strain (Kennedy et al., 1995). These complementation groups, termed UTH1-4, have formed the basis for many subsequent aging studies. In the course of analysis of the UTH mutants, cloning of UTH4 revealed it to be allelic to MPT5, and also led to the observation that the unmutagenized strain used in the screen actually contained a C-terminal truncation of MPT5, resulting in a hypomorphic Mpt5 protein product (Kennedy et al., 1997). Deletion of MPT5 shortens life span by 50%, and overexpression extends life span by up to 40% (Kennedy et al., 1995; Kennedy et al., 1997), underscoring the importance of this post-transcriptional regulator in the aging process. Cells lacking Puf5p/Mpt5p/Uth4p display increased telomeric silencing, decreased rDNA silencing, and an aberrant distribution of Sir3p in the absence of Sir4p (Gotta et al., 1997). In the absence of SSD1-V, mpt5 mutant cells also suffer from several deficiencies associated with a weakened cell wall, including sensitivity to calcofluor white, sensitivity to sodium
dodecyl sulfate (SDS), and sorbitol-remedial temperature sensitivity (Kaeberlein and Guarente, 2002).

Furthermore, in strains lacking SSD1-V, mpt5Δ is synthetically lethal in combination with deletion of either SBF or CCR4 (Kaeberlein and Guarente, 2002). SBF, a transcriptional activator consisting of the cell-cycle regulated proteins Swi4p and Swi6p, plays an important role in cell cycle-regulated transcription of the G1 cyclins CLN1 and CLN2 (Nasmyth and Dirick, 1991) and also acts downstream of protein kinase C (Pkc1p) to promote cell wall biosynthesis (Igual et al., 1996). Ccr4p, a component of the cytoplasmic deadenylase complex (Thore et al., 2003; Tucker et al., 2002) as well as a different transcriptional complex, is also required for PKC1-dependent transcriptional regulation of multiple genes required for proper cell wall structure (Chang et al., 1999). Genetic analysis suggests that Mpt5p, Ssd1p, and Pkc1p define three parallel pathways to ensure cell wall integrity (Kaeberlein and Guarente, 2002).

Mpt5p contains an RNA-binding domain homologous to that of the Drosophila PUMILIO protein, a translational repressor important for positional patterning in the developing embryo (Parisi and Lin, 2000). Of the five PUMILIO family members in yeast, Mpt5p was the first to be characterized as a post-transcriptional regulator (Tadauchi et al., 2001). Mpt5p has been shown to bind to sequence elements in the 3’ UTR of the HO endonuclease (Tadauchi et al., 2001), a protein catalyzing the critical DNA strand cleavage step of haploid mating type switching. The binding of Mpt5p results in repression of HO translation, and Mpt5p is believed to exert a similar effect on other mRNAs to which it binds (Gerber et al., 2004; Tadauchi et al., 2001). Indeed, a recent study systematically identified RNA targets of the yeast PUMILIO family of
proteins (Puf1-5p), uncovering a novel mechanism whereby specific RNA-binding proteins physically associate with functionally related groups of target RNA molecules to regulate their post-transcriptional properties (Gerber et al., 2004). This same study identified consensus binding sites for three of the five PUF proteins in yeast, finding that binding motifs typically resided within the 3'-UTRs of target genes, although a significant quantity were found within ORFs or, rarely, the 5'UTR.

**SSD1, another RNA binding protein**

Naturally occurring polymorphisms of the SSD1 gene fall into two phenotypic classes: active SSD1-V alleles and hypomorphic ssdl-d alleles, classified on the basis of their ability to suppress mutations in the Sit4p phosphatase (Sutton et al., 1991). SSD1-V, which suppresses sit4Δ, has also been isolated as a suppressor of diverse genetic mutations affecting the cell wall, PKA activity, TOR-signaling, and RNA metabolism (summarized in (Kaeberlein et al., 2004a)). Addition of SSD1-V dramatically extends lifespan of ssdl-d strains by a novel, as yet uncharacterized mechanism. This mechanism is Sir2p-independent, affecting neither rDNA silencing nor ERC formation, two processes linked to yeast aging (Kaeberlein et al., 2004a). Significantly, addition of SSD1-V is the only intervention ever shown to extend the life span of a sir2Δ fob1Δ double mutant. Although SSD1 is highly conserved from yeast to humans, little is known of its biochemical function. SSD1 is discussed in detail in Chapter 2 as well as Appendix A.
Homologs of MPT5 and SSD1 in multicellular organisms

Both Mpt5p and Ssd1p are very highly conserved proteins, although the metazoan homologs of Mpt5p are far better characterized than those of Ssd1p. Interestingly, there are several conceptual similarities linking Mpt5p to its Drosophila and C. elegans homologs PUMILIO and FBF. These three homologs are all translational repressors that bind conserved sequence elements found in the 3’-UTR. And all three proteins possess the eight consecutively repeated PUMILIO homology domains conferring RNA binding activity and defining the PUF family.

Early in embryogenesis, the Drosophila PUMILIO protein binds maternal hunchback mRNA to repress hunchback translation at the posterior pole by a mechanism also requiring the NANOS protein (Parisi and Lin, 2000). In addition to this developmental role, PUMILIO is required for the maintenance of germ line stem cells in Drosophila (Forbes and Lehmann, 1998; Lin and Spradling, 1997). C. elegans FBF, which regulates germ cell fate through a repressive interaction with GLD-1 mRNA, is also required for germ line stem cell maintenance in this organism (Crittenden et al., 2002). Thus, both PUMILIO and FBF promote continued cell divisions and delay the onset of an alternate state of differentiation and developmental potency. Intriguingly, Mpt5p also conforms to this common theme, using specific targeted translational repression to prolong cell divisions and maintain potential to produce young cells. The metazoan homologs of Ssd1p still await characterization and may provide likely candidates for regulators of development and cell fate analogous to those of the PUF family.
**Chronological Aging in Yeast**

An alternative means of defining and measuring yeast life span is to assess the survival of cells maintained in a non-dividing state. This parameter is known as chronological aging, as it correlates survival with time as measured in days rather than cell division cycles. The most common assay for chronological aging is a post-diauxic survival test in which cells grown to the post-diauxic phase are kept in culture, and viability is measured at successive time points by the plating of cells and measurement of colony formation. Aging under these conditions appears to be largely attributable to damage from reactive oxygen species (ROS), as mutations in catalase and superoxide dismutase accelerate the loss of viability (Longo et al., 1996; Longo et al., 1999) and mutations increasing paraquat resistance prolong survival under post-diauxic conditions (Fabrizio et al., 2003; Fabrizio et al., 2001). Only two genes have so far been reported to increase both post-diauxic aging and replicative aging, and these genes are *SCH9* and *CYR1* (Fabrizio et al., 2004; Fabrizio et al., 2001). *SCH9* encodes a serine/threonine kinase that acts in parallel to components of the PKA pathway to affect stress resistance and response to glucose. *CYR1* encodes adenylate cyclase, which produces cAMP in response to glucose uptake by a mechanism described above. This process is essential for PKA activity, a reduction of which is known to increase stress resistance by downstream activation of the Msn2p and Msn4p transcription factors (Rolland et al., 2001).

By this token, the dual effects of *cyr1* mutation on replicative and post-diauxic aging can be rationalized by downstream activation of stress response genes (predicted to prolong survival in non-dividing cells) and respiration-dependent activation of Sir2p
(predicted to prolong replicative life span). It is not clear whether sch9Δ requires Sir2p in order to extend replicative life span, but it has been shown that sch9 mutation extends chronological life span by a mechanism requiring Sod2p (Fabrizio et al., 2003). Interestingly, the effects of sch9Δ on replicative life span are very well conserved between several different yeast strains (Kaeberlein et al., 2005), and this gene, like FOB1, displays a defect in HOT1-dependent recombination when mutated (Defossez et al., 1999). It is therefore possible that sch9Δ might promote longevity by correcting a defect similar to that corrected by deletion of FOB1. Future studies should aim to integrate SCH9 into existing genetic pathways of aging.

CONSERVATION OF YEAST AGING PATHWAYS IN METAZOANS

A critical question facing the model systems approach to aging is to what extent findings from such model systems are conserved across phyla. Intriguingly, the last several years have provided numerous reports confirming that aging and longevity genes can, in fact, affect similar aspects of the aging process in very distantly related species.

Insulin/IGF-1 pathway

Sch9, like Sir2, has been shown to affect aging in at least two species whose last common ancestor existed a whopping one billion years ago (Guarente and Picard, 2005). The kinase domain of Sch9p is highly conserved, showing 47-49% identity to its closest worm homologs AKT-1 and AKT-2. AKT-1 and AKT-2 act downstream of the DAF-2 insulin receptor homolog, regulating longevity, stress resistance and the diapause state in
response to a conserved PI-3 kinase signaling cascade (Guarente and Kenyon, 2000; Paradis and Ruvkun, 1998). Depending on the severity of the mutation, defects in genes of the DAF-2 pathway cause either dauer formation or extended adult life span, with more mild mutations generally favoring the latter result (Johnson, 1990; Kenyon et al., 1993). The longevity of daf-2 mutants is known to require several genes, including DAF-16 (a forkhead/winged helix transcription factor), heat shock protein HSF-1, and the AMP-dependent kinase AAK-2 (Garigan et al., 2002; Henderson and Johnson, 2001; Hsu et al., 2003; Lee et al., 2001; Lin et al., 2001; Morley and Morimoto, 2004). The insulin/IGF-1 pathway, like the PKA and Sch9 pathways in yeast, normally downregulates nutrient storage and stress responses (Kenyon, 2005). It is therefore especially interesting that mutating such a pathway would extend life span in both species, as it implies not only conservation of specific aging genes but conservation of an overall approach to regulating the aging process (Figure 3).

Other mutations in the insulin/IGF-1 pathway have been shown to extend life span in worms, flies, and even mice. In Drosophila, mutations of the insulin/IGF-1 receptor increase life span by variable amounts of up to 80% (Tatar et al., 2001). Similarly, mutations in the downstream *chico* insulin receptor substrate (IRS-1) extend life span by up to 40% (Clancy et al., 2001; Tu et al., 2002a; Tu et al., 2002b). Although no experiments have yet linked these phenotypes to the DAF-16 homolog FOXO, it is strongly predicted that extension of life span in IRS and insulin/IGF-1 receptor mutants requires FOXO activation. FOXO overexpression has been shown to extend fly life span (Giannakou et al., 2004), and the FOXO transcription factor is known to lie downstream
of insulin/IGF-1 signaling for at least one other phenotype, namely reduced cell division (Junger et al., 2003).

In mammals, the analogous genetic pathways are far more complex, but similar results have nonetheless been reported. In mice, which have distinct receptors for insulin and IGF-1, female mutants heterozygous for the IGF-1 receptor live about 30% longer than wild type controls (Holzenberger et al., 2003). Also, mice lacking the insulin receptor in adipose tissue display an 18% life span increase compared to controls (Bluher et al., 2003). Taken together, these results suggest that through evolution both the insulin and IGF-1 pathways have retained an influence on life span regulation.

Similar to the case of Drosophila, a downstream connection to FOXO is also strongly suspected but has yet to be demonstrated for long-lived mouse insulin and IGF-1 mutants. FOXO transcription factors act downstream of insulin and IGF-1 to effect changes in metabolism (Burgering and Kops, 2002; Kops et al., 2002), so it is conceivable that they play a similar role in the pathways affecting aging. A recent study has shown that p66shc mutation, which increases stress resistance as well as organismal longevity (Migliaccio et al., 1999), requires FOXO proteins, at least in order to confer cellular resistance to stress (Nemoto and Finkel, 2002).

**SIR2 and Calorie Restriction**

In *C. elegans*, Sir2 ortholog Sir2.1 extends life span, albeit by a mechanism differing from the one at work in yeast cells. Worms likely do not age as a result of ERC accumulation, as no such accumulation has ever been observed in this predominantly post-mitotic organism (Guarente lab, unpublished data). Life span extension by Sir2.1
instead requires the forkhead transcription factor DAF-16, as DAF-16 mutants live the same length regardless of Sir2.1 levels (Tissenbaum and Guarente, 2001). DAF-16, a key downstream element in the insulin-like signaling pathway, is therefore likely to act downstream of Sir2.1 as well. Unpublished results from our lab indicate that this is indeed the case (Ala Berdichevsky, personal communication). These findings in worms are especially interesting in light of recent studies uncovering interactions between mammalian SIRT1 and FOXO proteins homologous to the C. elegans DAF-16 (Brunet et al., 2004; Motta et al., 2004; van der Horst et al., 2004). These results are discussed in detail later on in this chapter.

Studies from Drosophila melanogaster support a conserved role for Sir2 in the response to CR, a surprising finding considering the vast evolutionary distance between yeast and fruit flies. In flies, food restriction is accomplished by limiting the yeast content of the flies’ diet, and this intervention succeeds in extending life span significantly (Clancy et al., 2002). Several lines of evidence support the claim that Sir2 upregulation plays an indispensable role in the fly response to CR. Caloric restriction in flies increases Sir2 mRNA levels (Rogina et al., 2002), and the overexpression of Sir2 using a UAS/Gal4 driver is sufficient to extend life span (Rogina and Helfand, 2004). This extension of life span does not synergizes with CR, suggesting that the two treatments constitute only one pathway (Rogina and Helfand, 2004). Further support for this model comes from experiments with the Sir2-activator resveratrol, which, like CR, extends life span in wild type but not Sir2 mutant flies (Rogina and Helfand, 2004; Wood et al., 2004). Like overexpression of Sir2, resveratrol treatment fails to further extend the long life span of CR flies.
These findings hint at a conserved role for Sir2 proteins in life span regulation, especially in response to CR. A significant effort now exists to establish a role for Sir2 in the mammalian response to calorie restriction, and to determine to what extent, if any, findings from lower organisms apply to the more sophisticated mammalian systems. To that end, numerous studies have already defined a role for mammalian Sir2 in the promotion of cell survival and division in the face of potentially apoptotic stresses (reviewed in (Blander and Guarente, 2004; Guarente and Picard, 2005). Mammalian genomes contain seven Sir2 homologs, termed sirtuins (SIRTs). Of these, SIRT1, orthologous to the yeast Sir2, is the best characterized and has the broadest substrate specificity (Blander and Guarente, 2003). A nuclear protein, SIRT1 deacetylates several non-histone substrates in vivo, including MyoD, p53, and FOXO transcription factors, thereby affecting cell differentiation and survival under stress (Brunet et al., 2004; Motta et al., 2004; North and Verdin, 2004).

In mammals, FOXO transcription factors mediate the metabolic output of IGF-1 and insulin pathways and also play a key role in the apoptotic response to stress. SIRT1 has been shown by several groups to deacetylate FOXO1, FOXO3, and FOXO4 (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004), thereby repressing FOXO-mediated apoptosis. Strangely, there are also reports of FOXO target genes activated by SIRT1 (Brunet et al., 2004; Daitoku et al., 2004; van der Horst et al., 2004), although no mechanism has yet been established to explain these findings.

These FOXO studies are not the only reports linking SIRT1 to regulation of the insulin pathway in mice. Very recent work has also defined a role for SIRT1 in pancreatic β-cells, where it enhances insulin secretion in response to glucose, improving
glucose tolerance (Moynihan et al., 2005). It is not yet known whether these conditional mutants overexpressing SIRT1 in β-cells show an extension in life span.

Recently, mouse SIRT1 was reported to promote the mobilization of fatty acids in white adipose tissue by repressing PPARγ, linking Sir2 proteins to the physiology of caloric restriction in mammals (Picard et al., 2004). Several lines of evidence suggest that fat storage in the white adipose tissue (WAT) may play an important role in mammalian aging. Both the FIRKO mice (Bluher et al., 2003) and C/EBPβ knock-in mice (Chiu et al., 2004), both genetically engineered to be lean, live longer than controls. These observations can be rationalized in the context of CR where WAT, a known endocrine tissue secreting such important hormones as leptin and adiponectin, shrinks in size as cells lose fat in response to lowered food intake. WAT therefore provides a likely candidate for a diet sensor that could then secrete hormones adjusting the rate of aging in the organism as a whole.

Although appealing, this model is not without its inconsistencies. Notably, when leptin-deficient ob/ob mice are subjected to CR, these mice live as long as wild type CR animals, even though the ob/ob mice are fatter than not only wild type CR animals, but wild type animals fed an ad libitum diet (Harrison et al., 1984). Also, one report comparing animals within each CR or ad libitum group describes a slight positive correlation between body fat and longevity within each group (Bertrand et al., 1980). Both of these findings fail to support a key prediction of the WAT model of aging, which is that reduction of WAT should correlate with extended life span.

Nevertheless, SIRT1 has been shown to negatively regulate the fat cell differentiation of 3T3-L1 pre-adipocytes (Picard et al., 2004). This effect is explained
by the observation that SIRT1 binds to the PPARγ negative cofactors NCOR and SMRT and can be found at the promoters of fat-specific genes containing PPARγ binding sites (Picard et al., 2004). In animals, SIRT1 also plays a role in the mobilization of fat following fasting. In SIRT1 heterozygous knockouts, lipolysis of triglycerides and release of free fatty acids into the blood was reduced (Picard et al., 2004), although some mechanistic details of this process are still uncharacterized (Bordone and Guarente, 2005), such as how a decrease in food uptake activates SIRT1 in animals.

In cultured cells, acute nutrient withdrawal activates FOXO3- and p53-dependent transcription of SIRT1, whose levels consequently increase (Nemoto et al., 2004). Interestingly, p53 and FOXO3 physically interact in response to nutrient stress, and the induction of SIRT1 depends on two p53 binding sites located in the SIRT1 upstream sequence. In rats, caloric restriction increases levels of SIRT1 (Cohen et al., 2004b), inhibiting stress-induced apoptosis by a mechanism involving Ku70 and Bax. Specifically, deacetylation of Ku70 by SIRT1 allows this DNA repair protein to bind to and inactivate Bax, a proapoptotic factor (Cohen et al., 2004a; Cohen et al., 2004b).

Other sirtuins

Of the remaining SIRTs (SIRT2-7), an in vivo substrate has only been identified for the cytoplasmic SIRT2 (North et al., 2003). This substrate, β-tubulin, is specifically deacetylated by SIRT2 (North et al., 2003), although the biological consequences of this reaction are unclear. While the archetypal yeast Sir2p is generally believed to be a histone deacetylase, most mammalian sirtuins are non-nuclear (Michishita et al., 2005), strongly suggesting that they act on non-histone substrates.
Using sequence similarity, eukaryotic Sir2 genes have been divided into four broad phylogenetic classes (Frye, 2000), known as classes I-IV (Figure 1). SIRT1, SIRT2, and SIRT3 are class I, SIRT4 is class II, and SIRT5 falls within the predominantly prokaryotic class III. Finally, mammalian SIRT6 and SIRT7, are class IV sirtuins (Frye, 2000). In vitro studies indicate that human SIRT1, 2, 3, and 5 possess NAD-dependent histone deacetylase activity (North et al., 2003), whereas SIRT4, 6, and 7 fail to deacetylate $^3$H-labeled acetylated histone H4 peptide (North et al., 2003). The lack of detectable deacetylase activity in these SIRTs may result from their specificity for targets other than those tested, or it may indicate an enzymatic activity other than deacetylation inherent in these sirtuins.

To further our understanding of the Sir2 family of proteins in mammals, we have undertaken research on the molecular characteristics and functions of the two class IV sirtuins SIRT6 and SIRT7. As we present in Chapter 3 and Appendix C, SIRT6 and SIRT7 are both nuclear proteins, with SIRT7 found exclusively in the nucleolar subcompartment. While SIRT6 displays an auto-ADP-ribosyltransferase activity uncharacteristic of Sir2 family members, SIRT7 apparently lacks this activity. SIRT7, however, plays a unique role as an activator of RNA polymerase I transcription, physically interacting with the Pol I complex and promoting transcription through the greater association of this complex with the rDNA.
Summary and Conclusions

Many organisms appear to possess a genetically conserved mechanism by which to forestall aging in response to environmental cues. In yeast, this mechanism hinges on Sir2p, whose activity at the rDNA is essential for longevity. Interestingly, the characterization of Sir2 in yeast has led to several insights about aging in other organisms, and it now seems likely that Sir2 proteins play a conserved role in the response to calorie restriction in flies and even mammals.

And Sir2 is not the only longevity-associated gene implicated in a conserved pathway regulating aging. Notably, the insulin/IGF-1 hormonal signaling pathway can be mutated to forestall aging in worms, flies and mice, suggesting that current studies have only scratched the surface of a vast network of aging-regulatory genes that also coordinate energy homeostasis, stress resistance, and reproduction.

The mission of the field is now to continue the molecular characterization of longevity genes in model systems while simultaneously applying our current knowledge to mammalian systems. With this goal in mind, we present the research of this thesis. Chapter 2 and Appendix A aim to deepen the understanding of yeast aging, providing a model for the action of SSD1-V, one of only two natural polymorphisms known to affect this process. Appendix B visits the issue of Sir2p regulation by calorie restriction, providing evidence that an increase in the cellular NAD/NADH ratio, occurring via a decrease in NADH, results in activation of Sir2p and extension of life span.

Finally, Chapter 3 and Appendix C present the first characterizations of class IV sirtuins, SIRT6 and SIRT7. In light of the recent finding that multiple sirtuins can
collaborate to extend life span in yeast, it is especially interesting to know to what extent
the role of Sir2 has been conserved and distributed among its various homologs.
REFERENCES


FIGURES

Figure 1. Survival curves from distantly related species display similar shapes. Typical mortality data from *Homo sapiens* (Sinclair et al., 1998), *Mus musculus, Caenorhabditis elegans* (Tissenbaum and Guarente, 2001), and *S. cerevisiae* (Kaeberlein et al., 1999) are presented. This figure adapted from Matt Kaeberlein, Genetic Analysis of Longevity in Saccharomyces cerevisiae, Graduate Thesis, MIT 2002. Reproduced here by permission.
FIGURE 1
Figure 2. Caloric restriction (CR) in yeast increases budding life span by activating Sir2p. Two separate mechanisms relieve inhibition of Sir2p (and its homolog Hst2p) to promote rDNA stability and longevity. CR also induces a Sir2p- and Hst2p-independent extension of life span by an unknown mechanism.
Yeast Caloric Restriction

↑ Respiration

↑ NAD+/NADH Ratio

↑ Pnc1p

↓ Nicotinamide

↑ Sir2p (and Hst2) Activity

↑ rDNA Stability

↓ LONGLEVITY

FIGURE 2
Figure 3. Components of the insulin/IGF-1 signaling pathway promote life span in yeast, worms, flies, and mice. Details of each pathway are described in the text. Question marks indicate plausible regulatory connections within each pathway, also discussed in the text.
Chapter 2

Ssd1p Directly Represses Translation of Uth1p to Increase Longevity, Stress Resistance, and Cell Wall Stability in Saccharomyces cerevisiae

This chapter will be submitted for publication. The authors are Gregory Liszt, Matt Kaeberlein, Sarah Buckley, and Leonard Guarente. I contributed Figures 1c, Figure 4, Figure 5, Figure 6, and Figure 7, and all unpublished observations referred to in the text. I also developed the mechanistic model and wrote the manuscript.
SUMMARY

In yeast, natural polymorphisms at the SSD1 locus strongly affect aging, stress resistance, pathogenicity, and cell wall integrity. The active SSD1-V allele promotes longevity by a novel mechanism independent of rDNA recombination and extrachromosomal rDNA circle (ERC) formation, two processes tightly linked to known pathways of yeast aging. Here, we report that Ssd1p, an RNA-binding protein, strongly represses the in vivo translation of Uth1p, one of the original proteins implicated in the yeast aging process. Deletion of UTH1 from ssd1-d cells phenocopies the addition of SSD1-V, causing a long life span and unique stress resistance profile. Furthermore, SSD1-V and uth1Δ both suppress mutations in several apparently unrelated genes including MPT5, SIT4, and CCR4. Uth1p levels are significantly reduced in SSD1-V cells, although the level of the UTH1 message is not influenced by SSD1 under normal growth conditions. Ssd1p physically interacts with the UTH1 mRNA, and these molecules can be copurified from cells. Interestingly, this interaction depends on sequence elements in the 5'-UTR of UTH1. SSD1-V cells lacking the 5'-UTR of UTH1 fail to show repression of Uth1p and phenotypically resemble ssd1-d cells. These results demonstrate that the direct translational repression of Uth1p by Ssd1p is necessary and sufficient to account for several phenotypic hallmarks of SSD1-V cells. These results also suggest that many of the defects of ssd1-d cells arise from a misregulation of Uth1p.
INTRODUCTION

In *Saccharomyces cerevisiae*, dividing mother cells undergo an aging process that limits their replicative potential and induces pronounced changes in cell morphology, metabolism, genomic stability, transcriptional silencing, and the cell wall (Bitterman et al., 2003; Sinclair et al., 1998). Mutations that extend the yeast replicative life span have been used to study aging, and several of these mutations have led to the discovery of conserved mechanisms regulating aging in such diverse organisms as worms, flies, and even mice (Guarente and Picard, 2005; Kenyon, 2005).

With successive cell divisions, instability at the rDNA locus and extrachromosomal rDNA circle (ERC) accumulation exponentially increase the likelihood of senescence and mortality in yeast mother cells (Sinclair and Guarente, 1997). Aging can be forestalled by caloric restriction, a regimen that increases the activity of Sir2p (Anderson et al., 2003; Lin et al., 2000; Lin et al., 2004; Lin et al., 2002), an important regulator of aging in yeast and some metazoans (Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001; Wood et al., 2004).

*SSD1* is a polymorphic locus affecting diverse cellular processes including high temperature growth, cell integrity (Kaeberlein and Guarente, 2002), pathogenicity (Wheeler et al., 2003), and mother-cell aging (Kaeberlein et al., 2004a). Naturally occurring polymorphisms of the *SSD1* gene fall into two phenotypic classes: active *SSD1-V* alleles and inactive *ssd1-d* alleles, classified on the basis of their ability to suppress mutations in the Sit4p phosphatase. *SSD1-V*, which suppresses *sit4Δ* lethality, has also been isolated as a suppressor of diverse genetic mutations affecting the cell wall stability, protein kinase A activity, TOR-signaling, and mRNA transcription and
processing (summarized in (Kaeberlein et al., 2004a). Addition of SSD1-V dramatically extends lifespan of ssd1-d strains by a novel, as yet uncharacterized mechanism (Kaeberlein et al., 2004a). This mechanism is Sir2p-independent, affecting neither rDNA silencing nor ERC formation, two processes linked to yeast aging (Kaeberlein et al., 2004a). Although SSD1 is well conserved from yeast to humans, little is known of its biochemical function. A previous study has demonstrated that Ssd1p, a cytoplasmic protein, can bind RNA in vitro (Uesono et al., 1997), but it is not known whether this activity is physiologically significant.

RNA binding proteins have been shown to regulate a variety of post-transcriptional processes including mRNA localization and stability, translation and splicing (Kuersten and Goodwin, 2003). RNA-binding proteins feature prominently in metazoan development, often as repressors of translation (Dreyfuss et al., 2002; Johnstone and Lasko, 2001; Maniatis and Reed, 2002; Mazumder et al., 2003). For example, early in embryogenesis, the Drosophila PUMILIO protein binds maternal hunchback mRNA via sequence elements in the 3' untranslated region (UTR), repressing hunchback translation at the posterior pole by a mechanism also requiring the NANOS protein (Parisi and Lin, 2000). Similarly, the precise control of translation and localization results in highly regulated expression of other mRNAs including BICOID, OSCAR, and NANOS itself (Johnstone and Lasko, 2001). In C. elegans, the RNA-binding protein GLD-1 influences several germ cell fate decisions by repressing the translation of numerous maternal mRNAs, often in a spatially dependent manner (Jan et al., 1999; Lee and Schedl, 2001; Lee and Schedl, 2004; Marin and Evans, 2003; Mootz et al., 2004; Xu et al., 2001). Interestingly, GLD-1 also represses translation of CEP-1/p53
to affect germ cell apoptosis in response to DNA damage (Schumacher et al., 2005). The translation of the GLD-1 message is, in turn, regulated by the RNA-binding protein FBF (fem-3 mRNA Binding Factor; (Crittenden et al., 2002; Zhang et al., 1997). FBF consists of two nearly identical proteins, both of which contain the eight consecutively repeated PUMILIO-homology domains required for RNA-binding activity in the *Drosophila* protein (Zamore et al., 1997).

Notably, a recent study systematically identified RNA targets of the yeast PUMILIO-FBF family of proteins (Puf1-5p), uncovering a novel mechanism whereby specific RNA-binding proteins physically associate with functionally related groups of target RNA molecules to regulate their post-transcriptional properties (Gerber et al., 2004).

The best characterized of the five yeast Pumilio family members is Puf5/Mpt5/Uth4, a protein important for pheromone response (Chen and Kurjan, 1997), life span regulation (Kennedy et al., 1995; Kennedy et al., 1997), and cell wall integrity (Kaeberlein and Guarente, 2002). Deletion of *MPT5* shortens life span by 50%, and overexpression extends life span by up to 40% (Kennedy et al., 1997), underscoring the importance of this post-transcriptional regulator in the aging process. Cells lacking Puf5/Mpt5/Uth4 display increased telomeric silencing, decreased rDNA silencing, and an aberrant distribution of Sir3p in the absence of Sir4p (Gotta et al., 1997). In the absence of *SSDI-V*, *mpt5Δ* mutant cells also suffer from several deficiencies associated with a weakened cell wall, including sensitivity to calcofluor white (CFW), sensitivity to sodium dodecyl sulfate (SDS), and sorbitol-remedial temperature sensitivity (Kaeberlein
and Guarente, 2002). Genetic analysis suggests that Mpt5p, Ssd1p, and Pkc1p define three parallel pathways to ensure cell wall integrity.

Uth1p is a member of a family of proteins specific to fungi, known as the SUN family of proteins because its members include Sim1p, Uth1p, and Nca3p as well as the more recently identified Sun4p (Mouassite et al., 2000b). The UTH1 gene was originally identified as a loss-of-function suppressor of the short life span and stress sensitivity caused by a C-terminal truncation of Mpt5p (Kennedy et al., 1995). Subsequent studies have shown that deletion of UTH1 increases life span, resistance to peroxides (Bandara et al., 1998), and resistance to starvation- and rapamycin-induced autophagy (Kissova et al., 2004). Uth1p localizes to the outer mitochondrial membrane and the cell wall, and has been shown to be highly glycosylated (Velours et al., 2002).

We noticed that deletion of UTH1, like addition of SSD1-V, suppresses the cell wall defects and short life span of an mpt5Δ ssdl-d mutant. This similarity between SSD1-V and uth1Δ prompted us to further investigate the relationship between these two genes. Here, we present evidence that Ssd1p directly represses Uth1p, and by that repression effects large changes in stress resistance, life span, and cell wall integrity.

**MATERIALS AND METHODS**

**Strains and genetic techniques.** The strains used in this study are listed in Table 1. All strains were derived from either PSY316 (described in (Park et al., 1999)) or W303R (described in (Mills et al., 1999)). W303R has previously been shown to carry the ssd1-d2 allele (Sutton et al., 1991). PSY316 contains an ssd1-d nonsense mutation predicted to truncate Ssd1p at codon 132 (Nicholas Bishop, personal communication). Yeast transformations were accomplished using the
lithium acetate method (Gietz et al., 1992). Genetic crosses, sporulation, and tetrad analysis were carried out by standard techniques (Sherman and Hicks, 1991). Marker segregation in viable spore clones was used to infer the genotype of inviable spore clones from the same tetrad whenever possible. The ccr4::HIS3, swi6::TRP1, mpt5::LEU2, and mpt5::HIS3 disruptions were generated using plasmids described in (Kaeberlein and Guarente, 2002). All other gene deletions were done by transforming cells with PCR-amplified disruption constructs. For each gene disruption, the entire open reading frame (ORF) was removed except for SWI4 disruption, which was done by replacing the first 2kb of the SWI4 ORF with HIS3. All disruptions were verified phenotypically, by PCR, or both. The SSD1-V integrating plasmids p406SSD1 and p405SSD1, as well as the ARS-CEN variants of those plasmids (p416SSD1 and p415SSD1) were previously described (Kaeberlein and Guarente, 2002). Unless otherwise indicated, all SSD1-V strains contain SSD1-V integrated at the marker locus and still carry the ssd1-d allele at the native locus. Deletion of ssd1-d does not affect any of the phenotypes tested, including life span, growth at 30°C, 37°C, or 40°C, or sensitivity to calcofluor white, paraquat or hydrogen peroxide (Kaeberlein and Guarente, 2002; Kaeberlein et al., 2004b).

A yeast strain of the BY4741 background containing a tandem affinity purification (TAP) tag integrated in-frame at the 3' end of the SSD1 gene was obtained from OpenBiosystems (Ghaemmaghami et al., 2003). The following primers were used to PCR amplify a region of SSD1-TAP encompassing the final 1785 bp of the SSD1 ORF as well as the entire affinity tag:

\[
\text{T A A G G G A T A A C A A T T T T C T T (f o r w a r d \ p r i m e r) a n d T T T G C G G C C G C G A A A G A G T T A C T C A A G A A T A A A} \text{ (reverse primer). This PCR product was digested with BstBI and NotI and ligated into plasmid p416SSD1 cut with the two same enzymes. The resulting plasmid p416SSD1-TAP was transformed into PSY316AR, and expression of an active fusion protein was confirmed by phenotype and Western blot using a rabbit polyclonal anti-TAP antibody (OpenBiosystems).}
\]
The \textit{UTH1}\textsubscript{TERMINATOR}:\textit{ADH1}\textsubscript{TERMINATOR}:kanMX6 replacement was constructed by one-step PCR-mediated gene disruption as follows. Plasmid pFA6-3HA-kanMX6 was used as a template to generate the disruption construct, as this plasmid contains the \textit{ADH1}\textsubscript{TERMINATOR} sequence (Longtine et al., 1998). This sequence was amplified with the following primers incorporating regions flanking the \textit{UTH1} terminator sequence (indicated in uppercase):

\begin{verbatim}
TTCAGTTACTTCTGGTTCTGCTAACTTTGTCTTCTACTAGaccttaataagegaattt (forward)
TGTTATATAATATGCTATATATGATAATATCTAGTGGTATgaattcgctgttaaac (reverse).
\end{verbatim}

This construct was transformed into strain GLY3002 and gene replacement was verified by PCR.

\textbf{Spot Assays.} Calcofluor white (Fluorescence Brightener 28, Sigma), hydrogen peroxide, and paraquat (Methyl Viologen, Sigma) were added at the designated concentrations to cooled (<60°C), premixed YPD containing 3% agar (w/v). Plates were dried at 37°C and used for serial dilution spot assays the same day. These assays were performed by first growing cells overnight in YPD at 30°C, then diluting cultures back 50-fold in fresh YPD and allowing to grow for an additional 3-4 hours. For each strain, a series of 10-fold dilutions was prepared in fresh YEP over a range of concentrations from $10^{-4}$ to $10^{-5}$ relative to the initial culture. Spots of 5µL from each dilution series were then plated to the appropriate media using a multi-channel pipettor.

\textbf{Western Blotting.} Total protein extracts from yeast were prepared as described (Kushnirov, 2000) by brief NaOH treatment followed by boiling in optimized SDS sample buffer. From 2.5 O.D. units of cells, 50µL SDS-solubilized proteins were prepared, a 10µL quantity of which was used for Western analysis. Samples were resolved by SDS-PAGE on a 4-15% Tris-HCl gradient gel (Bio-Rad), transferred to nitrocellulose membrane and blocked using 4% nonfat dry milk in PBS with 0.1% Tween-20 (PBS-T). Uthlp was detected by incubation with rabbit polyclonal anti-Uthlp antisera diluted 1: 2,500 in PBS-T. This antibody, a generous gift from S. Manon, has been characterized previously (Velours et al., 2002). Peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Biosciences) was used at a dilution of 1: 10,000.
Chemiluminescent detection was achieved by incubation with the ECL reagent (Amersham Biosciences). Blots were stripped for 20 minutes in 50°C stripping buffer (62.5 mM Tris-HCl pH 7.5, 2% SDS, 100 mM beta-mercaptoethanol) and reprobed with anti-β tubulin as a loading control.

**Northern Blotting.** Total cellular RNA was prepared and purified using the Qiagen Rneasy kit according to the manufacture's instructions. Briefly, cells were grown to early log phase, and 2.5 O.D. units of cells were harvested and resuspended in 2 mL buffer Y1 (1M sorbitol, 0.1M EDTA pH 7.4, 0.1% β-mercaptoethanol, 100 U/mL zymolyase 100-T). Cells were incubated at 30°C for 25 minutes to create spheroplasts. Subsequently, spheroplasts were lysed, and the lysate was applied to a Qiagen Rneasy column, washed three times and eluted two times in 30 μL Rnase-free water each time. Final RNA concentrations were measured by spectrophotometer, and samples were concentrated in a speed-vac evaporator and resuspended in Rnase-free water at a final concentration of 3μg/μL. For each sample, 12 μg total RNA (4μL) was mixed with three volumes of formaldehyde loading buffer (Ambion) and denatured for 15 minutes at 65°C. Ethidium bromide was added to a final concentration of 10 μg/mL, and samples were run on a formaldehyde denaturing gel (1 % Agarose, 1x MOPS, 0.6 M formaldehyde). The gel was run at 100 V for 2.5 hours in gel running buffer (1x MOPS, 0.2 M formaldehyde), bands were visualized and photographed under UV light, and the gel was transferred overnight to GeneScreen membrane (NEN) by capillary transfer in 10X SSC buffer as described (Ausubel, 1999). Membranes were UV-crosslinked two times in a Stratagene Stratalinker and prehybridized for one hour at 42°C in pre-warmed UltraHybe solution (Ambion). During this period, the PrimeIt Random Primer labeling kit (Stratagene) was used to generate 32P radiolabeled probe corresponding to a 350 bp region of the *UTH1 ORF*. Blots were probed overnight and on the next day washed twice under both low stringency (2x SSC, 0.1% SDS; 5 minutes at room temperature) and high stringency (0.2x SSC, 0.1% SDS; 15 minutes at 42°C) conditions. Blots
were analyzed by autoradiography, stripped for 10 minutes in 5% SDS at 95° C, and reprobed for 
ADHI by the same method.

**Affinity purifications.** TAP purifications were conducted essentially as described (Gerber et al., 2004) with a few modifications. A 1 L YPD culture of cells grown to O.D. 0.7 at 30° C was harvested by centrifugation, washed twice with ice cold buffer A (20 mM Tris-HCl pH 8.0, 140 mM KCl, 1.8 mM MgCl2, 0.1% NP-40, 0.02 mg/mL heparin) and resuspended in 5 mL buffer B (buffer A plus 0.5mM DTT, 20 U/mL DNAse I, 100 U/mL RnaseOut (Invitrogen), 0.2 mg/mL heparin, and protease inhibitors (Roche)). Cells were subjected to glass bead lysis and whole cell extracts were incubated with 400μL (50% (v/v)) IgG-agarose beads (Sigma) for 4h at 4° C. Beads were washed four times for 15 min each wash at 4° C in buffer C (20 mM Tris-HCl pH 8.0, 140 mM KCl, 1.8 mM MgCl2, 0.5 mM DTT, 0.01% NP-40, 10 U/mL RnaseOut). TAP-tagged Ssd1p was released from the beads by cleavage with 100 U of TEV protease for 1.5 h at 16° C with gentle periodic agitation. RNA was isolated from final eluates as well as whole cell extracts using the Rneasy kit (Qiagen). Final RNA concentrations were determined by spectrophotometric measurement.

**RT-PCR.** One Step RT-PCR (Qiagen) was performed on RNA purified from whole cell extracts and final TEV eluates. Each reaction was first optimized on 500 ng whole cell RNA to determine the most effective number of PCR cycles for gene detection. The following primers were used:

**UTHI** forward-TCGGCGTCTCCCAGATCAAAG, **UTHI** reverse-CCGTCTGCTGCTGCTTAC

**ACT1** forward-ATTCTGAGGTTGCTGCTT, **ACT1** reverse-AACTCTCAATTCCGTTGTAG

**CBKI** forward-ATGTATAATAGCACCA, **CBKI** reverse-ATCATCTGATTTGAAGCTG

**NCA3** forward-TGCACCTGCTCCAGCGGACA, **NCA3** reverse-AGTAGTAGCCCATCCCTTAC

Reactions were carried out in a 50 μL volume of the following composition: 1X OneStep RT-PCR Buffer (Qiagen) containing 400 μM of each dNTP, 0.6 μM each primer, 5 units Rnasin, 500
ng template RNA (or equivalent volume from mock IP samples), and 2 µL OneStep RT-PCR
Enzyme Mix (Qiagen).

RT-PCR cycles were as follows: 30 min at 50°C (reverse transcription); 15 min at 95°C (initial
PCR activation step); either 30 (ACTI) or 35 (CBK1, UTH1, NCA3) cycles of the following three
steps: 30 sec at 94°C (denaturation); 1 min at 65°C (annealing); 1 minute at 72°C (extension); 10
minute final extension.
RESULTS

We have previously found that SSD1-V suppresses the short life span and cell wall defects caused by deletion of MPT5 in an sddl-d background (Kaeberlein and Guarente, 2002). To determine whether uth1Δ would have a similar effect on these mpt5Δ phenotypes, we deleted UTH1 from ssdl-d mpt5Δ cells. As expected, mpt5Δ alone severely impaired growth at 37°C and caused dose-dependent sensitivity to the cell wall-perturbing agent calcofluor white (CFW) (Figure 1A). Strikingly, when UTH1 was also deleted from these cells, they grew well at 37°C and nearly as well as wild type cells in the presence of CFW (Figure 1A). Similar to what is observed in UTH1 MPT5 SSD1-V cells (Kaeberlein and Guarente, 2002), uth1Δ MPT5 ssdl-d cells are more resistant to CFW than UTH1 MPT5 ssdl-d cells. Thus, deletion of UTH1 behaves phenotypically like addition of SSD1-V, with both modifications strongly promoting cell integrity.

Spurred by this finding, we sought to identify other phenotypic similarities between uth1Δ and SSD1-V cells. Uth1p was originally cloned by complementation of the sensitivity to paraquat displayed by uth1 mutant cells (Kennedy et al., 1995). Paraquat generates superoxide radicals, suggesting that cells lacking Uth1p are sensitive to oxidative stress. However, this view is complicated by the fact that uth1Δ also causes resistance to hydrogen peroxide (Bandara et al., 1998; Kennedy et al., 1995). We were interested in determining what effect, if any, SSD1-V would have on resistance to these oxidative agents. As expected, uth1Δ in strain PSY316 resulted in resistance to hydrogen peroxide and sensitivity to paraquat (Figure 2). Very similarly, addition of SSD1-V caused resistance to hydrogen peroxide and sensitivity to paraquat, demonstrating that both SSD1-V and Uth1p affect the oxidative stress response of cells.
During the course of our analysis, we observed that \textit{SSD1-V} and \textit{uth1Δ} cells both have a greater maximum growth temperature than \textit{ssd1-d} cells. Strain PSY316, which harbors the \textit{ssd1-d} allele, is unable to grow at 40°C; however, both \textit{SSD1-V} and \textit{uth1Δ} cells grow very well at this temperature (Figure 2). This phenotype could be the result of a strengthened cell wall, or could reflect some other function shared by these proteins.

We next examined the relative and combined effects of \textit{SSD1-V} and \textit{uth1Δ} on life span. Like \textit{SSD1-V}, \textit{uth1Δ} extends wild type \textit{ssd1-d} life span. \textit{SSD1-V UTH1} cells have a much longer life span than \textit{ssd1-d uth1} cells, suggesting that \textit{uth1Δ} has a weaker longevity-promoting effect than addition of \textit{SSD1-V} (Figure 1C). Interestingly, \textit{SSD1-V uth1Δ} cells have a life span comparable to \textit{ssd1-d uth1Δ} cells and shorter than those of the \textit{SSD1-V UTH1} genotype. These results indicate that the shorter-lived \textit{uth1} mutation is epistatic to \textit{SSD1-V} with respect to life span.

We sought to establish a genetic relationship between \textit{UTH1} and \textit{SSD1}. SBF, a transcriptional activator consisting of the cell-cycle regulated proteins Swi4p and Swi6p, acts downstream of protein kinase C (Pkc1p) to promote cell wall biosynthesis (Igual et al., 1996). Ccr4p, a component of the cytoplasmic deadenylase complex (Thore et al., 2003; Tucker et al., 2002) also functions in an RNA Polymerase II-containing complex required for PKC1-dependent transcriptional regulation of multiple genes required for proper cell wall structure (Chang et al., 1999). Mutation of either \textit{CCR4} or SBF is lethal in combination with mutation of \textit{MPT5} in an \textit{ssd1-d} or \textit{ssd1Δ} background (Kaeberlein and Guarente, 2002). In order to determine whether \textit{uth1Δ}, like addition of \textit{SSD1-V}, would suppress this synthetic lethality, we generated the following diploid mutants in a W303R (\textit{ssd1-d}) background: \textit{ccr4Δ/CCR4 mpt5Δ/MPT5 uth1Δ/UTH1; swi4Δ/SWI4}
mpt5Δ/MPT5 uth1Δ/UTH1; swi6Δ/SWI6 mpt5Δ/MPT5 uth1Δ/UTH1. These strains were sporulated, tetrads were dissected, and the genotypes of the surviving spores were determined. While ccr4Δ mpt5Δ ssdl-d UTH1, swi4Δ mpt5Δ ssdl-d UTH1 and swi6Δ mpt5Δ ssdl-d UTH1 spores were never viable, we were able to recover ccr4Δ mpt5Δ ssdl-d uth1Δ, swi4Δ mpt5Δ ssdl-d uth1Δ and swi6Δ mpt5Δ ssdl-d uth1Δ cells (Table 2). Deletion of UTH1 also partially rescued the growth defects and sensitivity to CFW caused by mutation of CCR4 or SBF (data not shown). These results suggest that Uth1p acts as a negative regulator of cell integrity in a pathway parallel to Mpt5p. Moreover, these results underline the striking similarity between mutation of UTH1 and addition of SSD1-V.

We therefore wished to determine whether this similarity extended to processes other than cell integrity and life span. SSD1-V was first defined based on its ability to confer viability to a strain carrying a mutation in SIT4. In order to test whether uth1Δ could also confer viability to a sit4Δ mutant, a sit4Δ/SIT4 ssdl-d/ssdl-d uth1Δ/UTH1 doubly heterozygous diploid was sporulated and tetrads were analyzed. The results are compiled in Table 2 and shown in Figure 3. As expected, 0/21 sit4Δ ssdl-d UTH1 spore clones were able to form viable colonies, confirming that sit4Δ and ssdl-d are synthetically lethal. In contrast, 14/14 sit4Δ ssdl-d uth1Δ spore clones formed colonies (Table 2), indicating that uth1Δ is an effective suppressor of the sit4Δ ssdl-d synthetic lethality. Like sit4Δ SSD1-V UTH1 cells, sit4Δ ssdl-d uth1Δ cells are slow growing, but are capable of vegetative growth indefinitely (Figure 3). Interestingly, maximal benefit to the sit4Δ cells appears to be obtained by either SSD1-V or mutation of UTH1, as sit4Δ
SSD1-V uth1Δ cells display no greater growth advantage compared to sit4Δ ssdl-d uth1Δ and sit4Δ SSD1-V UTH1 mutants (Figure 3).

Taken together, these results suggest a model whereby Ssd1p represses Uth1p to affect cell integrity, life span, and stress resistance. Using Western blot analysis, we tested this possibility by comparing Uth1p levels in SSD1-V and ssdl-d strains (Figure 4a). SSD1-V and ssdl-d cells grown in rich media were harvested during early-, mid-, and late-log phase, and protein extracts were subjected to Western blotting. As reported (Velours et al., 2002), a rabbit polyclonal antibody specific to Uth1p predominantly recognized a thick band of approximately 60 KDa corresponding to glycosylated Uth1p (Figure 4a). This band was absent from extracts of uth1Δ cells. Importantly, SSD1-V caused a dramatic reduction in Uth1p levels in all growth phases without significantly affecting the levels of β-tubulin. Similar results were obtained from cells grown at 37°C (data not shown), indicating that SSD1-V represses Uth1p under a wide variety of culture conditions. Interestingly, in the absence of SSD1-V, Uth1p was upregulated during late-log phase growth, while in the presence of SSD1-V Uth1p remained barely detectable as nutrients in the culture became exhausted (Figure 4a).

To determine whether SSD1-V repressed Uth1p at the transcriptional level, we performed Northern analysis on ssdl-d and SSD1-V cells. During log phase growth, UTH1 mRNA levels were unchanged by SSD1-V (Figure 4b), despite the fact that this allele caused a striking reduction in Uth1 protein. During late-log phase, as glucose was depleted from the growth medium, UTH1 mRNA levels decreased significantly in SSD1-V cells but not in ssdl-d cells (Figure 4b). All blots were stripped and reprobed for ADH1 expression to demonstrate equal RNA loading. From these data, we conclude that
under normal growth conditions, \textit{SSD1-V} reduces Uth1 protein expression without decreasing \textit{UTH1} mRNA levels. The repression of Uth1p by \textit{SSD1-V} under these conditions must therefore be post-transcriptional. Under conditions of high culture density and reduced glucose availability, \textit{SSD1-V} further reduces Uth1p expression by a mechanism that decreases the prevalence of the \textit{UTH1} mRNA.

As all phenotypic analyses (including life span assays) were conducted under early log phase growth conditions, we sought to explain the regulation of Uth1p by Ssd1p under these circumstances. Two possible models might account for the post-transcriptional repression of the Uth1 protein by Ssd1p. By the first model, Ssd1p would reduce Uth1p by lowering that protein's stability, making it more susceptible to degradation. To test this possibility, early-log phase \textit{ssd1-d} and \textit{SSD1-V} cells were treated with 100 \(\mu\text{g/mL}\) cycloheximide to inhibit protein synthesis. Proteins were extracted by boiling in modified SDS-sample buffer before cycloheximide treatment and every 70-seconds subsequently. Samples were then subjected to Western blotting, and Uth1p was detected using an anti-Uth1p polyclonal antibody. We observed that Uth1p decayed with identical kinetics in cycloheximide-treated cells of both the \textit{ssd1-d} and \textit{SSD1-V} genotypes (Figure 5), indicating that the observed decrease in Uth1p in log phase \textit{SSD1-V} cells was not the result of accelerated protein degradation. In both cases, an initially rapid period of degradation (Figure 5a) was followed by a very long period (>2hr) during which continued protein decay was undetectable (Figure 5b). Over the first 225 seconds, decay in both \textit{ssd1-d} and \textit{SSD1-V} strains occurred with a half-life time constant of approximately one minute, very short even for a yeast protein (Beyer et al., 2004; Varshavsky, 1996). Following this period, a fraction of the existing Uth1p in the
cell remained stable for hours. Importantly, this unusual protein stability profile applied to cells of both ssdl-d and SSD1-V genotypes, leading us to conclude that SSD1-V does not repress Uthlp at the level of protein stability.

A second model postulates that SSD1, an RNA-binding protein, might be acting as a post-transcriptional repressor by inhibiting the translation of the UTHI message. In order to purify ribonucleoparticles associated with Ssd1p, a single-copy plasmid containing the SSD1-V gene fused to an in-frame C-terminal tandem affinity purification (TAP) tag sequence was transformed into strain PSY316AR. The same plasmid lacking the TAP tag sequence was used to obtain transformants of strain PSY316AR to provide a control for non-specific enrichment during the purification process. Affinity purifications were performed from these two strains using agarose-IgG beads under conditions previously described to preserve protein-RNA interactions (Gerber et al., 2004). RNA was then isolated from purified TEV protease-eluted protein samples by column chromatography using the Qiagen Rneasy kit. From SSD1-TAP cells, we obtained between 0.2-0.6 \( \mu \text{g} \) RNA per 1 L of starting culture (data not shown), a result comparable to what has been reported for other yeast RNA binding proteins (Gerber et al., 2004). We did not obtain a significant quantity of RNA from the final elution of untagged control samples, suggesting that the RNA fraction obtained from Ssd1p-TAP cells consisted of RNA molecules specifically associated with the tagged protein.

To confirm this finding and to test for a specific physical association between Ssd1p-TAP and UTHI mRNA, purified RNA fractions were probed for several candidate genes using RT-PCR. Primers specific to \( \beta \)-actin, encoded by ACT1, detected that gene’s cDNA in input but not affinity-purified RNA preparations from both tagged and untagged
strains, indicating that this abundant transcript was not significantly associated with Ssd1p-TAP or control fractions. Similar results were obtained when extracts were probed for the *UTH1* family member *NCA3* and the unrelated *CBK1*, encoding a kinase whose protein product physically interacts with Ssd1p (Ho et al., 2002; Racki et al., 2000). When used to probe total cellular RNA fractions from Ssd1p-TAP and mock affinity purified eluates, each pair of primers detected a band, confirming the efficacy of these primer pairs in the RT-PCR reaction. However, of the four genes analyzed, only primers specific to *UTH1* succeeded in amplifying a product from Ssd1p-TAP purified fractions. This *UTH1* band was sequenced to confirm that it represented the predicted gene (not shown). This band was absent from the TEV eluate of untagged extracts. These results indicate that the *UTH1* mRNA physically associates with Ssd1p-TAP, and that this association is specific, as Ssd1p-TAP fails to stably associate with the abundant *ACT1* transcript or the *NCA3* or *CBK1* transcripts.

To identify the region of *UTH1* mediating this interaction and to ask whether this interaction is required for the regulation of Uth1p by Ssd1p, mutants were generated substituting the upstream region (including the 5'-UTR) or the downstream region (including the 3'-UTR) of the *ADH1* gene for that of *UTH1* (Figure 7). These mutations were first analyzed in an *SSD1-V* background to determine whether either substitution affected the regulation of Uth1p by Ssd1p. Surprisingly, substitution of the 3'-UTR of *UTH1* did not hinder the repressive effects of Ssd1p on Uth1p (Figure 7b), and *SSD1-V UTH1<sub>3UTR</sub>:ADH1<sub>3UTR</sub>* cells grew as well as *SSD1-V* cells at 40°C (Figure 7c). However, substitution of the promoter and 5'-UTR of *UTH1* with a truncated, less active form of the *ADH1* promoter and its 5'-UTR did abolish the repression by Ssd1p, also severely
limiting the ability of these cells to grow at 40°C (Figure 7). Therefore, we conclude that repression of Uth1p by Ssd1p requires the 5′ upstream region of UTH1, and that this repression is required for the SSD1-V phenotype of high temperature growth.

Preliminary results from affinity co-purification experiments indicate that the substitution of the UTH1 promoter and upstream region abolishes the physical interaction between Ssd1p and the UTH1 mRNA. Validation of these results will be included upon submission of this manuscript.

DISCUSSION

Here, for the first time, we provide a molecular mechanism to explain the action of Ssd1p, a highly conserved protein with diverse effects on yeast physiology. Several lines of evidence now lead us to conclude that Ssd1p directly represses Uth1p by a post-transcriptional mechanism, and this repression is necessary and sufficient to account for vast effects of Ssd1p on life span, cell integrity, high temperature growth, and stress resistance. First, deletion of UTH1 can effectively compensate for the null ssd1-d allele, restoring longevity, increased cell wall stability, resistance to high temperature, and the unusual combination of peroxide resistance and paraquat sensitivity. Second, deletion of UTH1, like addition of SSD1-V, suppresses several genetic mutations in an ssd1-d background, including the lethality caused by mpt5 swi4 ssd1-d, mpt5 swi6 ssd1-d, mpt5 ccr4 ssd1-d, and sit4 ssd1-d genotypes. These findings demonstrate that abrogation of Uth1p expression is sufficient to recapitulate widely varying phenotypes associated with SSD1-V.
Additional data show that not only is Uthlp reduction sufficient to vary stress resistance, life span, and cell wall stability in a manner reminiscent of SSDI-V, Uthlp is actually strongly reduced SSDI-V cells. This downregulation occurs without a significant effect on UTH1 mRNA levels under normal culture conditions, pointing to a post-transcriptional mechanism of repression. Indeed, Ssd1p-TAP forms a specific physical association with the UTH1 mRNA in vivo, and this association is required for the proper repression of Uthlp translation. SSDI-V cells in which this interaction has been abrogated by replacement of the UTH1 5′ UTR fail to resist oxidative stress and high temperature, indicating that downregulation of Uthlp is required for these phenotypic hallmarks of SSDI-V cells.

A previous study established that Ssd1p possesses an in vitro RNA binding activity, mediated by a central domain within the protein (Uesono et al., 1997). For the first time, our results link this activity to the biological function of Ssd1p, raising the possibility that Ssd1p may regulate other transcripts in addition to that of UTH1. Consistent with this possibility, Ssd1p-TAP co-purified with significant amounts of RNA even in mutants in which the interaction with UTH1 was apparently abolished (preliminary data, not shown). Future experiments should aim to systematically identify transcripts bound by Ssd1p in vivo, as these represent likely candidates for post-transcriptional co-regulation by Ssd1p.

The majority of mRNA-protein interactions characterized to date occur via sequence elements in the 3′ UTR of the bound mRNA (Gerber et al., 2004; Kuersten and Goodwin, 2003). However, our data indicate that the interaction between Ssd1p and UTH1 depends on sequence elements within the 5′ UTR of UTH1, and possibly
additional elements located elsewhere within the \textit{UTHI} mRNA. So far, we have been unable to identify the precise nucleotide sequence sufficient to direct Ssd1p binding to an mRNA (our unpublished observations), possibly because of a requirement for additional sequence or structural characteristics within the ORF or even the 3’ UTR. Interestingly, for the three yeast Pumilio family members for which consensus binding sites have been determined (Puf3-5), a significant fraction of these motifs reside within the open reading frames (ORFs) of target genes. We therefore cannot rule out the possibility that the \textit{UTHI} ORF contains additional sequence elements or structural characteristics required to direct Ssd1p binding to the mRNA. Perhaps a comprehensive identification of Ssd1p targets will also yield insight into the potentially complex issue of the sequence specificity of Ssd1p.

While Ssd1p likely acts as post-transcriptional repressor of Uth1p during normal growth conditions, presence of Ssd1p results in a dramatic reduction in \textit{UTHI} mRNA levels at the onset of the diauxic shift. Incidentally, we have also observed a similar reduction in \textit{UTHI} mRNA in \textit{SSD1-V} cells treated with rapamycin (G.L. and L.G., unpublished observations), used in some studies as a mimic of starvation (Albig and Decker, 2001; Reinke et al., 2004). This Ssd1p-induced reduction in \textit{UTHI} message could result from indirect effects of Ssd1p on transcription under these conditions. Alternatively, Ssd1p might specifically target \textit{UTHI} mRNA for decay as starvation sets in and the cellular mechanisms favoring RNA decay over synthesis and translation begin to predominate. Both starvation and rapamycin treatment have been shown to activate mRNA turnover via the nonsense mediated decay pathway, destabilizing particular RNAs.
Determination of the effect of Ssd1p on the half-life of *UTH1* mRNA would help distinguish between these two possibilities.

Mutation of *UTH1* results in a shorter life span extension than *SSD1-V*, and is epistatic to *SSD1-V* for life span. One possible explanation for this is that mutation of *UTH1* results in two opposing effects on life span, and a maximum life span is only obtained at an optimal level of the protein. Consistent with this model, *SSD1-V* cells do maintain a low level of Uth1p that might be responsible for their increased longevity compared to *uth1* null mutants. Although studies of Uth1p have primarily focused on the benefits conferred by the absence of this protein, there are reports indicating that *uth1Δ* mutants have subtle defects in mitochondrial function stemming from slightly reduced synthesis of critical mitochondrial proteins (Camougrand et al., 2000; Mouassite et al., 2000b). It is possible that these defects somehow begin to curtail life span past a certain very old age.

Remaining members of the SUN family of proteins can compensate for the loss of any individual SUN protein to some extent. This is evidenced by the fact that double mutations within this family typically show much more severe phenotypes than single mutations (Camougrand et al., 2000; Mouassite et al., 2000b). Consistent with this notion, we have observed by transcriptional profiling that the SUN family member *NCA3* is strongly induced by either *uth1Δ* or addition of *SSD1-V* (Kaeberlein et al., 2004a), possibly as a means of compensating for the loss of its homolog in both cases. It is interesting to note that Nca3p, although 63% identical to Uth1p, appears to be regulated very differently, showing induction rather than repression as a result of Ssd1p. This gene
therefore provided an excellent negative control in the purification of RBPs associated with Ssd1p-TAP in vivo.

Exactly how Uth1p affects so many cellular processes has been the subject of several studies (Bandara et al., 1998; Camougrand et al., 2003; Camougrand et al., 2000; Kissova et al., 2004), but still remains a significant mystery. Effects on cell wall stability and structure could conceivably be mediated directly by Uth1p, which is not only partially localized to the cell wall but also bears some homology to a β-glucosidase of Candida wickerhamii (Mouassite et al., 2000a; Velours et al., 2002). No enzymatic activity has yet been shown for Uth1p. And while effects of uth1Δ on stress resistance have been hypothesized to account for its extension of replicative life span (Camougrand et al., 2004), we feel this is unlikely to be the case, as genetic modifications made specifically to increase peroxide resistance are insufficient to extend life span (Van Zandycke et al., 2002). Moreover, superoxide sensitivity resulting from mutation of superoxide dismutase generally causes a shortened life span (Fabrizio et al., 2004). A deletion of UTH1 was found to increase resistance to death induced by the ectopic expression of the mammalian apoptosis protein Bax (Camougrand et al., 2003). As yeast have been shown to display a form of cell death resembling apoptosis in some ways (Madeo et al., 2002), it is possible that dysregulation of Uth1p in ssd1-d cells shortens life span by increasing susceptibility to this form of death in very old cells.

While Uth1p repression accounts for many phenotypes seen in SSD1-V cells, there is also evidence that Ssd1p has Uth1p-independent roles in the cell. Even in a UTH1 mutant with the ADHI 5’ UTR substituting for the endogenous 5’UTR, there still is a small effect on high temperature growth and stress resistance caused by SSD1-V,
despite the lack of Uth1p repression in this strain. This residual effect likely results from a parallel activity of Ssd1p affecting these two phenotypes. Furthermore, we have observed an $SSD1-V$ genetic interaction that is not recapitulated in $uth1A$ cells. Namely, deletion of components of the Cbk1p kinase complex, required for cell morphogenesis and the transcription of daughter-specific genes, is lethal in combination with $SSD1-V$ but not with $uth1A$ (G.L. and L.G., unpublished observations).

Ssd1p now becomes the second translational repressor implicated in the coordinated maintenance of yeast longevity, cell integrity, and stress resistance. The first such protein, Mpt5p, has many well-studied homologs in metazoans including Drosophila PUMILIO and C. elegans FBF, both of which are specific repressors of translation. Several studies now indicate a conserved role for these homologs in the maintenance of germ line stem cells in Drosophila (Forbes and Lehmann, 1998; Lin and Spradling, 1997) and C. elegans (Crittenden et al., 2002). Thus, both PUMILIO and FBF promote continued cell divisions and delay the onset of an alternate state of differentiation and developmental potency. Intriguingly, Mpt5p and Ssd1p also conform to this common theme, using specific targeted translational repression to prolong cell divisions and maintain potential to produce young cells. The metazoan homologs of Ssd1p still await characterization and may provide likely candidates for regulators of development and cell fate analogous to those of the PUF family.
References:


### TABLES AND FIGURES

Table 1. Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303R</td>
<td>MATa ADE2::RDNI ade2 his3 leu2 trp1 ura3 ssd1-d2 RAD5</td>
</tr>
<tr>
<td>MKY1324</td>
<td>W303R mpt5::LEU2</td>
</tr>
<tr>
<td>MKY641</td>
<td>W303R mpt5::LEU2 uth1::URA3</td>
</tr>
<tr>
<td>MKY643</td>
<td>W303R mpt5::LEU2 uth1::TRP1</td>
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<tr>
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<td>PSY316AR uth1::HIS3</td>
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</table>
**TABLE 2. Spore clone viability.** Tetrads were dissected onto YPD and incubated at 30°. Spore clones that failed to form visible colonies after 4 days were examined microscopically to verify inviability. Numbers in parentheses give the total number of representative spores analyzed.

<table>
<thead>
<tr>
<th>Spore Genotype</th>
<th>Spore Clone Percent Viability</th>
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</thead>
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<tr>
<td>mpt5 ccr4 ssd1-d UTH1</td>
<td>0 (23)</td>
</tr>
<tr>
<td>mpt5 ccr4 SSD1-V UTH1</td>
<td>83 (6)</td>
</tr>
<tr>
<td>mpt5 ccr4 ssd1-d uth1</td>
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</tr>
<tr>
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<td>0 (74)</td>
</tr>
<tr>
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</tr>
<tr>
<td>mpt5 swi4 ssd1-d uth1</td>
<td>30 (20)</td>
</tr>
<tr>
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<td>0 (65)</td>
</tr>
<tr>
<td>mpt5 swi6 SSD1-V UTH1</td>
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<tr>
<td>sit4 SSD1-V UTH1</td>
<td>100 (15)</td>
</tr>
<tr>
<td>sit4 ssd1-d uth1</td>
<td>100 (14)</td>
</tr>
<tr>
<td>sit4 SSD1-V uth1</td>
<td>100 (7)</td>
</tr>
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</table>
Figure 1. Effect of UTH1 on cell integrity and life span.
(A) 5 μL aliquots of 10-fold serial dilutions were plated onto the designated media and cultured at 30°C or 37°C for 2 days, as indicated.
(B) Life spans were determined for PSY316 (◆), PSY316 uth1 (■), PSY316 mpt5 (▲), and PSY316 mpt5 uth1 (X). Mean life spans and number of cells analyzed were: PSY316 21.9 (n=40), PSY316 uth1 26.4 (n=40), PSY316 mpt5 8.9 (n=40), and PSY316 mpt5 uth1 19.2 (40).
(C) Life spans were determined for PSY316 (◆), PSY316 uth1 (■), PSY316 SSD1-V (▲), and PSY316 uth1 SSD1-V (X). Mean life spans and number of cells analyzed were: PSY316 19.4 (n=40), PSY316 uth1 23.6 (n=40), PSY316 SSD1-V 34.0 (n=40), and PSY316 mpt5 SSD1-V 24.4 (40).
FIGURE 1
Figure 2. *SSD1-Vand UTH1 both regulate resistance to oxidative damage and growth at high temperature.* 10 μL aliquots of 10-fold serial dilutions were plated onto the designated media and cultured at 30°C or 40°C for 2 days, as indicated. H₂O₂ indicates YPD prepared with hydrogen peroxide. PQ indicates YPD prepared with paraquat.
<table>
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<th>ssd1-d</th>
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<td><img src="image" alt="SSD1-V 30°C" /></td>
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<tr>
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<td><img src="image" alt="uth1Δ 40°C" /></td>
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</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td><img src="image" alt="ssd1-d H₂O₂ 2 mM" /></td>
<td><img src="image" alt="uth1Δ H₂O₂ 2 mM" /></td>
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<tr>
<td><strong>2 mM</strong></td>
<td><img src="image" alt="ssd1-d H₂O₂ 2 mM" /></td>
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<td><img src="image" alt="uth1Δ PO 10 mM" /></td>
<td><img src="image" alt="SSD1-V PO 10 mM" /></td>
</tr>
</tbody>
</table>

**FIGURE 2**
Figure 3. Mutation of *UTH1* suppresses the lethality caused by deletion of *SIT4*.

(A) Strain MKD213 was sporulated and tetrads were dissected onto YPD. Growth after 4 days at 30°C is shown. Genotypes are shown for *sit4 ssd1-d UTH1* (○), *sit4 ssd1-d uth1* (△), *sit4 SSD1-V UTH1* (□), and *sit4 SSD1-V uth1* (<> spore clones.

(B) Spore clones were streaked onto YPD and cultured at 30°C. The pictures show cell growth after 3 days.
FIGURE 3
Figure 4. Effects of SSD1-V on Uth1p and UTH1 mRNA levels. Cells of the indicated genotypes were grown in YPD and harvested at early log phase (OD$_{600}$=0.6), mid-log phase (OD$_{600}$=2.0), or late-log phase (OD$_{600}$=4.5).

(A) Protein extracts were prepared and analyzed by SDS-PAGE followed by Western blotting. Blots were probed with rabbit polyclonal anti-Uth1p, then stripped and reprobed with mouse monoclonal antibody specific for beta-tubulin.

(B) Total RNA was extracted from the indicated samples and subjected to Northern blotting. Blots were probed with $^{32}$P-labelled cDNA corresponding to UTH1 (top panel) or ADH1 (bottom panel).
FIGURE 4
Figure 5. SSD1 does not affect Uth1p decay kinetics.

Early-log phase cells harboring the either the ssd1-d or SSD1-V allele were treated with 100 μg/mL cycloheximide to inhibit protein synthesis. Samples were harvested at the given time points, and extracts were prepared by boiling cells in modified SDS sample buffer (Kushnirov, 2000).

(A) Rate of Uth1p decay was assessed by Western blotting using rabbit antisera specific to Uth1p. Decay of Tub2p, included here as a control, was undetectable over this time period.

(B) Over 120 minute time course, a fraction of Uth1p resists degradation in both SSD1-V and ssd1-d cells.
FIGURE 5
**Figure 6.** Ssd1p specifically associates with *UTH1* mRNA in vivo.

Yeast cells expressing either Ssd1p-TAP or Ssd1p (mock IP) were grown to OD<sub>600</sub>=0.6 and harvested. Cell extracts were prepared by glass bead lysis and immunoprecipitated using rabbit IgG agarose beads. RNA purified from the supernatant and pellet fractions was subjected to RT-PCR using primers specific to *UTH1, ACT1, CBK1*, and *NCA3*. RT-PCR reactions were performed on two different starting volumes of RNA input to confirm that amplification occurred within a linear range.
Mock IP

UTH1

input | IP
--- | ---
1 | 5
1 | 5

μL input

SSD1-TAP

IP

UTH1

input | IP
--- | ---
1 | 5
1 | 5

μL input

ACT1

input | IP
--- | ---
1 | 5
1 | 5
1 | 5

μL input

CBK1

input | IP
--- | ---
1 | 5
1 | 5

μL input

NCA3

input | IP
--- | ---
1 | 5
1 | 5

μL input

SSD1-TAP

IP

FIGURE 6
Figure 7. Repression of Uth1p by Ssd1p is necessary for growth at high temperature and requires the UTH1 5' UTR. (A) Two mutations were constructed: the tUTH1::tADHI replacement substituted the downstream terminator sequence and 3'-UTR of ADH1 for that of UTH1. The pUTH1::pADHI* substitution replaced the promoter and 5'UTR of UTH1 with a truncated, weakened promoter from ADH1. (A) These mutants were assayed for growth at 41°C and (B) the effects of each mutation on Uth1p expression was compared in ssd1-d and SSD1-V cells.
FIGURE 7
Chapter 3

Mouse Sir2 Homolog SIRT6 is a Nuclear ADP-Ribosyltransferase

This chapter was published in the Journal of Biological Chemistry, Volume 280, Issue 22, pages 21313-20. The authors were Gregory Liszt, Ethan Ford, Martin Kurtev, and Leonard Guarente. I contributed all figures except Figure 2a and Figure 4a. I also wrote the manuscript.
SUMMARY

Members of the Sir2 family of NAD-dependent protein deacetylases regulate diverse cellular processes including aging, gene silencing, and cellular differentiation. Here, we report that the distant mammalian Sir2 homolog SIRT6 is a broadly expressed, predominantly nuclear protein. Northern analysis of embryonic samples and multiple adult tissues reveals mSIRT6 mRNA peaks at day E11, persisting into adulthood in all eight tissues examined. At the protein level, mSIRT6 is readily detectable in the same eight tissue types, with the highest levels in muscle, brain and heart. Subcellular localization studies using both C- and N-terminal GFP fusion proteins show mSIRT6-GFP to be a predominantly nuclear protein. Indirect immunofluorescence using antibodies to two different mSIRT6 epitopes confirms that endogenous mSIRT6 is also largely nuclear. Consistent with previous findings, we do not observe any NAD+-dependent protein deacetylase activity in preparations of mSIRT6. However, purified recombinant mSIRT6 does catalyze the robust transfer of radiolabel from $^{32}$P-NAD to mSIRT6. Two highly conserved residues within the catalytic core of the protein are required for this reaction. This reaction is most likely mono-ADP ribosylation, as only the modified form of the protein is recognized by an antibody specific mono-ADP-ribose. Surprisingly, we observe that the catalytic mechanism of this reaction is intra-molecular, with individual molecules of mSIRT6 directing their own modification. These results provide the first characterization of a Sir2 protein from phylogenetic class IV.
INTRODUCTION

Members of the Sir2 family of enzymes, conserved from bacteria to man, utilize oxidized nicotinamide adenine dinucleotide (NAD⁺) as a cosubstrate in the deacetylation of a wide variety of proteins, thereby regulating diverse processes including aging, genomic silencing, recombination, cell fate, and metabolism (Blander and Guarente, 2004). In yeast, the archetypal Sir2p localizes to chromatin sites at the telomeres, rDNA, and silent mating type loci, facilitating genomic silencing through the deacetylation of lysines within the histone H3 and H4 tails (Gasser and Cockell, 2001). In this context, yeast Sir2p promotes mother cell longevity by repressing rDNA recombination and formation of toxic extra-chromosomal rDNA circles (Bitterman et al., 2003; Hekimi and Guarente, 2003). Overexpression of Sir2p extends yeast lifespan (Kaeberlein et al., 1999), and Sir2p activity increases in response to caloric restriction (Lin et al., 2004), contributing to the resultant extension of life span (Lin et al., 2000). Intriguingly, Sir2 orthologues in worm and fly also promote longevity, though probably by different molecular mechanisms (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001; Wood et al., 2004).

Mammalian genomes contain seven Sir2 homologs, termed sirtuins (SIRTs). Of these, SIRT1, orthologous to the yeast Sir2, is the best characterized and has the broadest substrate specificity (North and Verdin, 2004). A nuclear protein, SIRT1 deacetylates several substrates in vivo, including MyoD, p53, and FOXO transcription factors, thereby affecting cell differentiation and survival under stress (Brunet et al., 2004; Motta et al., 2004; North and Verdin, 2004). Recently, mouse SIRT1 was reported to promote the mobilization of fatty acids in white adipose tissue by repressing PPARγ,
linking Sir2 proteins to the physiology of caloric restriction in mammals (Picard et al., 2004).

Of the remaining SIRTs (SIRT2-7), an in vivo substrate has only been identified for the cytoplasmic SIRT2 (North et al., 2003). This substrate, β-tubulin, is specifically deacetylated by SIRT2 (North et al., 2003), although the biological consequences of this reaction are unclear.

Yeast Sir2 and its orthologues, including mouse SIRT1 and bacterial CobB, catalyze the tightly-coupled cleavage of NAD+ and protein deacetylation, producing nicotinamide and 2-O-acetyl-ADP-ribose reaction products (Sauve and Schramm, 2004). While Sir2 proteins are generally thought to be NAD-dependent protein deacetylases, most also display a less robust mono-ADP-ribosyltransferase activity in vitro (Frye, 1999; Tanner et al., 2000; Tanny et al., 1999; Tanny and Moazed, 2001). Mutations in phylogenetically conserved residues within the catalytic core of Sir2 and SIRT1 have failed to separate the two enzymatic activities (Armstrong et al., 2002). The initial observation that ADP-ribosylation by Sir2 is stronger on acetylated substrates (Imai et al., 2000), combined with subsequent mechanistic studies (Tanner et al., 2000; Tanny and Moazed, 2001), has led to the conclusion that the deacetylase and phosphoribosyltransferase activities of Sir2 proteins are coupled (Sauve and Schramm, 2004).

Using sequence similarity, eukaryotic Sir2 genes have been divided into four broad phylogenetic classes (Frye, 2000), known as classes I-IV (Figure 1). SIRT1, SIRT2, and SIRT3 are class I, SIRT4 is class II, and SIRT5 falls within the predominantly prokaryotic class III. Finally, mammalian SIRT6 and SIRT7, are class IV
sirtuins (Frye, 2000). In vitro studies indicate that human SIRT1, 2, 3, and 5 possess NAD-dependent histone deacetylase activity (North et al., 2003), whereas SIRT4, 6, and 7 fail to deacetylate $^3$H-labeled acetylated histone H4 peptide (North et al., 2003). The lack of detectable deacetylase activity in these SIRTs may result from their specificity for targets other than those tested, or it may indicate an enzymatic activity other than deacetylation inherent in these sirtuins.

Mono-ADP-ribosylation is emerging as a common mechanism of reversible protein modification within mammalian cells. Originally described as the acting mechanism for specific bacterial toxins, ADP-ribosylation is typically performed by separate families of intra- and extracellular enzymes in vertebrates (Corda and Di Girolamo, 2003). Known targets of the intracellular class of enzymes include molecular chaperone GRP78/BiP, translational elongation factor 2, and β-subunit of heterotrimeric G-proteins (Corda and Di Girolamo, 2003). Extracellular mammalian ADP-ribosyltransferases, generally found in cells of the immune system, modify substrates important for the immune response, as well as integrin α7 and the antimicrobial peptide defensin (Corda and Di Girolamo, 2003). In most known cases, ADP-ribosylation of arginine residues results in reversible inactivation of the substrate protein (Riese et al., 2002), although this modification may enhance certain enzymatic functions within the substrate (Weng et al., 1999).

In this report, we characterize the tissue-specific expression, subcellular localization, and in vitro enzymatic activity of mouse SIRT6. This investigation, the first detailed study of a Class IV sirtuin, reveals mouse SIRT6 to be a widely expressed, predominantly nuclear protein with a robust auto-ADP-ribosyltransferase activity.
MATERIALS AND METHODS

Multiple sequence alignments —

Sequences of mouse proteins SIRT1, SIRT4, SIRT5, and SIRT6, as well as yeast Sir2p were obtained from the Proteome BioKnowledge® Library (https://proteome.incyte.com/control/tools/proteome). Core domains were aligned by ClustalW using software from the DNASTAR package.

Plasmid construction —

IMAGE clone 1259892, which contains the mouse SIRT6 cDNA, was obtained from ATCC. The mSIRT6 coding sequence was amplified by PCR and cloned into the EcoRI and BamHI sites of pEGFP-N1 and the BglII and EcoRI sites of pEGFP-C2, creating pmSIRT6-EGFP and pEGFP-mSIRT6, respectively. Similarly, the mSIRT6 coding sequence was amplified by PCR and cloned into the NheI and BamHI sites of pET28a (Novagen) and pET-GST (gift from Robert Marciniak,) creating pET28a-6xHis-SIRT6 and pET-GST-SIRT6. pET28a-6xHis-mSIRT6-S56A and pET28a-6his-SIRT6-H133Y were created using the Stratagene QuikChange site directed mutagenesis kit according to the manufacturer’s instructions. PET-GST-SIRT6 (274-355) was created by amplifying the portion of the mSIRT6 cDNA corresponding to amino acids 274-355 and ligating into pET-GST cut with NheI and BamHI.

Multiple tissue Northern blots —

Probe containing the SIRT6 open reading frame was created using the PrimeIt Random Primer Labeling kit (Stratagene) according to the manufacturer’s instructions. This 32P-labeled fragment was used to probe Stratagene Multiple Tissue Northern Blots.
(Stratagene) according to the manufacturer’s instructions. The blots were then stripped and reprobed with the actin probe included in the kit.

*Antibody production —*

pET28a-6xHis-mSIRT6 and pET-GST-mSIRT6 (274-355) were transformed into BL21(DE3)-Codon Plus-RP cells (Stratagene), and inoculated into 4 or 2 liter cultures of LB (50 mg/ml kanamycin, 35 mg/ml chloramphenicol), respectively. The cells were grown at 37°C to an optical density of 0.7. Protein expression was induced by the addition of IPTG to a final concentration of 0.4 mM for 2 h, and the cells were harvested by centrifugation.

The cells containing the pET28a-6xHis-mSIRT6 plasmid were resuspended in lysis buffer (0.1 M NaH₂PO₄, 10 mM Tris, 8 M urea, pH 8.0) and lysed by freezing in liquid N₂. The lysate was centrifuged at 19,000 rpm in a Sorvall SS-34 rotor. The supernatant was incubated with 4 ml of Ni-NTA agarose (Stratagene) for 3 h at 4°C, loaded onto a column, and washed extensively with wash buffer (0.1 M NaH₂PO₄, 10 mM Tris, 8 M urea, pH 6.4). The purified protein was eluted with elution buffer (0.1 M NaH₂PO₄, 10 mM Tris, 8 M urea, pH 3.0) in 1.5 ml fractions. The pH was neutralized by the immediate addition of 100 ml 1 M Tris base. The fractions containing protein were pooled and dialyzed against PBS. This protein was sent to Covance Reseach Bioproducts for antibody production.

The cells containing the pET-GST-SIRT6 (274-355) plasmid were resuspended in PBSTG (PBS, 0.1% Tween-20, 10% glycerol, 1 mM DTT) and 0.1 g lysozyme, incubated at 37°C for 20 min, and frozen in liquid N₂. The lysis was completed by sonication and the lysate was centrifuged at 19,000 rpm for 30 min. The supernatant was
incubated with 4 ml GST-agarose (Sigma) for 3 h at 4° C and loaded onto a column. The column was washed extensively with PBSTG and the protein was eluted with elution buffer (50 mM Tris, 10 mM glutathione, pH 7.5). Fractions containing protein were dialyzed against PBS and sent to Covance Research Bioproducts for antibody production.

The antibodies were affinity purified as follows. Aliquots of the proteins used to generate the antibodies were attached to 1 ml NHS-activated sepharose columns (Amersham) according to the manufacturer’s instructions. 10 ml of the final bleed of each antibody was combined with 10 ml antibody wash buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and applied to the column. Each column was washed extensively with antibody wash buffer and the antibody was eluted with 100 mM glycine pH 2.5. The pH was neutralized by the addition of 1/10 volume of 1 M Hepes-KOH, pH 7.5. The antibodies were then subjected to a second round of affinity purification, but this time the anti-mSIRT6 (full length) antibody was applied to the SIRT6 (274-355) column and the anti-mSIRT6(274-355) antibody was applied to the SIRT6(full length) column.

Western blot analysis —

Homogenized mouse tissues from four mice of the FVB genetic background were obtained as a generous gift from L. Bordone, MIT. From these samples, SDS-solubilized proteins were prepared (Kain et al., 1994), equal amounts of which were used for Western analysis.

For Western detection, samples were resolved on 4-15% Tris-HCl gradient gels (Bio-Rad), transferred to PVDF membrane, and blocked using 4% nonfat dry milk in PBS with 0.1% Tween-20 (PBS-T). Primary anti-mSIRT6 antibody was diluted 1:400 in
PBS-T before incubation with membranes. Rabbit anti-ADP-ribose antibody (Kain et al., 1994), a generous gift from H. Hilz, was used at a 1:100 dilution in PBS-T. Anti-actin C4 (Sigma) was used as a loading control (1:5,000) in multiple tissue Western blots. Secondary antibodies were goat anti-rabbit or sheep anti-mouse horseradish peroxidase-conjugated antibodies (1:10,000). Chemiluminescent detection was achieved by incubation with the ECL reagent (Amersham Biosciences).

**Immunofluorescence and GFP microscopy**

NIH 3T3 cells were grown in Dulbecco’s Modified Eagle’s Medium for 48 hours on gelatin-treated 18mm circular glass coverslips in 6-well plates. Cells were fixed for 10 minutes with 4% paraformaldehyde and washed twice with PBS, then permeablized by 3 minute treatment with 0.2% Triton X-100. Coverslips were gently washed twice with PBS and blocked with 10% BSA for 15 minutes, transferred to a rack, and washed in PBS. Affinity purified primary antibody was added at a 1:100 dilution. Staining proceeded for 45 minutes, and cells were then washed twice for 5 minutes in fresh PBS. FITC-conjugated chicken anti-rabbit IgG was diluted in 1% BSA and incubated with coverslips for 45 minutes. Coverslips were mounted with VectaShield mounting media containing 4',6-diamidino-2-phenyindole, dilactate (Vector, Burlingame, CA). Microscopy was performed on a Nikon Eclipse E500 fluorescence microscope. Digital images were obtained using a SPOT RT CCD camera and software using identical exposure times for comparable samples.

NIH 3T3 cells were transfected with 5 μg of each GFP construct using FuGene 6 (Roche) according to manufacturer’s instructions. Cells were grown for 48 hours on glass coverslips. Samples were fixed, DAPI-stained, and visualized as described above.
Purification of recombinant SIRT6 protein

BL21(DE3)-Codon Plus-RP *E. coli* (Stratagene) were transformed with the mSIRT6 expression plasmids. Protein purifications were performed essentially as described for antibody purification. Briefly, fusion protein expression was induced in 0.4 mM IPTG at 37°C for 1 h. The induced 6 x His-tagged proteins were purified with Ni-NTA agarose beads (Qiagen) under native conditions. The control eluate was prepared from a bacterial clone carrying pET28a vector alone. GST-tagged proteins were purified by a similar method, with affinity purification accomplished with glutathione agarose beads (Pierce). Control eluate for GST-tagged proteins was prepared from bacteria carrying pET28-GST. Aliquots of recombinant proteins were stored at -70°C.

**ADP-ribosylation assays and detection of ADP-ribosylated proteins —**

ADP ribosylation assays were performed essentially as described (Imai et al., 2000), with minor modifications. Reactions were performed in 50 μL total volume containing 5 μg recombinant SIRT6 in 50 mM Tris-HCl, pH 8.0 (at 22°C), 150 mM NaCl, 10 mM DTT, 1 μM unlabeled NAD, and 8 μCi of NAD 5’-[-32P]triphosphate (800 Ci mmol⁻¹, Amersham Pharmacia). Reactions also contained 2.5 μg core histones as indicated. For reactions containing both His-tagged and GST-tagged recombinant SIRT6 protein, 2.5 μg of each protein were added. Prior to SDS-PAGE analysis, all reactions were purified from unincorporated 32P-NAD using Micro Bio-Spin chromatography columns (Bio-Rad) with a 6,000 Da exclusion limit. Gels were transferred to PVDF membrane before autoradiography and subsequent immunoblot analysis. Blots were stained with Coomassie, and the Coomassie-stained bands and Western signals were aligned to verify that the radioactive bands were SIRT6.
Quantitation of ADP-ribosylation—

ADP-ribosylation reactions were performed as above, but with the following modifications. Total reaction volume was 40 μL, including approximately 0.5 μg protein, 0.8 μL of 6.25 μM \(^{32}\)P-NAD, and 4 μL 10 μM NAD. Purified reaction products were separated by SDS-PAGE, and BSA standards of 5 μg, 2 μg, 0.8 μg, 0.4 μg, and 0.1 μg were included on the same gel. SIRT6 protein concentrations in each lane were determined by comparison to these standards upon Coomassie staining. Gel was analyzed by autoradiography, and radioactive SIRT6 bands were excised. Radioactivity was determined by scintillation counting on a Beckman LS 6500 Liquid Scintillation Counter. A standard curve relating NAD quantity to radioactivity was generated by scintillation counting of known pmol quantities of \(^{32}\)P-NAD diluted to reflect the ratio of unlabeled to labeled NAD in the original reaction. This curve was used to estimate pmol NAD incorporated in SIRT6 bands. For each band, pmol of NAD was divided by pmol of SIRT6. This final value reflects the molar ratio of total incorporated ADP-ribose to SIRT6.

RESULTS

mSIRT6 expression in mice and embryos

To determine the expression pattern of mSIRT6, we analyzed RNA extracts from eight adult mouse tissues by Northern blotting (Fig. 2a). Using a cDNA probe corresponding to the 5’ region of mSIRT6, expression of mSIRT6 mRNA was observed in every tissue type tested (Fig. 2a). Confirming the predictions of sequence analysis
(Frye, 2000), mSIRT6 was detected as a single 1.0 Kb transcript, suggesting that this gene is not found in alternate splice forms. When normalized to actin, the highest levels of mSIRT6 mRNA were seen in the brain, heart, and liver, with the lowest expression level observed in skeletal muscle.

To evaluate mSIRT6 levels during development, we probed Northern blots of RNA from four mouse embryonic stages from E7 to E17 (Fig. 2b). Mouse SIRT6 transcript was readily detectable in all embryonic samples, reaching a peak at E11.

Having established the prevalence of mSIRT6 message in embryonic and adult tissues, we wished to determine the distribution of mSIRT6 protein. Affinity-purified antiserum specific to mSIRT6 was used to probe Western blots of proteins isolated from eight adult murine tissue types (Fig. 2c). For each tissue type, samples from four individual animals were obtained and analyzed by immunoblot. As illustrated in Figure 2c, mSIRT6 protein was detectable in all tissue types as a single 40 KDa band. As expected from Northern analysis, levels were high in brain, liver, and heart when normalized to actin protein levels. Surprisingly, mSIRT6 protein was most strongly expressed in muscle (Fig. 2c), despite the relative paucity of its transcript in this tissue. This observation might indicate increased mSIRT6 transcript stability in muscle, or an enhanced rate of translation in this tissue type relative to others tested.

**Visualization of mSIRT6-GFP in nuclei**

In order to observe the subcellular localization of mSIRT6, we engineered two GFP-fusion expression vectors under the control of the CMV promoter. Both a C-terminal (mSIRT6-GFP) and an N-terminal (GFP-mSIRT6) fusion construct were transfected into NIH 3T3 cells grown on glass coverslips. Samples were cultured for 48
hours, at which point they were fixed, washed and DAPI-stained to reveal nucleic acids. Fluorescence microscopy of cells harboring each GFP-fusion construct showed strong localization of mSIRT6-GFP to the nucleus, accompanied by diffuse cytoplasmic staining, as judged by colocalization of DAPI-stained nuclei (Fig. 3a). As expected, transfection of GFP alone caused intense fluorescence throughout the cytoplasm and nucleus.

**Indirect immunofluorescence of mSIRT6**

To visualize endogenous levels of mSIRT6, affinity-purified rabbit antisera to two different mSIRT6 antigens were used to probe NIH 3T3 cells by indirect immunofluorescence. As a control, cells were incubated without primary antibody and subsequently stained with DAPI and Cy-3 conjugated anti-rabbit IgG. Very faint background staining was seen throughout the cytoplasm and nucleus in these control samples (Fig. 3b). Strikingly, mSIRT6 showed strong staining predominantly in the nucleus (Fig. 3b), regardless of which anti-mSIRT6 antibody was used in the primary incubation. In both cases, endogenous mSIRT6 appeared to be excluded from the nucleolus.

Importantly, immunofluorescence and GFP-localization studies revealed the same predominantly nuclear localization pattern for mSIRT6. We therefore conclude that the majority of mSIRT6 is found in the nucleus, while a small fraction may be localized to the cytoplasm.

**mSIRT6 catalyzes the transfer of $^{32}$P from a $^{32}$P--NAD donor**

Of the seven mammalian Sir2 homologs, only SIRT1, SIRT2, SIRT3, and SIRT5 have been shown to have NAD$^+$-dependent protein deacetylase activity *in vitro* (North et
al., 2003). Consistent with published reports, we did not observe any protein deacetylase activity of mouse or human SIRT6 using a variety of experimental conditions and potential substrates (data not shown).

Several sirtuins also possess a mono-ADP-ribosyltransferase activity (Tanny et al., 1999). We therefore sought to test whether mSIRT6 could catalyze the transfer of radiolabel from $^{32}$P--NAD$^+$ to histones, an assay of ADP-ribosyltransferase activity. Six-histidine-tagged human SIRT1 and mSIRT6 were expressed in *E. coli* and purified by chromatography. Equal amounts of hSIRT1 and mSIRT6 were incubated with $^{32}$P-NAD$^+$ in the presence and absence of core histones (Fig. 4a). Whereas hSIRT1 transferred label efficiently to histones, mSIRT6 failed to do so (Fig. 4a). However, mSIRT6 catalyzed the robust transfer of label to itself, regardless of whether histones were present in the reaction (Fig. 4a). Control extracts purified from *E. coli* containing vector alone possessed no detectable activity under these conditions (data not shown).

We wished to determine if $^{32}$P transfer stimulated by mSIRT6 required the catalytic activity of the enzyme. Two point mutations in highly-conserved residues within mSIRT6 core domain were introduced by site-directed mutagenesis (Figure 1). The histidine residue at position 133 was changed to tyrosine (mSIRT6-H133Y), a mutation previously demonstrated to abolish enzymatic activity in human and yeast Sir2 proteins (Frye, 1999; Tanny et al., 1999). In a separate construct, the phylogenetically invariant serine residue at position 56 was mutated to alanine (mSIRT6-S56A). Serine 56 is buried deep in the active site of the enzyme, and does not appear to be structurally important (Finnin et al., 2001). Both mutant proteins were expressed in *E coli*, purified, and incubated in the presence of $^{32}$P-NAD$^+$ as described above. Unincorporated NAD
was removed by chromatography column. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and exposed to film. Whereas wild type mSIRT6 efficiently transferred $^{32}\text{P}$ radiolabel to itself, no label transfer was detected in mutants mSIRT6-S56A and mSIRT6-H133Y (Fig. 4c). Presence of mutant proteins on the membrane was verified by Western blotting using affinity-purified antibody to mSIRT6 (Fig. 4b).

Based on this requirement for two catalytic core residues, we conclude that mSIRT6 catalyzes the transfer of radiolabel from $^{32}\text{P}$-NAD by an enzymatic mechanism.

Labeling of proteins with $^{32}\text{P}$-NAD$^+$ can also occur nonenzymatically by the covalent binding of NAD. Such labeling, as observed in the case of GAPDH (Zhang and Snyder, 1992), can be misinterpreted as ADP-ribosylation of specific amino acid acceptors (Itoga et al., 1997; Zhang and Snyder, 1992). We have observed low levels of nonspecific radiolabeling of proteins incubated in the presence of $^{32}\text{P}$-NAD$^+$. Under our reaction conditions, such modification occurs in core histones and enzymatically-inactive Sir2 point mutants, generally not labeling more than 0.001% of total protein after 1-hour incubation at 30° C (Fig. 5), as determined by scintillation counting of protein bands. In contrast, in the case of mSIRT6 we observed incorporation of a molar quantity of ADP-ribose equivalent to 15% of total moles of mSIRT6 present in the reaction (Fig 5). For this reason, and because of the difference in labeling efficiency between wild type and point-mutant varieties of mSIRT6, we conclude that the transfer of $^{32}\text{P}$-radiolabel occurs via a robust enzymatic mechanism.
Auto-ADP-ribosylation of mSIRT6

To test the hypothesis that mSIRT6 catalyzed the mono-ADP-ribosylation of itself, we probed the reaction products from the above label-transfer reactions with an antibody specific to mono-ADP-ribose (Meyer and Hilz, 1986). This antibody recognized a band corresponding to the radiolabeled mSIRT6 following incubation with $^{32}$P--NAD (Fig. 6a). No band was present at the corresponding position in preparations from mutant mSIRT6 (Fig. 6a), demonstrating the specificity of the antibody for the modified form of mSIRT6. This evidence strongly suggests that this modified form of mSIRT6 is mono-ADP-ribosylated. While our results do not rule out the possibility that the modification of wild type mSIRT6 occurs in *E. coli* before purification, the absence of signal in point mutant SIRT6 indicates that mono-ADP ribosylation depends on the catalytic activity of mSIRT6.

Having confirmed mono-ADP-ribosylation as the nature of the modification of mSIRT6, we wished to ascertain whether this modification was catalyzed in an inter- or intra-molecular fashion. mSIRT6 and two different mutants (H133Y and S56A) were used in a molecular cis/trans test for enzymatic activity. GST-mSIRT6 was purified from *E. coli* and incubated in combination with His-tagged mutant and wild-type mSIRT6 in the presence of $^{32}$P--NAD. Reactions were purified as before and subjected to autoradiography. Labeled GST-mSIRT6 migrated as an approximately 80 KDa band in all reactions (Fig. 6b). Six histidine-tagged mSIRT6 migrated as a strongly labeled 39 KDa band (Fig. 6b). However, this band was absent from the corresponding reactions of both His-tagged mutants, demonstrating that GST-mSIRT6 was unable to ADP-ribosylate those proteins. Coomassie staining of the gel prior to film exposure revealed similar
amounts of protein in all three lanes (data not shown). We therefore conclude that the auto-ADP-ribosylation reaction catalyzed by mSIRT6 is intra-molecular.

DISCUSSION

Here we provide the first characterization of a class IV sirtuin, mouse SIRT6. This sirtuin shows a relatively weak sequence homology to the yeast Sir2 (25%), suggesting a significant divergence in function and enzymatic activity. Consistent with published findings, we failed to detect any NAD-dependent protein deacetylase activity in preparations of mSIRT6 expressed in bacterial and mammalian cells. Surprisingly, mSIRT6 did show a robust ADP-ribosyltransferase activity in vitro, indicating that recombinant bacterial preparations indeed contained active protein.

We conclude that mSIRT6 is a mono-ADP-ribosyltransferase for the following reasons. First, the purified protein can catalyze the transfer of radiolabel from $^{32}$P--NAD, a reaction also catalyzed by other Sir2 family members (Frye, 1999; Imai et al., 2000; Tanny et al., 1999) and previously shown to be mono-ADP-ribosylation (Tanny et al., 1999). Second, this activity requires the catalytic function of mSIRT6, as two different point mutations in phylogenetically invariant residues predicted to abolish enzymatic activity eliminated the transfer of label from NAD to mSIRT6. Third, only the enzymatically-modified form of mSIRT6 is recognized by an anti-ADP ribose antibody specific to mono-ADP-ribosylated proteins. Finally, the transfer of label from $^{32}$P--NAD to mSIRT6 is accomplished in by an intra-molecular mechanism, indicating that SIRT6 may use ADP-ribosylation as a way to auto-regulate its activity.
Recombinant mSIRT6 expressed in E. coli and incubated with NAD was able to incorporate a quantity of ADP-ribose equivalent to 15% of the moles mSIRT6 present in the reaction. This level of ADP-ribosylation is impressive considering the intramolecular nature of the reaction, by which a molecule of SIRT6 is only active on a single substrate molecule. We failed to observe a band shift resulting from ADP-ribosylation, suggesting that the number of amino acid residues ADP-ribosylated on mSIRT6 is very small. Assuming a single ADP-ribosylation per molecule of SIRT6, our results indicate a strong 15% activity in our recombinant protein preparations.

Mouse SIRT6 is broadly expressed at the RNA and protein levels throughout development and during adulthood in at least eight different tissue types, indicating broad expression throughout the organism. The SIRT6 protein is particularly abundant in brain and liver. Interestingly, mSIRT6 protein expression is highest in muscle, even though mRNA levels in this tissue are low relative to those of actin. This might indicate increased SIRT6 protein stability in muscle compared to other tissue types. Alternatively, the disproportionate amount of SIRT6 protein observed in muscle may result from an increased rate of translation of SIRT6 mRNA in this tissue type.

Localization studies of mSIRT6 using N- and C-terminal GFP fusions as well as antibodies to two different epitopes showed that expression of mSIRT6 was largely nuclear, with only a diffuse presence in cytoplasm. Interestingly, endogenous levels of SIRT6 do not permeate certain intra-nuclear regions, probably corresponding to the nucleolus (Figure 3b). The only other mouse class IV sirtuin, mSIRT7, is localized to the nucleolus (E. F. and L. G., unpublished data). We therefore speculate that mSIRT6 and mSIRT7, despite extensive sequence homology (39%), have non-overlapping functions
within the cell. Like SIRT6, SIRT7 has not been shown to possess any \textit{in vitro} protein deacetylase activity (North et al., 2003).

Several ADP-ribosyltransferases are capable of reversible auto-modification resulting in altered enzymatic activity. \textit{Pseudomonas} ExoS, a bifunctional enzyme, is a Rho GTPase-activating protein (GAP) as well as an ADP-ribosyltransferase (Riese et al., 2002). Auto-ADP-ribosylation at arginine-146, observed \textit{in vitro} and \textit{in vivo}, reduces GAP activity (Riese et al., 2002), suggesting a mechanism for intramolecular regulation. In mammals, auto-modification of ADP-ribosyltransferase 5 (ART5) converts the protein from NADase to transferase (Weng et al., 1999), again suggesting a mechanism for regulation of enzyme activity. We speculate that SIRT6 might regulate its own activity \textit{in vivo} by ADP-ribosylation of specific residues required for activity. At this time, we have not yet identified the physiological targets of SIRT6. Unlike class I sirtuins including yeast Sir2 and SIRT1, SIRT6 appears to be highly specific \textit{in vitro}, even failing to catalyze ADP-ribosylation of SIRT6 molecules in \textit{trans}. The discovery of auto-ADP-ribosyltransferase activity in preparations of recombinant mouse SIRT6 is the first report of enzymatic activity for any class IV sirtuin. Future experiments will aim to identify physiological substrates ADP-ribosylated by SIRT6.
FOOTNOTES

*We thank H. Hilz (Hamburg, Germany) for the ADPR antibody. We also thank Laura Bordone (Guarente lab, MIT) for her kind contribution of mouse tissue protein samples. Finally, we would like to thank Marcia Haigis and Gil Blander for scientific input and critical discussions.

Abbreviations used in this study: mSIRT6, mouse SIRT6; GFP, Green Fluorescent Protein; DAPI, 4',6-diamidino-2-phenyindole; ADPR, ADP-ribose.

REFERENCES


FIGURES

Figure 1. *Sequence alignment of mouse sirtuin core domains.* Conserved core domains of mouse SIRT1, SIRT4, SIRT5, and SIRT6 were aligned with the yeast Sir2 core using ClustalW. In the sirtuin phylogenetic tree, SIRT1 is class I, SIRT4 is class II, SIRT5 is class III, and SIRT6 is class IV. At each position of the alignment, identical residues are boxed and shaded in gray. Amino acids filled in black correspond to mSIRT6 residues targeted for mutagenesis in this study.
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**Figure 2.** Analysis of mouse SIRT6 and actin expression in adult tissues and developing embryos. (A) RNA was isolated from the indicated tissues in wild-type adult mice, resolved by electrophoresis, and subjected to Northern blotting. Blots were probed with $^{32}$P-labeled cDNA specific to mSIRT6 (*top panel*) or actin (*lower panel*). Muscle actin found in heart and skeletal muscle samples migrated as a distinct band. (B) RNA from 7-day, 11-day, 15-day, and 17-day mouse embryos was analyzed by Northern blotting as in A. (C) Protein extracts from the indicated wild-type mouse tissues were resolved by SDS-PAGE, and analyzed by Western blotting using antibodies specific to mSIRT6 (*top panel*) or actin (*lower panel*). For each tissue type, samples from four different mice were included.
**Figure 3.** *Subcellular localization of mSIRT6 by GFP and indirect immunofluorescence microscopy.* (A) NIH 3T3 cells were transfected with GFP (top row), an N-terminal eGFP-mSIRT6 fusion (middle row), or a C-terminal mSIRT6-eGFP fusion (bottom row). Transfections were performed on glass coverslips in six-well plates. 48 hours post-transfection, cells were washed, fixed with paraformaldehyde, stained with DAPI, and analyzed by fluorescence microscopy. The FITC channel (left column) shows GFP fluorescence, and the DAPI channel (middle column) reveals nucleic acid localization. Images from both channels are merged in the rightmost column. For each transfection, a representative field of cells is pictured. (B) NIH 3T3 cells grown on coverslips were stained with affinity-purified rabbit polyclonal antibodies to two different mSIRT6 epitopes (full-length mSIRT6, middle row; mSIRT6 amino acids 274-355, bottom row). Samples were then stained with DAPI and Cy3-conjugated anti-rabbit IgG and visualized by fluorescence microscopy. Cy3 fluorescence is shown in the left column, DAPI-stained nucleic acids are shown in the middle column, and the merged image is shown in the right column.
Figure 4. NAD⁺-dependent modification of mSIRT6. (A) Recombinant hSIRT1 (left) and mSIRT6 (right) were incubated in the presence of ^32P-NAD⁺ with and without core histones. Samples were purified by gel filtration chromatography, subjected to SDS-PAGE, and exposed to film for 2 hrs. (B) Wild-type and two mutant forms of mSIRT6 (S56A; H133Y) were incubated with ^32P-NAD⁺, purified, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and developed by autoradiography (right panel). Presence of wild type and mutant SIRT6 was confirmed by probing membrane with affinity-purified anti-mSIRT6 rabbit polyclonal antibody (left panel).
A. mSIRT1 mSIRT6

Histones: - +

mSIRT6

Histones

B. SIRT6 SIRT6-S56A SIRT6-H133Y

anti-mSIRT6

C. SIRT6 SIRT6-S56A SIRT6-H133Y

32p
Figure 5. Quantification of auto-ADP ribosylation by mSIRT6. (A) 0.5 μL recombinant wild-type SIRT6 and two mutant forms (S56A, H133Y) were incubated with $^{32}$P-NAD, purified by gel filtration chromatography, and analyzed by SDS-PAGE. The gel was stained with Coomassie and photographed. SIRT6 quantities were determined by comparison to BSA standards run on the same gel. Wild type SIRT6 was estimated at 0.250 μg, or 6.4 pmol. (B) The stained gel from (A) was analyzed by autoradiography to confirm radiolabeling of SIRT6. (C) SIRT6 bands were excised from the gel and $^{32}$P radioactivity was quantitated by scintillation counting. Results are normalized to measurements from the corresponding region excised from pET28a vector control lanes. (D) An NAD standard curve was generated by scintillation counting of known quantities of $^{32}$P-NAD diluted to reflect the ratio of unlabeled NAD to $^{32}$P-NAD in the reaction. (E) Ratio of incorporated ADP-ribose to SIRT6 was determined as follows. Radioactivity measured in SIRT6 bands was converted to pmol NAD by comparison to the standard curve. Pmol of NAD was divided by pmol of SIRT6 to attain the fraction of labeled mSIRT6.
A. 

![Coomassie gel image](image1)

B. 

![32P gel image](image2)

C. 

![Bar graph](image3)

D. 

![Graph](image4)

E. 

![Bar graph](image5)
Figure 6. Auto-ADP-ribosylation of mSIRT6. (A) Equal amounts of recombinant mSIRT6 and mutant mSIRT6-H133Y protein were incubated with $^{32}$P-NAD$^+$ and blotted to nitrocellulose as described in Fig. 4. The blot was probed with antibody specific to mono-ADP-ribose, stripped, and reprobed with antibody specific to mSIRT6 as a control. (B) GST-SIRT6 was incubated with $^{32}$P-NAD$^+$ and either mSIRT6 (lane 1), mSIRT6-S56A (lane 2), or mSIRT6-H133Y (lane 3). Reactions were analyzed by autoradiography as in Fig. 4b. Coomassie staining of the gel prior to film exposure revealed similar amounts of recombinant protein in all three lanes (not shown).
Chapter 4

Conclusions


**SSD1 and UTH1**

Naturally occurring polymorphisms can account for significant effects on longevity and the rate of aging, not only in yeast but in humans, where a locus linked to extreme long life span has been mapped to chromosome 4 (Puca et al., 2001). Different alleles of *S. cerevisiae SSD1* have been isolated from both laboratory and wild yeast strains, indicating that this is a true polymorphic locus and suggesting that variation of *SSD1* positively impacts survival under certain environmental conditions (Sutton et al., 1991; Wheeler et al., 2003). The observation that *SSD1-V* affects aging adds to an already long list of cellular processes influenced by this gene. A summary of *SSD1*’s reported genetic interactions, included in Appendix A, Table 1, lists more than 50 genes whose phenotypes are modified by *SSD1*.

It is therefore notable that we present the first mechanistic study of the action of Ssd1p in cells, establishing this protein as a direct repressor of translation of at least one key target mRNA. Ssd1, like Mpt5/Puf5/Uth4, is likely to have a conserved regulatory role of some sort, probably in its capacity as an RNA binding protein and repressor of translation. However, the key downstream target of Ssd1p, Uth1p, is a protein specific to fungi. Interestingly, several genes associated with yeast longevity play a conserved role in evolution, despite the fact that yeast aging is predominantly controlled by a mechanism specific to yeast cells (discussed in Chapter 1). It is therefore possible that Ssd1 proteins play a role in maintaining life span and stress resistance in other organisms.

Exactly how Ssd1p and Uth1p affect life span is unclear, but genetic data clearly indicate that the mechanism is distinct from the main yeast aging pathways discussed in detail in Chapter 1. *SSD1-V* extends the life span of *sir2Δ* and *sir2Δ foB1Δ* strains,
strongly suggesting that the longevity caused by Ssd1p is independent of Sir2p activity. Indeed, Ssd1p does not reduce ERC accumulation or rDNA marker loss, strongly supporting this conclusion. A recent report indicates that the Sir2p homolog Hst2p promotes longevity during caloric restriction by a mechanism very similar to the one already described for Sir2p (Lamming et al., 2005). This effect, while genetically independent of SIR2, still occurs through a decrease in rDNA silencing and a reduction in ERC formation and accumulation. For this reason, we believe that Hst2p is an unlikely candidate to account for the Sir2p-independent extension of life span by Ssd1p.

Ssd1p exerts a profound effect on cell wall stability and cell integrity. Although the bud scar hypothesis of aging has been widely discredited, it is nevertheless possible that cell wall stability becomes a limiting factor for extremely old cells. Senescence is typically accompanied by eventual cell lysis, and it is therefore possible that stabilizing the cell wall might prevent this lysis and allow cell division to continue.

A small portion of the extension of life span observed in SSD1-V cells is attributable to an increase in Nca3p, a mitochondrial homolog of Uth1p. NCA3 mRNA is upregulated in both SSD1-V and UTHI cells, possibly as a means of compensating for a reduction in Uth1p function in both cases. Overexpression of NCA3 slightly extends life span, and deletion of this gene slightly shortens life span in an SSD1-V background. Despite extensive homology to UTHI, NCA3 does not appear to associate with Ssd1p in cells, and is regulated quite differently in response to SSD1-V. NCA3 has been reported to affect mitochondrial biogenesis (Pelissier et al., 1995), and might exert its modest effects on aging through an influence on metabolism.
Ssd1p is the second translational repressor found to strongly affect yeast aging. The first, Mpt5/Uth4/Puf5, also plays a conserved role in germ line stem cell development in *C. elegans* and *Drosophila* (Crittenden et al., 2002; Lin and Spradling, 1997). It will be interesting to see if Ssd1 and Mpt5 homologs have co-evolved with similar roles in these two organisms. In yeast, the similarities between Ssd1p and Mpt5 are striking. Both are large polymorphic repressors of RNA translation affecting cell wall stability, stress resistance, and life span through the regulation of critical target molecules. The discovery of these two genes as key polymorphic regulators suggests that control of RNA translation and stability is a key mechanism controlling phenotypic variation in the wild.

Very little is known of the homologs of *SSD1* in higher organisms, although *SSD1* is a conserved gene. The finding, presented in Chapter 2, that Ssd1p can act as a translational repressor should provide a starting point for studies of Ssd1p-related proteins in other organisms. We believe the next major step in the research of Ssd1p will be to systematically identify the RNA molecules associated with Ssd1p *in vivo*, as these represent excellent targets for regulation by Ssd1p. This approach has succeeded in identifying targets of several RNA-binding proteins in yeast and worms (Gerber et al., 2004; Lee and Schedl, 2001), and shows promise in other systems as well. It will be interesting to see if the other targets of Ssd1p are connected by a common theme, such as cell wall regulation or metabolism. It will also be interesting to see whether Ssd1 homologs regulate similar targets in different species.
**SIR2 and CR**

In Appendix B, we discuss the mechanism by which CR increases Sir2p activity to promote rDNA stability and longevity. The results presented in this thesis support the model wherein CR increases respiration, effectively increasing the NAD/NADH ratio through a reduction in NADH levels. By this model, Sir2p activity is increased by relief of competitive inhibition by NADH. An opposing model posits that Pnc1p, a nicotinamidase involved in NAD synthesis, is increased by CR, thereby lowering the levels of nicotinamide that normally limit Sir2p function (Anderson et al., 2003).

There is also a third possible model that has not been explicitly addressed by any published studies. Although Sir2p is a nuclear protein, there is reason to believe that NAD is freely diffusible between the nucleus and the cytoplasm. It is therefore possible that the major NAD-utilizing glycolytic enzymes of the cytosol compete with Sir2p for NAD binding, and it is the reduction of these enzymes during CR that activates Sir2p by increasing available (but not total cellular) NAD. Consistent with this model, deletion of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which converts glyceraldehydes-3-phosphate to 1,3-diphosphoglycerate while reducing one molecule of NAD to NADH, extends life span in a Sir2p-dependent manner (Lin et al., 2000). Overexpression of GAPDH isoform *TDH3* decreases Sir2p activity and rescues toxicity resulting from extremely high *SIR2* overexpression (Matecic et al., 2002). Future studies should investigate whether reduction of major NAD-utilizing enzymes of glycolysis is sufficient to activate Sir2p, and whether this activation might result from increased NAD availability to Sir2p.
SIRT6 and SIRT7

In Chapter 3 and Appendix C, we present the first characterization of class IV members of the Sir2 phylogenetic tree (Frye, 2000). Interestingly, despite extensive conservation within the catalytic core, both SIRT6 and SIRT7 fail to demonstrate an *in vitro* NAD-dependent deacetylase activity. Mouse SIRT6 does, however, catalyze a robust auto-ADP-ribosyltransferase reaction, the discovery of which counters the assumption, widely held in the field, that Sir2 proteins are deacetylases first and foremost, only weakly catalyzing ADP-ribosylation. It remains to be seen what is the biological significance of this ADP-ribosylation reaction. In most known cases, ADP-ribosylation of arginine residues results in reversible inactivation of the substrate protein (Riese et al., 2002), although this modification may enhance certain enzymatic functions within the substrate (Weng et al., 1999). Future studies should identify ADP-ribosylated targets of SIRT6.

SIRT7, is a broadly expressed nucleolar protein that strongly activates RNA polymerase I transcription by an enzymatic mechanism. It remains to be seen what the exact molecular target of SIRT7 is, but it is likely a member of the enormous Pol I complex. SIRT7 displays neither deacetylase activity nor ADP-ribosyltransferase *in vitro*, but this may result from substrate specificity issues rather than an inherent lack of activity. SIRT1, orthologous to the yeast Sir2, has the broadest substrate specificity of the mammalian sirtuins (North and Verdin, 2004) and is also the best characterized (reviewed in Chapter 1). The discovery of SIRT7 as an activator of rDNA transcription provides a potential link between energy metabolism, NAD availability, and ribosome biogenesis, all of which one would expect to be tightly linked. It will therefore be
important and interesting to establish a model of SIRT7 regulation analogous to those established for the yeast Sir2p.
REFERENCES


Appendix A

Saccharomyces cerevisiae SSD1-V Promotes Longevity by a Sir2p-Independent Mechanism

This chapter was published in Genetics Volume 166 Issue 4 pages 1661-1672. The authors were Matt Kaeberlein, Alex Andalis, Gregory Liszt, Gerald Fink and Leonard Guarente. I contributed Table 3, Figure 3, and Figure 4.
SUMMARY

The SSD1 gene of *Saccharomyces cerevisiae* affects diverse cellular processes including cell integrity, cell cycle progression, and growth at high temperature. We show here that the SSD1-V allele is necessary for cells to achieve extremely long life span. Furthermore, we demonstrate that addition of SSD1-V to cells can increase longevity independently of SIR2 or calorie restriction. Microarray analysis differentiates SSD1-V from four other long-lived cell types and suggests that the presence of SSD1-V results in altered transcript levels for genes involved in many cellular processes including cell wall biosynthesis, DNA repair and stress response, and RNA processing. We propose that SSD1-V defines a previously undescribed pathway affecting cellular longevity and aging.
INTRODUCTION

Aging in *Saccharomyces cerevisiae* can be studied by mutations that extend the replicative life span of mother cells, defined as the number of daughters produced by a given mother cell prior to senescence. We report here a new longevity-promoting factor, *SSD1*-V, that extends mean life span up to 85% and promotes longevity in the absence of Sir2p.

One cause of aging in yeast is the accumulation of extrachromosomal ribosomal DNA circles (ERCs) (SINCLAIR and GUARENTE 1997). The yeast ribosomal DNA (rDNA) is organized into a tandem array of 100-200 copies of a 9.1 kb repeat (PETES and BOTSTEIN 1977; PHILIPPSEN *et al.* 1978; RUSTCHENKO and SHERMAN 1994). Homologous recombination between adjacent repeats can result in excision of an ERC. ERCs are self-replicating and asymmetrically segregated to the mother cell nucleus during S-phase, resulting in an exponential increase in ERC copy number with age (SINCLAIR and GUARENTE 1997). Once a threshold level of ERCs is achieved, it is thought that a cell senescence pathway is activated that culminates in cell death.

A central regulator of life span in yeast is the Sir2 protein (KAEBERLEIN *et al.* 1999). Sir2p is an NAD-dependent histone deacetylase (IMAI *et al.* 2000; LANDRY *et al.* 2000; SMITH *et al.* 2000) required for transcriptional silencing at telomeres (GOTTSCHLING *et al.* 1990), silent mating (HM) loci (IVY *et al.* 1986; RINE and HERSKOWITZ 1987), and the rDNA (BRYK *et al.* 1997; SMITH and BOEKE 1997). Mutation of SIR2 results in increased rDNA recombination (GOTTLIEB and ESPOSITO 1989), increased ERC formation (KAEBERLEIN *et al.* 1999), and decreased life span (KENNEDY *et al.* 1995), whereas overexpression extends life span by 30-40% (KAEBERLEIN *et al.* 1999). Sir2p is also required for life span extension by calorie restriction (CR), demonstrating the importance of this protein as a central regulator of longevity (LIN *et al.* 2000).

Intriguingly, overexpression of a Sir2p homolog, Sir-2.1, was found to extend life span in the nematode *C. elegans*, suggesting that Sir2 proteins also regulate aging in higher eukaryotes (TISSENBAUM and GUARENTE 2001).
Genetic interaction between \textit{MPT5} and \textit{SSD1}: \textit{SSD1} is a polymorphic locus that affects diverse cellular processes. Two allele classes have been identified for \textit{SSD1}, designated \textit{SSD1-V} and \textit{ssd1-d}. \textit{SSD1-V} alleles confer viability in the absence of the Sit4 protein phosphatase and code for functional Ssd1 protein. In contrast, strains carrying \textit{ssd1-d} alleles are inviable in the absence of Sit4p (SUTTON \textit{et al.} 1991), and d-type alleles are likely null for Ssd1p function. Both V and d type alleles have been found in natural isolates as well as laboratory strains of \textit{S. cerevisiae}. A recent report (WHEELER \textit{et al.} 2003) suggests that \textit{SSD1} allele type affects pathogenicity of yeasts, suggesting that the \textit{SSD1} locus is susceptible to strong selective pressure under certain environmental conditions.

A potential role for \textit{SSD1-V} as a regulator of cell life span was suggested by the observation that \textit{SSD1-V} suppresses many phenotypes associated with mutation of the \textit{MPT5/UTH4} gene (KAEBERLEIN and GUARENTE 2002). \textit{MPT5} is a post-transcriptional regulator (TADAUCHI \textit{et al.} 2001) involved in regulating pheromone response (CHEN and KURJAN 1997), cell wall stability (KAEBERLEIN and GUARENTE 2002), telomere silencing (COCKELL \textit{et al.} 1998), and longevity (KENNEDY \textit{et al.} 1995). Like \textit{SIR2}, \textit{MPT5} is a limiting factor for longevity: overexpression of \textit{MPT5} extends life span, while deletion has the opposite effect (KENNEDY \textit{et al.} 1997).

\textit{SSD1-V} suppresses the temperature sensitive growth defect caused by mutation of Mpt5p as well as the sensitivity to calcofluor white (CFW) and sodium dodecyl sulfate (SDS)(KAEBERLEIN and GUARENTE 2002). Furthermore, in strains lacking \textit{SSD1-V}, deletion of \textit{MPT5} is synthetically lethal in combination with loss of function in either SBF or \textit{CCR4} transcriptional complexes (KAEBERLEIN and GUARENTE 2002), both of which function downstream of protein kinase C (Pkc1p) to promote cell wall biosynthesis (CHANG \textit{et al.} 1999; IGUAL \textit{et al.} 1996; MADDEN \textit{et al.} 1997). These
results were interpreted to suggest that Mpt5p, Ssd1p, and Pkc1p define three parallel pathways that function to ensure cell integrity (KAEBERLEIN and GUARENTE 2002).

In addition to suppressing the cell integrity defects, SSD1-V suppresses the shortened life span caused by deletion of MPT5 (KAEBERLEIN and GUARENTE 2002). We were therefore interested in examining the possibility that SSD1-V might also promote longevity in wild type cells. Here we show that addition of a single copy of SSD1-V to ssd1-d wild type cells extends life span in at least two different strain backgrounds. Furthermore, life span extension by SSD1-V does not require the Sir2 protein, although the presence of both SSD1-V and SIR2 is necessary for maximal longevity.

**MATERIALS AND METHODS**

**Strains and genetic techniques:** The strains used in this study are listed in Table 2. All strains were derived from W303R (described in MILLS et al. 1999), PSY316 (described in PARK et al. 1999), or BKY5 (described in KENNEDY et al. 1995). Genetic crosses, sporulation, and tetrad analysis were carried out as described (SHERMAN and HICKS 1991). The genotype of inviable spore clones was inferred when possible based on marker segregation in viable spore clones from the same tetrad. Unless otherwise noted, cells were cultured in YPD or synthetic media prepared using conventional methods (GUTHRIE and FINK 1991). Yeast transformation was accomplished by the lithium acetate method (GIETZ et al. 1992). The sir2 fob1 strain was constructed as described (LIN et al. 2000). All other gene deletions were generated by transforming cells with PCR amplified disruption cassettes as described (KAEBERLEIN et al. 1999). In each case, the entire open reading frame was removed. All disruptions were verified phenotypically or by PCR. The SSD1-V integrating plasmids p406SSD1 and p405SSD1 were previously described (KAEBERLEIN and GUARENTE 2002). Unless otherwise indicated, all SSD1-V strains contain SSD1-V integrated at the marker locus and still carry the ssd1-d allele at the SSD1 locus. Deletion of ssd1-d does not affect any of the phenotypes tested, including life span, growth at 30°, 37°, or 40°, or sensitivity to calcofluor white.
**Determination of SSD1 allele:** In order to determine which SSD1 allele was present in PSY316, we deleted one copy of the SIT4 gene in diploid cells. Sporulation of these cells revealed that deletion of SIT4 always resulted in lethality (n>20 sit4Δ spore clones). This lethality was suppressed by integration of a single copy of SSD1-V at the URA3 locus. Therefore, we conclude that in PSY316, the SSD1 allele is a ssdl-d allele.

**Life span, recombination and ERC analysis:** Life spans were performed as described (KAEBERLEIN and GUARENTE 2002). Statistical significance was determined by a Wilcoxon rank sum test. Average life span is stated to be different for p<0.05. Each figure represents data derived from a single experiment, unless otherwise stated. ERC levels were determined as described (KAEBERLEIN et al. 1999; DEFOSSEZ et al. 1999). ERCs were separated on a 0.6% agarose gel without addition of ethidium bromide at 1 Volt/cm for 48 hr. rDNA recombination rate was determined as described (KAEBERLEIN et al. 1999).

**Microarray analysis:** RNA isolation and microarray analysis was performed essentially as described (LIN et al. 2002). Candidate genes with altered transcript levels in SSD1-V cells relative to wild type were defined as such if the ratio of SSD1-V (Cy5) to ssdl-d (Cy3) was greater than 1.5 or less than 0.667 (1.5-fold decrease) in 2/2 independent experiments. All such genes are listed in the supplemental data (Supplemental Table 1). As a control, microarray analysis was performed on ssdlA (Cy5) cells relative to ssdl-d (Cy3) cells. The 124 genes defined as regulated by CR represent the subset of genes found to show significant changes in mRNA expression both in cells lacking HXK2 and in cells grown on 0.5% glucose (LIN et al. 2002). Cluster analysis was performed using Cluster and visualized with TreeView (EISEN et al. 1998). Statistical significance of the overlap between regulated genes in different experiments shown in Figure 4 was calculated using a hypergeometric distribution. P-values were obtained from the online hypergeometric distribution calculator at http://www.alewand.de/stattab/tabdiske.htm. Supplemental Table 1 as well as the entire microarray data sets for all experiments presented in this paper are available on the World Wide Web at

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RESULTS

SSDI-V extends life span and improves growth at high temperature: Mpt5p is a limiting factor for longevity and functions in a pathway parallel to SSDI-V for cell integrity (KAEBERLEIN and GUARENTE 2002). Based on this genetic interaction, we hypothesized that SSDI-V might also regulate longevity. We first determined that our wild type strain PSY316 carries the ssdl-d allele at the SSD1 locus, based on inviability caused by deletion of SIT4 in this background (see Materials and Methods). A single copy of SSDI-V integrated at the URA3 locus results in an approximately 50% increase in mean life span (Figure 1A). Integration of SSDI-V at the SSD1 locus has a similar effect on life span (not shown). Deletion of the chromosomal ssdl-d allele of PSY316 has no effect on life span and does not affect life span extension by SSDI-V (Figure 1A). Therefore ssdl-d is a null allele with respect to life span. All subsequent experiments were carried out in the parental ssdl-d background.

PSY316 is a moderately long-lived yeast strain, however many yeast aging studies have been carried out in short lived strain backgrounds having mean life spans of 10-15 generations. In order to determine whether the life span extension by SSDI-V was strain specific, we integrated SSDI-V into the short-lived strain BKY5. We verified that BKY5 carries an ssdl-d allele (see Materials and Methods) as well as a previously identified C-terminal truncated allele of MPT5 (KENNEDY et al. 1997). Addition of SSDI-V to BKY5 results in an 85% increase in mean life span (Figure 1B). Thus, SSDI-V promotes long life span in at least two different ssdl-d strain backgrounds.

We had previously observed that cells from strain PSY316 grow normally at 37°, but are incapable of sustained growth at 40°. Cells grown at 40° generally arrest as large budded cells with a significant fraction undergoing lysis (data not shown), consistent with a loss
of cell wall integrity at the restrictive temperature. Addition of SSD1-V fully suppresses these phenotypes and allows growth of PSY316 at 40° (Figure 1C). Addition of SSD1-V to PSY316 also improves growth in the presence of the cell wall perturbing agents CFW and SDS (data not shown), as previously reported for strain W303R (KAEBERLEIN and GUARENTE 2002).

**SSD1-V extends life span in the absence of SIR2:** The Sir2 protein is a central regulator of yeast longevity, necessary for life span extension in response to environmental signals such as reduced nutrient availability (LIN et al. 2000) and osmotic stress (KAEBERLEIN et al. 2002). In order to place SSD1-V into a genetic pathway relative to Sir2p, we integrated the SSD1-V allele into a sir2 fob1 double mutant. The sir2 fob1 double mutant was utilized because it has a nearly wild-type life span, rather than the extremely short life span observed for sir2 FOB1 cells (KAEBERLEIN et al. 1999). As predicted by our model previously proposed model (LIN et al. 2000; LIN et al. 2002), calorie restriction (CR) by growth on low glucose fails to extend life span in the absence of SIR2 (Figure 2A). In contrast, sir2 fob1 SSD1-V cells grown on 2% glucose have a life span that is significantly longer than sir2 fob1 ssdl-d cells. However, sir2 fob1 SSD1-V cells do not live as long as SIR2 FOB1 SSD1-V cells (Figure 2B). Therefore, SSD1-V acts in a novel, SIR2-independent pathway for longevity, although Sir2p is required for maximum longevity in SSD1-V cells.
The Sir2-dependent life span extension caused by CR is the result of a metabolic shift from fermentation to respiration (LIN et al. 2002). It is possible that a portion of the longevity conferred by SSD1-V is achieved by causing the cell to undergo a similar metabolic shift. To address this possibility, the effect of SSD1-V on life span was examined in a respiration deficient strain lacking the CYT1 gene encoding cytochrome c1. Mutation of CYT1 prevents life span extension by CR or by overexpression of the Hap4p transcription factor (LIN et al. 2002). In contrast, SSD1-V extends the life span of cells lacking CYT1 to the same extent as wild type cells (Figure 2C), suggesting that the mechanism of life span extension by SSD1-V is independent of mitochondrial function and respiration.

**SSD1-V acts independently of ERC formation or accumulation:** Sir2p and calorie restriction promote longevity by decreasing the formation and accumulation of ERCs in mother cells (KAEBERLEIN et al. 1999; LIN et al. 2000). In order to determine whether the long life span of SSD1-V cells is also due to fewer ERCs, we measured the rate of ERC formation and the amount of ERCs present in ssd1-d and SSD1-V cells. ERC formation was estimated by determining the frequency at which an ADE2 marker integrated into the rDNA is lost, as demonstrated by the presence of half-sector colonies (KAEBERLEIN et al. 1999). No difference was observed between SSD1-V and ssd1-d cells by this assay (Figure 3A). Upon direct quantitation of ERCs from unsorted cells, we observed that SSD1-V cells often had higher levels of ERCs than ssd1-d cells (Figure 3B), indicating that the life span extension caused by SSD1-V is unlikely to be the result of decreased ERC formation or accumulation. This is consistent with the observation that SSD1-V does not require Sir2p to extend life span.

**Transcriptional analysis of SSD1-V:** In order to further understand the effect of SSD1-V on cell physiology and longevity, we used microarray analysis to examine the transcriptional profile of SSD1-V cells relative to wild type ssd1-d cells. In 2/2 independent experiments, 83 genes were down-regulated and 217 genes were up-regulated at least 1.5-fold by SSD1-V (see Supplemental Table 1).
The most common phenotype associated with Ssd1p is the ability to suppress temperature sensitivity caused by mutations that affect the cell wall (Table 1). Recently, SSD1-V has been found to increase resistance to the cell wall perturbing agents CFW and SDS (KAEBERLEIN and GUARENTE 2002) and to alter the amount of chitin, β-1,6-glucan, β-1,3-glucan, and mannan present in the cell wall (WHEELER et al. 2003). Microarray analysis suggests that these observations can be at least partially explained by altered expression of cell wall genes in SSD1-V cells. Messenger RNA levels for several cell wall proteins, including the Chs5p chitin synthase and Kre5p, a protein required for β-1,6-glucan biosynthesis, are elevated by SSD1-V. Interestingly, we also observe increased transcript levels for several proteins involved in sporulation and spore wall formation. In particular, SWM1 and TEP1, two genes involved in assembly of the spore cell wall are up-regulated 2.2-fold and 2.1-fold respectively, perhaps suggesting a fundamental alteration in the cell wall structure of vegetatively growing SSD1-V cells. We have previously demonstrated a correlation between cell wall stability and longevity (KAEBERLEIN and GUARENTE 2002), suggesting that enhanced cell wall integrity may be necessary for cells to attain extreme longevity.

SSD1-V has also recently been reported to promote resistance to the plant antifungal osmotin (IBEAS et al. 2000; IBEAS et al. 2001). It has been suggested that this increased resistance is due to an altered composition of cell wall mannoproteins in SSD1-V cells. Indeed, we find that mRNA coding for the cell wall mannoprotein, Cwp1p, is decreased 2.4-fold in SSD1-V cells. Cwp1 is known to directly bind osmotin (IBEAS et al. 2000), suggesting that reduction of Cwp1p might promote the osmotin resistance observed in SSD1-V cells.

SSD1-V has also been found to suppress the temperature sensitivity of several splicing defective mutations (LUUKKONEN and SERAPHIN 1999). Consistent with these observations, we find that several genes involved in RNA processing are differentially transcribed in SSD1-V cells relative to ssd1-d cells, perhaps accounting for this phenotype. In addition to these changes, SSD1-V cells also show altered levels of mRNA coding for proteins involved in DNA repair, stress response, mating, and mitochondrial
function. Reasonable hypotheses could be made as to why each of these functional categories might be important for the effect of SSD1-V on life span. Therefore, further experimental analysis of specific candidate genes will be required in order to determine which are important for longevity.

One particularly interesting candidate gene that shows altered mRNA levels in SSD1-V cells is NCA3. Nca3p is a member of the SUN family of proteins (Sim1, Uth1, Nca3, and Sun4) and functions to promote maturation of the mitochondrially encoded ATP8-ATP6 co-transcript (PELISSIER et al. 1995). Up-regulation (3.4-fold) of NCA3 by SSD1-V is striking because Nca3p shares extensive homology (60%) with the aging protein Uth1p. Interestingly, we find that NCA3 mRNA is increased 4-fold in long-lived cells lacking Uth1p (data not shown). Overexpression of NCA3 from the ADH1 promoter results in a slight, but reproducible increase in life span (Figure 4A), suggesting that Nca3p dosage can affect longevity. However, NCA3 is not required for the majority of the life-span extension seen in SSD1-V cells, as demonstrated by the finding that nca3 SSD1-V cells have a life span comparable to NCA3 SSD1-V cells (Figure 4B). Therefore, we conclude that increased transcription of NCA3 accounts for, at most, a minor fraction of the longevity promoting activity of SSD1-V.

Gene expression analysis of long-lived cell types: We have previously shown that calorie restriction by growth in 0.5% glucose, which promotes long life span, causes characteristic changes in gene expression that are reproduced in two genetic models of CR, namely overexpression of HAP4 and deletion of HXK2 (LIN et al. 2002). More recently, we demonstrated that growth in the presence of high external osmolarity (HEO) also extends life span in a SIR2-dependent manner and results in a gene expression profile with significant similarity to calorically restricted cells (KAEBERLEIN et al. 2002). We were therefore interested in determining how closely the changes in gene expression caused by addition of SSD1-V would match those caused by CR and HEO.

Two dimensional cluster analysis (EISEN et al. 1998) of the SSD1-V gene expression data and previously published data sets for 0.5% glucose, HAP4-overexpression, HXK2
deletion (LIN et al. 2002), and 20% glucose (KAEBERLEIN et al. 2002) was performed over the set of genes previously identified as being regulated by CR (see Materials and Methods; LIN et al. 2002). Two independently derived data sets were included for each condition, except for cells lacking HXX2, for which only one microarray experiment was performed. In each case, the two data sets corresponding to a particular condition cluster together (Figure 5A).

As expected, cells grown in 0.5% glucose cluster most closely with cells lacking HXX2 and define the CR group (Figure 5B). Cells overexpressing HAP4 also cluster with this group, although on a separate branch. Cells exposed to HEO cluster with the HAP4/CR group on the next level of the tree, and together these four cell types comprise the SIR2-dependent longevity group. Strikingly, SSD1-V cells cluster separately from all the other data sets at the highest level of the tree.

This analysis is further supported by a comparison of genes co-regulated by each of the longevity promoting conditions above. Of the 124 genes regulated by CR, 55 are similarly regulated by HAP4-overexpression and 48 are similarly regulated by growth in 20% glucose (Figure 5C). In contrast, only 8/290 genes differentially transcribed by addition of SSD1-V overlap with the CR-regulated genes (Figure 5C). Thus, the gene expression changes observed by microarray analysis strongly support the genetic data and suggest that SSD1-V promotes longevity by a novel mechanism unrelated to CR.

**MPT5 and SIR2 affect longevity in a pathway parallel to SSD1-V:** We have previously demonstrated that MPT5 and SSD1-V act in parallel pathways to promote cell integrity and that SSD1-V suppresses the short life span caused by deletion of MPT5 in the W303R strain background (KAEBERLEIN AND GUARENTE 2002). As expected, addition of SSD1-V also suppresses the short life span of the mpt5 deletion in PSY316 (Figure 6A). It is interesting to note, however, that mpt5 SSD1-V cells have a life span intermediate between cells lacking both MPT5 and SSD1-V and cells with functional copies of both genes. In fact, mpt5 SSD1-V cells have a life span not significantly different than the wild type MPT5 ssd1-d strain, suggesting that MPT5 and SSD1-V have
additive effects on longevity, as would be expected for genes functioning in parallel pathways.

Like SSD1-V, overexpression of MPT5 increases mother cell life span (KENNEDY et al. 1997). Since SSD1-V is capable of extending the life span of cells lacking SIR2, we wished to determine whether overexpression of MPT5 would have a similar effect. In contrast to SSD1-V, MPT5-overexpression fails to extend the life span of sir2 fob1 cells (Figure 6B), suggesting that MPT5 and SIR2 act in the same pathway to promote longevity.

It had been previously observed that cells with altered dosage of MPT5 display changes in telomeric and rDNA silencing (KENNEDY 1996), suggesting a further link between MPT5 and SIR2. Overexpression of MPT5 increases rDNA silencing and decreases telomeric silencing, while deletion has an opposite effect (Table 3). Integration of SSD1-V, in contrast, has no detectable effect on silencing at either locus. Consistent with the inability of MPT5 overexpression to extend life span in the absence of SIR2, the enhanced rDNA silencing observed in cells overexpressing MPT5 is fully suppressed by deletion of SIR2 (Table 3). Based on these results, we propose that overexpression of MPT5 increases life span by relocalizing Sir2p from telomeres to the rDNA (see Discussion), thus enhancing ability of Sir2p to inhibit ERC accumulation in aging mother cells.

**DISCUSSION**

One cause of aging in yeast is the accumulation of ERCs (SINCLAIR and GUARENTE 1997). A central regulator of ERC formation and longevity is the Sir2p histone deacetylase (KAEBERLEIN et al. 1999). Several genes that regulate yeast life span act by altering Sir2p activity or dosage (KAEBERLEIN et al. 1999; LIN et al. 2000; LIN et al. 2002; KAEBERLEIN et al. 2002). Here we present evidence that SSD1-V defines a novel Sir2p-independent pathway necessary for cells to achieve extreme longevity.
Two pathways promoting longevity: We initially began studying **SSD1-V** based on its ability to suppress the temperature sensitivity caused by mutation of the **UTH4/MPT5** gene. Like Sir2p, Mpt5p is limiting for life span in wild type cells (KENNEDY et al. 1997). Overexpression of Mpt5p increases life span and rDNA silencing in a Sir2p-dependent manner (Figure 6B, Table 3), suggesting that Mpt5p promotes longevity by increasing Sir2p activity at the rDNA.

In contrast to overexpression of **MPT5**, addition of a single copy of **SSD1-V** extends life span in both **SIR2** wild type and **sir2 fo81** double mutant cells (Figure 2C). However, the **sir2 fo81 SSD1-V** strain has a life span that is shorter than the **SIR2 FOB1 SSD1-V** strain, demonstrating that Sir2p is required for maximum longevity in **SSD1-V** cells. This is consistent with the observation that **MPT5 SSD1-V** cells have a longer life span than **mpt5 SSD1-V** cells (Figure 6A), and suggests a model whereby Mpt5p and Sir2p function in one pathway to increase life span while Ssd1p functions in a parallel pathway (Figure 7).

Mechanism of life span extension by **SSD1-V**: How is **SSD1-V** acting to extend life span? The effect of Sir2p on life span is, at least partially, due to its ability to deacetylate rDNA histones and inhibit ERC formation (KAEBERLEIN et al. 1999). We have observed no evidence to suggest that **SSD1-V** affects the rate of ERC formation or accumulation. Addition of **SSD1-V** had no effect on rDNA recombination (Figure 3A) or on rDNA silencing (Table 3) in PSY316, and we failed to detect a decrease in ERC levels in **SSD1-V** cells relative to **ssd1-d** cells (Figure 3B). While it is still possible that **SSD1-V** affects ERC replication or segregation specifically in aged cells, we feel that this is unlikely to be the case. An alternative possibility is that **SSD1-V** makes cells more resistant to ERCs, rather than reducing ERC levels. In support of this hypothesis, we often observed that steady-state ERC levels were increased in unsorted **SSD1-V** cells relative to wild-type **ssd1-d** cells (Figure 3B), although this was not always the case. The mechanism by which ERCs induce senescence is currently unknown. One hypothesis is that ERCs bind to and titrate key cellular replication or transcription factors away from
their normal targets. Alternatively, the rapid amplification of rDNA sequence could alter rRNA transcription and/or processing, resulting in ribosome dysregulation. Ssd1p has been shown to bind RNA and is predicted to have RNase activity (UESONO et al. 1997). Perhaps, SSD1-V somehow alters rRNA or ribosome biogenesis in a manner that makes cells more resistant to ERCs.

One attractive hypothesis is that SSD1-V promotes longevity by increasing cell wall stability and cell integrity. SSD1-V suppresses several temperature sensitive mutations that weaken the cell wall (Table 1) and has been found to directly affect cell wall composition (WHEELER et al. 2003). Furthermore, microarray analysis suggests that mRNA transcripts coding for cell wall regulatory and biosynthetic proteins are differentially expressed in SSD1-V cells (Supplemental Table 1). SSD1-V also improves resistance to the cell wall perturbing agents CFW and SDS (KAEBERLEIN and GUARENTE 2002), and increases the maximum temperature at which PSY316 is capable of growth (Figure 1C). Perhaps, the cell wall becomes limiting in very old cells and SSD1-V extends life span by stabilizing it. How might cell wall stability limit replicative life span? The terminal phenotype of yeast cells in the life span assay is cell cycle arrest often accompanied by cell lysis (MCVEY et al. 2002). Enhanced cell wall stability may prevent cell lysis late in life and allow additional cell divisions to occur.
Genetic diversity and the study of aging: The data presented here identify a genetic polymorphism that has a profound effect on mother cell life span. Genetic polymorphisms have also been proposed to affect the likelihood of achieving extreme longevity in human populations (PUCA et al. 2001), as well as other model systems. Since both ssd1-d and SSD1-V allele types have been isolated from natural yeast populations, the SSD1 locus represents a true polymorphic locus affecting longevity. In the past, researchers studying aging in yeast have tended to avoid using long-lived wild type backgrounds. We speculate that the majority (if not all) of these shorter lived yeast strains carry ssd1-d alleles. A comprehensive reevaluation of previously identified mutations affecting life span in a long-lived SSD1-V background would be of value to the field.

SSD1-V is a polymorphic locus that confers extreme longevity on yeast mother cells by a pathway independent of Sir2p. Sir2p has been found to extend life span in animals and, like SIR2, SSD1 homologs are present in yeast, worms, flies, and mammals. Might SSD1 family members also promote longevity outside of yeast? The mechanism by which SSD1-V cells achieve up to 85% longer life span is still unknown. Further work should be devoted to testing candidate longevity genes regulated by SSD1-V and to defining the molecular function of Ssd1p in cells.
REFERENCES


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Kennedy, B. K., 1996 Genetic and Molecular Analysis of Aging in Yeast, pp. 233 in Biology. Massachusetts Institute of Technology, Boston.


TABLE 1.

Reported genetic interactions with SSD1-V.

<table>
<thead>
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<th>Function</th>
<th>Genetic Interaction</th>
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<td>COSTIGAN et al. 1992</td>
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<td>GTPase activating protein required for polarized cell growth</td>
<td>ts</td>
<td>KIM et al. 1994</td>
</tr>
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<td>Protein that binds ubiquitin ligase.</td>
<td>ts</td>
<td>YASHIRODA et al. 1996</td>
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<td>sl(mpt5), ts</td>
<td>DU and NOVICK 2002</td>
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<td>CCR4</td>
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<td>G</td>
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175
<table>
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Abbreviations for genetic interactions are as follows: ts = suppresses temperature sensitivity, sl(x) = suppresses synthetic lethality between given gene and gene x, G = improves growth, L = suppresses lethality, D = causes death.
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<td>MKY590</td>
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TABLE 3.

Effects of \textit{MPT5}, \textit{SIR2}, and \textit{SSD1-V} on silencing

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<td>\textit{sir2A}</td>
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<td>\textit{ssd1Δ}</td>
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<td>↔</td>
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<tr>
<td>\textit{SSD1-V}</td>
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</table>

Stated effects on silencing are relative to silencing in the wild type \textit{ssd1-d} background. Telomere and rDNA silencing were measured by color formation using an \textit{ADE2} marker integrated into subtelomeric or ribosomal DNA. In order to detect decreased rDNA silencing relative to wild type in the \textit{sir2A} strain, an \textit{rpd3Δ} mutation was introduced into the wild type background.
Figure 1 - *SSD1-V* extends life span and improves cell integrity. (A) Life spans were determined for PSY316 (◆), PSY316 *SSD1-V* (■), PSY316 *ssdlA::HIS3* (X), and PSY316 *ssdlA::HIS3  SSD1-V* (▲). Mean life spans and number of cells analyzed were: PSY316 22.2 (n=40), PSY316 *SSD1-V* 36.2 (n=40), PSY316 *ssdlA::HIS3* 21.9 (n=40), and PSY316 *ssdlA::HIS3  SSD1-V* 38.0 (40). (B) Life spans were determined for BKY5 (◆) and BKY5 *SSD1-V* (▲). Mean life spans and number of cells analyzed were: BKY5 13.0 (n=40) and BKY5 *SSD1-V* 24.3 (n=40). (C) PSY316 cells are unable to grow at temperatures greater than 39° unless *SSD1-V* is present. 10-fold serial dilutions of a log phase culture plated onto YPD and incubated at either 30° or 40° for 48 hours are shown.
Figure 2. - SSD1-V and Sir2p act in different pathways to promote longevity. (A) Life spans were determined for PSY316 (◆), PSY316 sir2 fob1 (■), PSY316 0.5% glucose (▲), and PSY316 sir2 fob1 0.5% glucose (X). Mean life spans and number of cells analyzed were: PSY316 22.8 (n=40), PSY316 sir2 fob1 21.2 (n=39), PSY316 0.5% glucose 27.0 (n=40), and PSY316 sir2 fob1 0.5% glucose 21.4 (40). (B) Life spans were determined for PSY316 (◆), PSY316 sir2 fob1 (■), PSY316 SSD1-V (▲), and PSY316 sir2 fob1 SSD1-V (X). Mean life spans and number of cells analyzed were: PSY316 22.8 (n=40), PSY316 sir2 fob1 21.2 (n=39), PSY316 SSD1-V 37.3 (n=40), and PSY316 sir2 fob1 SSD1-V 30.0 (40). (C) Life spans were determined for PSY316 (◆), PSY316 SSD1-V (■), PSY316 cyt1 (▲), and PSY316 SSD1-V cyt1 (X). Mean life spans and number of cells analyzed were: PSY316 21.7 (n=40), PSY316 SSD1-V 39.3 (n=40), PSY316 cyt1 22.2 (n=40), and PSY316 SSD1-V cyt1 37.9 (40).
Figure 3. - *SSD1*-V does not extend life span by decreasing ERC levels. (A) *SSD1*-V has no detectable effect on rDNA recombination. rDNA recombination was measured by the frequency at which an *ADE2* marker integrated into the rDNA is lost. A total of 33,000 colonies from 3 independent derived isolates was examined for each strain. (B) DNA from unsorted cells was isolated and electrophoresed as described (Kaeberlein et al. 1999). The gel was transferred and probed with sequence homologous to the rDNA. Extrachromosomal rDNA circles (ERCs) are denoted by arrowheads. The dark band present in both lanes corresponds to genomic rDNA. In this isolate, the long-lived *SSD1*-V cells have a greater steady-state amount of ERCs than *ssd1*-d cells, as quantitated by the ratio of ERC DNA to genomic rDNA. However, in one independently derived *SSD1*-V transformant we were unable to detect a significant difference in ERC levels relative to wild type cells. In no case, did we detect fewer ERCs in *SSD1*-V cells.
Figure 4. - Effect of NCA3 cell life span. (A) Life spans were determined for PSY316 (◆) and PSY316 pADH_NCA3 (■). Mean life spans and number of cells analyzed were: PSY316 23.4 (n=80) and PSY316 pADH_NCA3 27.6 (n=120). This figure represents data pooled from two different experiments. (B) Life spans were determined for PSY316 (◆), PSY316 nca3 (■), PSY316 SSDI-V (▲) and PSY316 nca3 SSDI-V (X). Mean life spans and number of cells analyzed were: PSY316 23.3 (n=40), PSY316 nca3 22.3 (n=40), PSY316 SSDI-V 37.5 (n=40) and PSY316 nca3 SSDI-V 32.8 (n=40).
Figure 5. - Microarray Analysis of long-lived cells. (A) Gene expression profiles for long-lived cells were clustered over the 55 most highly expressed genes previously identified as being regulated by CR (Lin et al. 2002). (B) Identical clustering as in A, except over all 124 genes regulated by CR. The CR group clusters most closely together and consists of cells grown on 0.5% glucose or deleted for HXK2. Life span extension by CR, HAP4-overexpression or 20% glucose is SIR2-dependent, and these data sets cluster together on one of the two primary branches. Cells containing a single copy of SSD1-V cluster separately from the other long-lived cell types. (C) Comparison of the overlap in co-regulated genes between long-lived cell types. 55/124 genes transcriptionally altered by CR are similarly altered by overexpression of HAP4. This overlap is statistically significant (p < 10^{-40}). 48/124 genes transcriptionally altered by CR are similarly altered by growth in 20% glucose. This overlap is statistically significant (p < 10^{-50}). 8/124 genes transcriptionally altered by CR are similarly altered by introduction of SSD1-V. This overlap is not statistically significant (p = 0.1).
Figure 6. - *MPT5* and *SIR2* determine longevity in a pathway parallel to *SSD1-V*. (A) Life spans were determined for PSY316 (◆), PSY316 *SSD1-V* (■), PSY316 *mpt5* (▲), and PSY316 *mpt5 SSD1-V* (X). Mean life spans and number of cells analyzed were: PSY316 24.3 (n=40), PSY316 *SSD1-V* 35.5 (n=40), PSY316 *mpt5* 15.3 (n=40), and PSY316 *mpt5 SSD1-V* 22.9 (40).

(B) Life spans were determined for W303R (◆), W303R *pADH_MPT5* (■), W303R *sir2* (▲), W303R *sir2 pADH_MPT5* (X), W303R *SIR2/URA3* (●), and W303R *SIR2/URA3 pADH_MPT5* (●). This experiment was performed in the W303R strain background because overexpression of *MPT5* from the *pADH_MPT5* plasmid causes slow growth and decreased viability in strain PSY316. Mean life spans and number of cells analyzed were: W303R 20.9 (n=41), W303R *pADH_MPT5* 25.8 (n=70), W303R *sir2* 11.2 (n=41), W303R *sir2 pADH_MPT5* 10.1 (n=70), W303R *SIR2/URA3* 27.4 (n=40), and W303R *SIR2/URA3 pADH_MPT5* 25.8 (n=40).
Figure 7. - Genetic model for Ssd1p as a regulator of longevity.
Ssd1p acts parallel to Sir2p to extend life span. This could involve increasing the cell’s resistance to ERCs or could represent an ERC-independent longevity promoting function. One likely possibility is that *SSDI-V* results in an altered cell wall structure that allows mother cells to achieve extreme old age.
Caloric Restriction

Mpt5p

Sir2p

Ssd1p

ERC

Longevity

Cell wall?
APPENDIX B

Caloric Restriction Extends Yeast Life Span
By Lowering the Level of NADH

This chapter was published in Genes and Development, Volume 18, Issue 1, pages 12-16.
The authors were Su-Ju Lin, Ethan Ford, Marcia Haigis, Gregory Liszt, and Leonard Guarente. I contributed to the generation of the data presented in Figure 4.
SUMMARY

Calorie restriction extends life span in a wide variety of species. Previously, we showed that calorie restriction increases the replicative life span in yeast by activating Sir2, a highly conserved NAD-dependent deacetylase. Here we test whether CR activates Sir2 by increasing the NAD/NADH ratio or by regulating the level of nicotinamide, a known inhibitor of Sir2. We show that CR decreases NADH levels, and that NADH is a competitive inhibitor of Sir2. A genetic intervention that specifically decreases NADH levels increases life span, validating the model that NADH regulates yeast longevity in response to CR.
INTRODUCTION

Calorie restriction (CR) extends life span in a wide spectrum of organisms and for decades was the only regimen known to promote longevity in mammals (Weindruch and Walford 1998; Roth et al. 2001). CR has also been shown to delay the onset or reduce the incidence of many age-related diseases including cancer and diabetes (Weindruch and Walford 1998; Roth et al. 2001). Although it has been suggested that CR may work by reducing the levels of reactive oxygen species due to a slowing in metabolism (Weindruch and Walford 1998), the mechanism by which CR extends life span is still uncertain. To study the mechanism by which CR extends life span, we established a model of CR in the budding yeast *Saccharomyces cerevisiae*. In this system, life span can be extended by limiting glucose content in the media from 2% to 0.5% or by reducing the activity of the glucose-sensing cAMP-dependent kinase (PKA) (Lin et al. 2000).

The benefit of CR requires NAD (nicotinamide adenine dinucleotide, oxidized form) and Sir2 (Lin et al. 2000; Lin et al. 2002), a key regulator of life span in both yeast and animals (Kaeberlein et al. 1999; Tissenbaum and Guarente 2001). Sir2 is an NAD-dependent histone deacetylase and is required for chromatin silencing and life span extension (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). The requirement of NAD for Sir2 deacetylase activity suggested CR may activate Sir2 by increasing the available pool of NAD for Sir2 (Guarente 2000).

Our previous studies suggested that there was a second mechanism to activate
Sir2 and extend the life span in yeast – osmotic stress (Kaeberlein and Guarente 2002). This stress mechanism was genetically distinguishable from CR. Mutations knocking out electron transport prevented CR from extending life span, but had no effect on the longevity conferred by osmotic stress (Lin et al. 2002; Kaeberlein and Guarente 2002). This requirement for electron transport during CR is because of a shunting of carbon metabolism from fermentation to the mitochondrial TCA cycle. The concomitant increase in respiration is necessary and sufficient for the activation of Sir2-mediating silencing and extension in life span (Lin et al. 2002). The fact that respiration produces NAD from NADH (Bakker et al. 2001), reinforces the idea that changes in the NAD/NADH ratio regulates Sir2 during CR.

A recent report, however, challenged this model by claiming that both stress and CR activated Sir2 by a different mechanism, namely by decreasing intracellular levels of nicotinamide (Anderson et al., 2003), a non-competitive inhibitor of Sir2 (Bitterman et al. 2002). This change in nicotinamide levels was triggered by activation of the PNC1 gene, encoding a nicotinamidase in the NAD salvage pathway (Ghislain et al. 2002). This gene was strongly activated by osmotic stress, but to a lesser degree by CR. Also consistent with this model, PNC1 was shown to be required for the extension in life span by CR (Anderson et al. 2003). The authors conclude that CR reduces nicotinamide levels by up-regulating PNC1, and this reduction activates Sir2 and extends the life span.

In this report we attempt to determine whether changes in nicotinamide, the NAD/NADH ratio, or both up-regulate Sir2 during CR. We show that NADH is a...
competitive inhibitor of Sir2, and that CR reduces the level of NADH in cells. These findings provide a simple model for activation of Sir2 and extension of the life span by CR, which we further support by genetic experiments.

**RESULTS AND DISCUSSION**

To study whether CR activates Sir2 by increasing the NAD/NADH ratio, we first measured the NAD and NADH levels in cells grown in 2% and 0.5% glucose (CR). Surprisingly, the NAD levels in cells under CR were not changed (Fig. 1A), whereas the NADH levels were decreased to 50% of non-CR cells, (Fig. 1B). To validate the efficacy and sensitivity of our assay, we measured the NAD levels of the npt1Δ mutant. It has been shown that cells lacking the NPT1 gene (encoding nicotinic acid phosphoribosyl transferase in the NAD salvage pathway) exhibit a 40% to 60% decrease in NAD levels (Smith et al. 2000; Anderson et al. 2002). Consistent with previous reports (Smith et al. 2000; Anderson et al. 2002), we detected a similar decrease in the NAD levels in npt1Δ mutants. As a further test, we measured NAD and NADH levels in cells that over-express the transcription factor Hap4, which activates nuclear genes encoding mitochondrial proteins (Forsburg and Guarente 1989; de Winde and Grivell 1993). This manipulation was shown to extend life span in a Sir2 dependent manner (Lin et al. 2002). Similar to CR, Hap4 over-expression causes a switch of metabolism from fermentation toward respiration (Blom et al. 2000) and, as shown in Fig. 1B, triggered a 50% decrease in NADH levels without altering NAD concentration (Fig. 1A). Since respiration is required for life span extension in CR, we tested whether a functional electron transport chain is required for the decrease in NADH levels. As shown in Fig. 1D, deleting the
\textit{CYTI} gene, which encodes the cytochrome c1, abolished the decrease in NADH levels induced by CR. All these studies suggest that CR increases the intracellular NAD/NADH ratio by up-regulating respiration thereby decreasing NADH levels. We also measured NAD and NADH levels in a \textit{sir2A} mutant grown in 2\% and 0.5\% glucose. The \textit{sir2A} mutant exhibited the normal reduction in NADH levels in response to CR (shown in Fig. 1C, D), indicating that it functions downstream of the metabolic changes.

Since NADH levels responded to CR but NAD levels did not, it seemed possible that NADH, and not NAD, regulated Sir2, perhaps by inhibiting its activity. To test this hypothesis, we measured activity of Sir2 in the presence of various concentrations of NADH using purified recombinant GST-tagged Sir2 proteins. Sir2 activity was determined by quantitating the NAD-dependent deacetylation of histone H4 peptides labeled with [\textsuperscript{3}H]-acetyl coenzyme A (Armstrong et al. 2002). If NADH is indeed a competitive inhibitor of Sir2 activity, the presence of NADH should increase the apparent \(K_m\) (the Michaelis-Menten binding constant) of Sir2 for NAD without affecting the \(V_{\text{max}}\) (maximum velocity) (Stryer 1995). Kinetic analysis with a wide range of substrate (NAD) concentrations is shown as a Lineweaver-Burk double reciprocal plot (Fig. 2A). This analysis estimates the \(K_m\) (Fig. 2A, X intercept) of Sir2 to be 30 \(\mu\)M, consistent with previous report (Imai et al. 2000; Tanner et al. 2000). As NADH concentrations were stepped up, the apparent \(K_m\) for NAD was increased, reaching an increase of 3 fold at 250 \(\mu\)M NADH. The \(V_{\text{max}}\) (Fig. 2A, Y intercept) of Sir2 activity was not significantly changed in the presence of NADH. These data suggest NADH functions as a competitive
inhibitor of Sir2. As shown in Fig. 2B, NADH also competitively inhibited the human SIRT1. In the presence of 300 μM NADH, the $K_m$ of SIRT1 increased 3 fold.

Intracellular concentrations of total NAD plus NADH of about 1mM have been reported (de Koning and van Dam 1992; Richard et al. 1993). We have calculated values for NAD and NADH from the data in Fig 1, assuming a yeast cell size of 70 mm³ (Guthrie and Fink 2002). Table 1 shows our values and those of other recent reports. The data are in reasonable agreement with the previous reports and with each other, and small differences may by partly due to different estimates of cell size. The concentration of free NADH in cells must be less than the values in Table 1, and appears to be in a sensitive range to regulate Sir2 activity.

We sought genetic data to support the idea that the increase in the NAD/NADH ratio is what extends life span in CR. During respiratory growth, both cytosolic and mitochondrial NADH are re-oxidized, in part by the NADH dehydrogenases in the respiratory chain (Bakker et al. 2001). To determine whether yeast life span could be extended by simply activating NADH dehydrogenase, we over-expressed two related mitochondrial NADH dehydrogenases, Nde1 and Nde2 (Luttik et al. 1998). Similar to CR, over-expressing Nde1 and Nde2 significantly decreased the NADH levels without changing the NAD levels (Fig. 3A). Moreover, cells over-expressing Nde1 and Nde2 exhibited a longer life span on 2% glucose to a degree similar to cells grown on 0.5% glucose (Fig. 3B). Further, 0.5% glucose did not extend the life span of cells over-expressing the NADH dehydrogenases (Fig. 3B), suggesting that CR and the NADH
dehydrogenases function in the same pathway, i.e. to decrease NADH levels and extend the life span.

Our findings appear to challenge the claim that the Pnc1 nicotinamidase triggers the life span extension in CR (Anderson et al. 2003). To address this paradox, we first repeated life span analysis of the pnc1Δ mutants on 2% and 0.5% glucose. Consistent with the previous report, the pnc1Δ mutation largely prevented the life span extension by 0.5% glucose (at best a 10-15% increase) (Fig. 4A) when compared with wild type cells (~30% increase) (Fig. 4B). Since the above data indicated that CR functions by decreasing NADH, we surmised that hyper-accumulation of nicotinamide in the pnc1Δ mutants might mask regulation by NADH. Nnt1, a putative nicotinamide methyl transferase, appears to modify nicotinamide in yeast (Anderson et al. 2003). Thus, we over expressed Nnt1 to reduce nicotinamide levels in the pnc1Δ mutant. Strikingly, over-expressing Nnt1 restored the ability of CR to extend life span (~30%) in pnc1Δ mutants (Fig. 4B). These data show that the reduction in NADH can activate Sir2 and give a full extension of the life span in a pnc1Δ mutant, as long as the excess nicotinamide is depleted. This result shows that PNC1, the NAD salvage pathway, and glucose-dependent changes in nicotinamide levels are not required for the extension of life span by CR.

Our studies show that a switch to oxidative metabolism during CR increases the NAD/NADH ratio by decreasing NADH levels. NADH is a competitive inhibitor of Sir2, implying that a reduction in this dinucleotide activates Sir2 to extend the life span in
Indeed, over-expression of the NADH dehydrogenase specifically lowers NADH levels and extends the life span, providing strong support for this hypothesis. Regulation of the life span by NADH is also consistent with the earlier finding that electron transport is required for longevity during CR (Lin et al., 2002). The NAD/NADH ratio reflects the intracellular redox state and is a read out of metabolic activity. Our findings suggest that this ratio can serve a critical regulatory function, namely the determination of the life span of yeast mother cells. It remains to be seen whether this ratio will serve related regulatory functions in higher organisms.

MATERIALS AND METHODS

Yeast strain PSY316 MATa ura3-52 leu2-3, 112 his3-D200 ade2-101 lys2-801 RDN1::ADE2 has been previously described (Park et al. 1999). Rich media YPD and synthetic media were made as described (Sherman et al. 1978). The ADHI-driven integrating ppp35 (URA3) vector is a derivative of ppp81 (LEU2) vector (Lin et al. 2002) made by Peter Park. Over-expression constructs of Nde1, Nde2 and Nnt1 were made as described previously (Lin et al. 2002) using ppp81 (Nde1) or ppp35 (Nde2 and Nnt1). All constructs made for this study were verified by sequencing. The yeast Sir2 expression construct pGEX-SIR2 was a gift from J. Tanny and D. Moazed at Harvard Medical School (Tanny et al. 1999). The human SIRT1 expression construct pGEX-GST-hSIRT1 was a gift from F. Ishikawa at Tokyo Institute of Technology, Japan (Takata and Ishikawa 2003). All gene deletions in this study were done by replacing the wild type genes with the Kanr marker as described in (Guldener et al. 1996) and verified by Polymerase Chain Reaction (PCR) using oligonucleotides flanking the genes of interest.
Life span analyses were carried out as previously described (Lin et al. 2000). All life span analyses in this study were carried out on YPD plates at least twice independently with more than 45 cells per strain per experiment. Results from a single experiment are shown.

Yeast GST-Sir2 and human GST-SIRT1 fusion proteins were made using the T7 expression system as previously described (Ford and Hernandez 1997) with the following modifications. pGEX-SIR2 and pGEX-GST-hSIRT1 were transformed into the E. coli strain BL21(DE3) Codon-Plus-RIL (Stratagene) and BL21(DE3) respectively. In addition, the procedure for yeast GST-Sir2 was scaled up to a 10 liter culture and 5 ml of glutathione-agarose resin. The purified protein was concentrated on a Centriprep-30 (Amacon) column.

The deacetylation assay was carried out as described previously (Armstrong et al. 2002). In brief, histone deacetylase activity was measured using a peptide corresponding to the N-terminal tail of the histone H4 (Upstate Biotechnology) labeled with tritiated Acetyl Coenzyme A (PerkinElmer). Reactions were carried out in 50 μl buffer containing 50 mM Tris-HCl, pH 8, 4 mM MgCl₂, 0.2 mM DTT and a variable concentration of NAD and NADH at 30°C for 2.5 hours (yeast Sir2) or 37°C for 2 hours (human SIRT1).

Measurement of the NAD and NADH nucleotides were performed as described previously (Lin et al. 2001) with a few modifications. In brief, cells were grown to an OD₆₀₀ of 0.5 then 10⁷ cells were harvested in duplicates by centrifugation in 2 x 1.5 ml tubes. Acid extraction was performed in one tube to obtain NAD and alkali extraction was performed in the other to obtain NADH. 2 μl of neutralized cell extract (~10⁵ cells) was used for enzymatic cycling reaction as previously described (Lin et al. 2001). The concentration of nucleotides was measured fluorometrically with excitation at 365 nm.
and emission monitored at 460 nm. Standard curves for determining NAD and NADH concentrations were obtained as follows: NAD and NADH were added into the acid and alkali buffer to a final concentration of 0 μM, 2.5 μM, 5 μM, 7.5 μM which were then treated with the same procedure along with other samples. The fluorometer was calibrated each time before use with 0 μM, 5 μM, 10 μM, 20 μM, 30 μM and 40 μM NADH to ensure the detection was within a linear range.

Acknowledgment

We thank members of the Guarente laboratory for discussions; J. Gordon for advice with the NAD nucleotide measurements; J. Tanny and D. Moazed for the yeast Sir2 expression construct and discussions; F. Ishikawa for the human SIRT1 expression construct. Supported by NIH, the Ellison Medical Foundation, the Seaver Institute, and the Howard and Linda Stern Fund (LG); Individual National Research Service Award (S.-J. L., E. F., M. H.).
REFERENCES


Table 1: Intracellular concentrations of NAD and NADH.

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<th>Normal condition (mM)</th>
<th>Calorie restriction (mM)</th>
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^a Reported unit was 1.5x10^4 pmole/cell.
^b Reported unit was about 80 amole/cell.
^c Reported units were 23.7 amole/pg protein and 9.3 amole/pg protein for NAD and NADH respectively.
^d We converted reported units (a to c) into mM (per cell) assuming a yeast cell size of 70 femtoliter (70 μm^3) and the total protein of 6 pg/cell (Guthrie and Fink 2002).
^d Numbers show mean ± standard deviations derived from data shown in Fig 1.
**Figure 1.** Calorie restriction decreases intracellular NADH levels. Measurements of intracellular levels of NAD (A and C) and NADH (B and D) in various yeast strains grown in 2% or 0.5% glucose. Results show average of 3 independent experiments each conducted in duplicates. Error bars denote standard deviations. NAD and NADH levels are shown as (μM/μg): levels of NAD or NADH (μM) normalized to the concentrations of proteins from the extracts (μg) present in the reaction. Reactions contain cell extracts from $10^5$ cells. WT, wild type yeast strain PSY316; +Hap4-oe: wild type cells over-expressing Hap4; 2%: cells grown in 2% glucose; 0.5%: cells grown in 0.5% glucose. nptΔ, cytΔ and sir2Δhmr1Δ are isogenic derivatives of PSY316.
Figure 2. NADH inhibits Sir2 NAD-dependent histone deacetylase activity. (A) Kinetic analysis of the Sir2 NAD-dependent histone deacetylase activities in the presence of 0 μM (■), 50 μM (□), 100 μM (●) or 250 μM (○) NADH. 150 ng of recombinant GST-tagged yeast Sir2 protein was assayed with various concentrations of NAD and NADH at 30°C for 2.5 hrs. Data are shown as a Lineweaver-Burk double reciprocal plot of 1/V (CPM/hr) versus 1/[NAD] (μM). Results show average of three independent experiments each measured in duplicates. (B) Kinetic analysis of the human SIRT1 NAD-dependent histone deacetylase activities in the presence of 0 μM (■), 50 μM (□), 150 μM (●) or 300 μM (○) NADH. Experiments were carried out as in (A), except that 50 ng of recombinant GST-tagged human SIRT1 protein (Takata and Ishikawa 2003) was used and reaction was carried out at 37°C for 2 hrs.
Figure 3. Over-expressing NADH dehydrogenase increases the intracellular NAD/NADH ratio and life span. (A) Measurements of NADH (closed bars, left panel) and NAD (open bars, right panel) levels in cells over-expressing Nde1 and Nde2. Results show average of 4 independent experiments each conducted in duplicates. Error bars denote standard deviations. (B) Life span analysis of cells over expressing Nde1 and Nde2 grown in 2% and 0.5% glucose. Average life spans on 2% glucose: +vectors, 19.4; +Nde1-oe, +Nde2-oe: 27.2. Average life spans on 0.5% glucose: +vectors, 25.8; +Nde1-oe, +Nde2-oe: 27.1. + vectors: cells carrying control vectors ppp35 and ppp81; +Nde1-oe, +Nde2-oe: cells over-expressing Nde1 and Nde2.
Figure 4. Calorie restriction in \( pnc1\Delta \) mutants. (A) Calorie restriction slightly extends life span in a \( pnc1\Delta \) mutant. (B) Over-expressing Nnt1 restores the full life span extension by calorie restriction in a \( pnc1\Delta \) mutant. Average life spans on 2% glucose: \( pnc1\Delta \) + vector, 20.9; WT + vector, 20.16; \( \Delta pnc1\) + Nnt1-oe, 21.6. Average life spans on 0.5% glucose: \( pnc1\Delta \) + vector 23.9; WT + vector, 27.3; \( pnc1\Delta \) +Nnt1-oe, 28. WT: wild type PSY316 cells; + vector: cells carrying a control vector ppp35; + Nnt1-oe: cells over-expressing Nnt1. \( pnc1\Delta \) was derived from PSY316.
APPENDIX C

The Mammalian Sir2 Protein SIRT7 is an Activator of RNA Polymerase I Transcription

This chapter has been submitted for publication. The authors are Ethan Ford, Renate Voit, Gregory Liszt and Leonard Guarente. My contributions include Figure 1 and Supplemental Figure S1, as well as contributing to the writing of the paper.
SUMMARY

The yeast silent information regulator Sir2 is a highly conserved NAD\(^+\)-dependent protein deacetylase. Here, we show that one mammalian homolog, SIRT7, is preferentially localized in nucleoli, where it is associated with the coding regions of the rRNA genes (rDNA). SIRT7 interacts with RNA polymerase I (Pol I) as well as with histones. Overexpression of SIRT7 upregulates Pol I transcription, whereas knock-down of SIRT7 via siRNAs decreases Pol I transcription. SIRT7 increases the amount of Pol I at the coding region of the rDNA. Our results establish SIRT7 as a novel activating component of the mammalian Pol I transcription apparatus.
RESULTS AND DISCUSSION

RNA polymerase I (Pol I) is the most efficient of the mammalian RNA polymerases, accounting for up to 65% of total transcription in metabolically active mammalian cells (Grummt, 2003). To attain this high level of ribosomal RNA (rRNA) synthesis, the ribosomal DNA (rDNA) comprises approximately 200 tandemly repeated rRNA genes. Each of the 43 kb gene repeats encodes a single rRNA precursor transcript that is later cleaved and processed into the mature 18S, 28S, and 5.8S rRNAs. Intergenic spacer regions (IGS) separate adjacent transcribed rDNA sequences. In all eukaryotes Pol I transcription is highly coordinated with cellular metabolism and cell proliferation (Grummt, 1999). Nutrient starvation, aging, growth factor deprivation, DNA damage, and other conditions that slow down cellular division cause a dramatic decrease in pre-rRNA synthesis (Grummt, 2003). Along these lines, the tumor suppressors pRb and p53 downregulate Pol I transcription (Budde and Grummt, 1999; Cavanaugh et al., 1995; Voit et al., 1997; Zhai and Comai, 2000). Likewise, conditions that stimulate cellular division increase pre-rRNA synthesis (Zhao et al., 2003).

The Sir2 family of enzymes, termed sirtuins, is conserved from bacteria to humans and regulates a wide range of processes including gene silencing, aging, cellular differentiation, and metabolism (Blander and Guarente, 2004). Sirtuins contain a conserved catalytic core domain flanked by divergent amino- and carboxy-terminal regions (Frye, 2000). The catalytic domain confers NAD⁺-dependent protein deacetylase as well as ADP-ribosyltransferase activity. The mammalian genome encodes seven homologs of the yeast SIR2 gene, termed SIRT1-7 (Frye, 2000). While several
physiologically significant SIRT1 substrates have been identified including p53 (Luo et al., 2001; Vaziri et al., 2001), MyoD (Fulco et al., 2003), FOXO3 (Brunet et al., 2004; Motta et al., 2004), PPARγ (Picard et al., 2004), and NF-κB (Yeung et al., 2004), little is known about the biological functions of the other mammalian sirtuins. SIRT2 is a cytoplasmic protein and catalyzes together with HDAC6 the deacetylation of tubulin (North et al., 2003), whereas so far no target has been identified for SIRT3, which is located in mitochondria (Onyango et al., 2002).

To begin to address the function of SIRT7, the expression pattern of SIRT7 was investigated by Northern and Western blot analysis using mRNA and cell extracts derived from eight different adult mouse tissues. Antibodies were raised against the N-terminus of mSIRT7, which is not conserved in SIRT1-6. These antibodies recognized one single polypeptide of 45 kD in nuclear extracts, but not in cytoplasmic fractions of mouse and human cells (Fig. 1A). SIRT7 mRNA was detected in each sample except skeletal muscle, and showed highest levels in the liver (Fig. 1B). In addition, SIRT7 mRNAs were readily detectable in samples taken from different mouse embryonic stages between day E7 and day E17. SIRT7 protein was also broadly expressed except in heart and muscle (Fig. 1C).

Next, we investigated the subcellular localization of human SIRT7 using GFP-tagged proteins and indirect immunofluorescence. SIRT7-GFP was located almost exclusively in nucleoli (Fig. 1D). Moreover, the SIRT7-specific antibodies detected endogenous SIRT7 within distinct nuclear foci, which were co-stained with antibodies against Pol I and therefore represent nucleoli (Fig. 1E). Nucleolar localization was observed throughout interphase. During M-phase, when nucleoli dissolve, SIRT7 was
not retained at the Nucleolus Organizer Region (NOR), but distributed over the entire condensed chromatin (Fig. 1F).

The nucleolar localization raised the possibility that SIRT7 is associated with rDNA. We investigated this by chromatin immunoprecipitation (ChIP). Crosslinked chromatin from 293T cells was immunoprecipitated with anti-SIRT7 antibodies, and precipitated DNA was analyzed by PCR using four primer sets that amplify specific regions of the 43 kb human rDNA repeat except for primer set H23/H27 which recognizes a repeat in the intergenic spacer (IGS) region. (Fig. 2A). ChIP-PCR revealed that anti-SIRT7 antibodies specifically precipitated rDNA when tested with primer sets that amplify two parts of the transcribed region (H1 and H13) and the promoter sequence (H0) (Fig. 2B top). No signal above the IgG control was observed with the H23/H27 primer set that amplifies sequences in the IGS, demonstrating the specificity of the interaction of SIRT7 with the transcribed region of the rDNA. The distribution of SIRT7 on rDNA was almost identical to that of Pol I, viewed by precipitation with anti-RPA116 antibodies, which recognize the second largest subunit of Pol I (Fig. 2B middle). As expected, acetylated histone H4 was present across the entire rDNA repeat including the IGS (Fig. 2B bottom).

Since SIRT7 and Pol I were detected at the same rDNA regions, we next tested whether the two proteins interact. Lysates from 293T cells overexpressing FLAG-tagged SIRT7 or mock-transfected 293T cells were immunoprecipitated with anti-FLAG (M2) antibodies, and co-precipitation of Pol I was analyzed by Western blot with RPA116 antibodies. While equal amounts of RPA116 were present in both cell lysates, it was only detected in immunoprecipitates from cells expressing FLAG-tagged SIRT7 (Fig. 216
2C). Similarly, RNA polymerase I remains associated with SIRT7 after tandem affinity purification (TAP)(Fig. 2D). We next tested whether SIRT7 and Pol I interact in vivo at their endogenous levels. Antibodies against Pol I, but not control antibodies, co-immunoprecipitated endogenous SIRT7 along with Pol I from a partially purified mouse nuclear extract (Fig. 2E). Thus, SIRT7 and Pol I associate in vivo at the rDNA. Interestingly, like the yeast SIR protein complex, SIRT7 fused to GST also interacts with histones isolated from HeLa cells in vitro (Fig. 2F).

To test whether SIRT7 modulates Pol I transcription, a human rDNA minigene reporter (Voit et al., 1999) and an expression plasmid for FLAG-tagged SIRT7 were cotransfected into human 293T cells. Transcripts derived from the rDNA reporter gene were analyzed by Northern blot. Overexpression of SIRT7 stimulated transcription of the reporter gene up to six-fold over basal levels in a dose-dependent manner (Fig. 3A). A comparable activation of Pol I transcription was also observed in human U2OS cells and mouse 3T3 fibroblasts that were overexpressing SIRT7 (data not shown). This stimulation was Pol I-specific, since overexpression of SIRT7 did not activate transcription of a Pol II-driven luciferase reporter gene (data not shown). These results demonstrate that SIRT7 specifically increases transcription by Pol I in vivo.

To gain further evidence that SIRT7 functions in transcriptional activation, cells were depleted of SIRT7 by RNA interference (RNAi). Western blots from cells transfected with anti-SIRT7 small inhibitory RNAs (siRNAs) showed that SIRT7, but not actin protein levels were efficiently decreased by anti-SIRT7 siRNAs (Fig. 3B left). Pre-rRNA levels were measured using RT-PCR and pairs of primers that amplify part of the 5' external transcribed spacer of the pre-rRNA. Downregulation of SIRT7 by specific
siRNAs resulted in significant reduction of pre-rRNA synthesis, whereas expression of GAPDH was not affected (Fig. 3B right). These results indicate that depletion of SIRT7 reduces cellular rRNA synthesis and substantiate the claim that SIRT7 is a positive regulator of Pol I transcription.

To determine whether activation of Pol I transcription by SIRT7 is functionally linked to the enzymatic activities of sirtuins, we generated point mutants, replacing serine 112 with alanine and histidine 188 with tyrosine (fig. S1). These residues within the catalytic domain are invariant among sirtuins. Moreover, the homologous mutations have been shown to be required for enzymatic activity in SIRT1 and yeast Sir2 (Frye, 1999; Liszt et al., 2005; Vaziri et al., 2001). Wild-type and mutant proteins were transiently expressed in human 293T cells, and 45S pre-rRNA levels were analyzed on Northern blots. Notably, a two- to three-fold increase of the level of pre-rRNA was observed in cells expressing wild-type FLAG-SIRT7, but not in cells overexpressing SIRT7S112A or SIRT7H188Y (Fig. 3C). Both mutant proteins were correctly localized to the nucleolus (Fig 3D). These findings indicate that an NAD+-dependent enzymatic activity of SIRT7 is required for transcriptional activation but not for nucleolar localization.

In addition, pre-rRNA levels were determined in cells treated with nicotinamide, which is a potent inhibitor of sirtuins. As expected, treatment of NIH3T3 cells with 5 mM nicotinamide strongly inhibited pre-rRNA synthesis (Fig. 3E). In contrast, TSA, an inhibitor of class I and II HDACs, did not affect the level of pre-rRNA synthesis, suggesting that SIRT7, but not the class I and II HDACs, supports Pol I transcription in vivo. Inhibition by nicotinamide is not due to cellular redistribution of SIRT7, since the localization of GFP-SIRT7 is not altered in the presence or absence of nicotinamide (Fig. 218).
3F). In contrast, addition of actinomycin D at a concentration that selectively inhibits Pol I transcription released GFP-SIRT7 from the nucleoli resulting in redistribution throughout the nucleus. These results suggest that nucleolar localization of SIRT7 is tightly linked to ongoing Pol I transcription.

Based on the observation that SIRT7 is (i) bound to rDNA, (ii) interacts with Pol I, and (iii) stimulates Pol I transcription, we asked whether SIRT7 changes the occupancy of Pol I at the rDNA. To investigate this, we performed ChIP using anti-RPA116 antibodies and cell lines stably expressing FLAG-SIRT7 wild-type or mutant proteins. Western blot analysis demonstrated equivalent overexpression of SIRT7\textsuperscript{wt}, SIRT7\textsuperscript{S112A}, and SIRT7\textsuperscript{H188Y} (Fig. 4A). Both input chromatin and chromatin precipitated with anti-RPA116 antibodies were used as template for PCR with primers corresponding to the rDNA promoter (H0), coding region (H1 and H13), and IGS (H23/H27). Notably, the amount of rDNA precipitated with Pol I-specific antibodies was significantly higher in SIRT7-overexpressing cells when compared to control cells that had been transfected with the empty vector (Fig. 4B). However, when cell lines overexpressing the SIRT7 point mutants S112A and H188Y were analyzed, the levels of precipitated rDNA was similar to that of the empty vector (Fig. 4B). When a primer pair to the IGS was used, a region in which Pol I is not bound, the background ChIP signal was identical in all four cell lines, demonstrating that the differences between the cell lines seen with primer pairs H0, H1, and H13 is not due to variations in the preparation of the chromatin. These data demonstrate that overexpression of SIRT7 increases the amount of Pol I at the rDNA. In addition, enhancement of association of Pol I with the rDNA requires conserved residues within the catalytic domain of SIRT7.
In a converse experiment, we analyzed the association of Pol I with rDNA in cells transfected with nonspecific or SIRT7-specific siRNAs. Western blot analysis demonstrated significant reduction of SIRT7 protein levels by SIRT7-specific siRNA, whereas the levels of actin were the unaffected (Fig. 4C). Crosslinked chromatin was precipitated from cells treated with nonspecific and SIRT7-specific siRNAs using anti-RPA116 antibodies, and quantified by PCR with the rDNA-specific primer sets H0, H1, H13, and H23/H27. Anti-RPA116 antibodies precipitated significantly less rDNA from cells treated with anti-SIRT7 siRNAs compared to cells treated with nonspecific siRNAs (Fig. 4D). This demonstrates that reduction of cellular SIRT7 protein levels by RNAi concomitantly reduced the amount of Pol I associated with rDNA. These results are in agreement with our observation that SIRT7 is critical for activation of Pol I transcription.

Here, we establish that SIRT7 is an activating component of the RNA polymerase I transcriptional machinery. SIRT7 strongly stimulates Pol I transcription in vivo by an enzymatic mechanism. Importantly, these data suggest a novel mechanism by which mammalian cells may regulate rRNA transcription. In yeast, a change in the NAD+/NADH ratio translates the metabolic shift of calorie restriction into lifespan extension via Sir2p (Lin et al., 2004). Such a model is also believed to explain the regulation of SIRT1 in liver and muscle cells, where changes in the NAD+/NADH ratio can arise from diet or exercise and influence SIRT1 activity (Fulco et al., 2003; Rodgers et al., 2005). This paradigm suggests that diet-induced changes in the NAD+/NADH ratio may regulate SIRT7 to couple changing energy status with levels of rRNA transcription and ribosome production.
Sir2 homologs positively regulate longevity in yeast, worms, and flies despite extraordinary phylogenetic distance between these organisms (Hekimi and Guarente, 2003). In yeast, Sir2p promotes longevity predominantly through its silencing role at the rDNA (Kaeberlein et al., 1999; Sinclair and Guarente, 1997). It is interesting that the effect of sirtuins at the rDNA has been inverted in mammals. SIRT7 activates RNA polymerase I transcription, which is typically proportional to the requirement for cell growth. We suspect that a link between SIRT7 and growth may be part of a larger set of physiological changes involving all seven sirtuins that is induced by diet.
MATERIALS AND METHODS

Plasmid constructs, antibodies and antibody production

The human SIRT7 cDNA was purchased from ATCC (IMAGE 222518), amplified by PCR, and cloned into pCMV-Tag4a (Stratagene) cut with EcoRI and XhoI to make the plasmid pCMV-FLAG-SIRT7. The plasmid pCMV-FLAG-SIRT7 served as template for site directed mutagenesis using the Stratagene Quick Change mutagenesis kit to produce the plasmids pCMV-FLAG-SIRT7S112A and pCMV-FLAG-SIRT7H188Y. pEGFP-SIRT7 was constructed by amplifying the SIRT7 coding sequence by PCR and cloning it into the EcoRI and BamHI sites of pEGFP-N1 (Clontech). pET-GST-SIRT7 was created by cloning the SIRT7 coding sequence into the NheI and BamHI sites of the vector pET-GST (kind gift from R. Marciniak, University of Texas, San Antonio). To generate TAP-tagged SIRT7, the stop codon of the SIRT7 cDNA was removed by site directed mutagenesis and substituted by a BglII site, and the coding sequence was cloned into the Bam HI site of the vector pZome-1C (Cellzome).

The N-terminal 81 amino acids of mouse SIRT7 were expressed in E. coli as a glutathione S-transferase (GST)-fusion and purified over a glutathione-Sepharose column. The purified protein was injected into NZW rabbits by Covance Bioproducts to create SIRT7 antiserum. Full-length SIRT7 and SIRT7(1-81) proteins were bound to NHS-Sepharose according to manufacturer instructions (Amersham). The crude serum was first passed over the SIRT7(1-81)-NHS-Sepharose resin. The resin was washed extensively. The purified antibody was eluted with 0.1 M glycine, pH 2.5, and the pH was neutralized by the addition of 1/10th volume of 1 M Hepes, pH 7.9. The process was repeated a second time using the full-length SIRT7-NHS-Sepharose resin.

Other antibodies used were anti-actin C4 (ICN), anti-Flag M2 (Sigma), anti-acetylated histone H4 (Upstate), and anti-histone H4 (Upstate), a purified rabbit polyclonal antibody against RPA-116, and human autoimmune serum against the largest subunit of Pol I (provided by U. Scheer, University of Wuerzburg).

Multiple tissue Northern and Western blots

A hybridization probe containing the SIRT7 open reading frame was created using the Prime-It Random Primer Labeling kit (Stratagene) and was used to probe Multiple Tissue Northern Blots (Stratagene) according to the manufacturer’s instructions. The blots were then stripped and reprobed with the actin probe included in the kit.
Homogenized mouse tissues from two mice of the FVB genetic background were obtained as a generous gift from L. Bordone, MIT. From these samples, SDS-solubilized proteins were prepared, separated by SDS-PAGE, and analyzed by Western blotting.

**Cell culture, transfections and RNA analysis**

293T, U2OS and NIH3T3 cells were cultured in DMEM supplemented with 10% FCS. Where indicated cells were treated with 0.050 μg/ml actinomycin D, 5 mM nicotinamide, or 40 nM TSA. For transient expression, 293T or U2OS cells were transfected in 60 mm dishes with the calcium phosphate precipitation technique using different amounts of pCMV-SIRT7 plasmids and 2 μg rDNA reporter plasmid pHr-P-BH, which contains a fragment from −401 to +378 (+1, transcription start site) of the human rDNA repeat fused to a BamHI-HinfI fragment encompassing the transcription termination sites T1 and T2. Reporter transcripts were analyzed on Northern blots as described (Voit et al., 1999). To analyze pre-rRNA synthesis, cellular RNA was isolated and subjected to Northern blot analysis using a 32P-labeled antisense RNA encompassing the 5’-terminal rDNA sequences from −57 to +183. To normalize for gel loading, the blots were reprobed with radiolabeled riboprobes against cytochrome c oxidase 1 (cox 1).

For stable expression of TAP-tagged SIRT7, U2OS cells were transfected with pZome-SIRT7 or pZome alone followed by selection with 2 μg/ml of puromycin. Similarly, for stable expression of FLAG-tagged SIRT7 cell lines, 293T cells were transfected with pCMV-TAG-4a (Stratagene), pCMV-FLAG-SIRT7, pCMV-FLAG-SIRT7S112A, or pCMV-FLAG-SIRT7H188Y in combination with pBABE-PURO at a 10:1 ratio and selected with 2 mg/ml puromycin.

**Immunofluorescence**

U2OS cells were fixed with paraformaldehyde as described before (Zatsepina et al., 1993) and stained with purified anti-SIRT7 antibodies, anti-Pol I antibodies, anti-rabbit Alexa-488 antibodies (MoBiTec), anti-human Cy3 antibodies (Dianova), and Hoechst 33342. Images were visualized using a Zeiss Axiophot microscope. Localization of GFP-tagged proteins was visualized with a Nikon microscope in live cells that had been treated with the dye Hoechst 33342.

**Immunoprecipitations**

One 10 cm plate of 293T cells stably transfected with pCMV-Tag4a and pCMV-FLAG-SIRT7 was resuspended in 1 ml NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-
40) and transferred to a 1.5 ml tube. The lysis reaction was allowed to proceed for 30 min on ice and centrifuged for 15 min at 14,000 rpm. 10 µl of FLAG(M2)-protein A-Sepharose beads were added to the lysates and incubated for 1 hr. After extensive washing the beads were boiled in SDS loading buffer and the eluted proteins were analyzed by SDS-PAGE and Western blotting.

Chromatin immunoprecipitations

Chromatin immunoprecipitation (ChIP) experiments were performed essentially as described (Weinmann et al., 2001) with a few modifications. For each ChIP, one 15 cm plate of 293T cells was grown to 75% confluency. Formaldehyde was added directly to the media to a final concentration of 1% and incubated for 10 min at room temperature. The reaction was stopped by adding glycine to a final concentration of 0.125 M for 5 min. Cells were washed twice with cold PBS, incubated with 2 ml of 2.5% trypsin at 37°C for 10 min, harvested with 10 ml of PBS plus Complete Protease Inhibitors (Roche), and washed twice with PBS plus protease inhibitors. The final cell pellet was resuspended in cell lysis buffer (5 mM HEPES pH 8.0, 85 mM KCl, 0.5% NP-40, and protease inhibitors), incubated on ice for 5 min, and centrifuged at 5,000 rpm in a microfuge for 10 min. The pellet was resuspended in nuclei lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors) and incubated on ice for 10 min. The chromatin was sonicated to an average length of approximately 500 bp as visualized by ethidium bromide agarose gel electrophoresis. The resulting chromatin was cleared of insoluble material by spinning at 14,000 rpm for 15 min in a microfuge and diluted 5-fold in IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl). Chromatin was pre-cleared by adding 80 µl of salmon sperm DNA/BSA/protein-A slurry (0.4 mg/ml salmon sperm DNA, 1 mg/ml BSA, 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 50% protein A sepharose beads). In separate 1.5 ml tubes, antibody beads were prepared by incubating 10 µl of specific antibodies with protein-A Sepharose in 500 ml IP dilution buffer plus 50 mg/ml BSA and 0.4 mg/ml ssDNA. After 1 hour the supernatant was removed from the antibody beads. The pre-cleared chromatin was added and incubated for 3 hours at 4°C. The beads were washed two times with each of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 500 mM NaCl), LiCl wash buffer (1% deoxycholic acid, 1% NP-40, 1 mM EDTA, 10 mM Tris-Cl pH 8.0, 250 mM LiCl), and TE. After the last wash the chromatin was eluted from the beads by incubating twice for 15 minutes with 150 µl IP elution buffer (100 mM NaHCO₃, 1% SDS). Formaldehyde crosslinking was reversed by incubating at 65°C for 4 hours in the presence of 0.3 M NaCl and 0.1 mg/ml RNase A. DNA was
ethanol precipitated and resuspended in 100 μl of H₂O. 2 μl 0.5 M EDTA, 4 μl 1 M Tris-Cl pH 6.5, and 1.5 μl of 20 mg/ml Proteinase K were added and incubated for 2 hours at 45° C. Samples were extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated. Pellets were washed with 70% ethanol, dried and resuspended in 30 μl TE/10 (10 mM Tris-Cl pH 7.5, 0.1 mM EDTA). Increasing amounts of precipitated chromatin was used as template in quantitative PCR reactions as previously described (O'Sullivan et al., 2002).

Purification of proteins and protein-protein interactions
GST and recombinant GST-tagged SIRT7 were expressed in E. coli Codon Plus-RP cells (Stratagene) and affinity purified over glutathione-Sepharose according to standard protocols. Acetylated core histones were purified from butyric acid treated HeLa cells by hydroxylapatite chromatography essentially as described (Ausubel et al., 1997). To assay for interaction of SIRT7 and histones, GST and GST-SIRT7 were immobilized on glutathione-Sepharose and incubated with 2 μg of purified core histones for 2 h at 4° C in buffer AM-400 (400 mM KCl, 20 mM Tris-Cl pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.5 mM EDTA) supplemented with 0.2% NP-40 and protease inhibitors. After five washes in incubation buffer, bound proteins were eluted with SDS-loading buffer, separated on 15% polyacrylamide gels by SDS-PAGE, and visualized by staining with Coomassie.

U2OS cells stably transfected with pZome or pZome-SIRT7 were grown on ten 150 mm dishes to 80% confluency. Cells were harvested in buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, protease inhibitors), incubated for 15 min at 4° C, and the nuclei pelleted by centrifugation. The nuclei were resuspended in buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA, protease inhibitors), homogenized, and incubated at 4° C for 30 min. The lysate was cleared by centrifugation at 13000 rpm for 30 min at 4° C, and the extract was dialysed against buffer AM-100 (100 mM KCl, 20 mM Tris-Cl pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.5 mM EDTA, 0.5 mM PMSF). Batch purification of the IgG binding domain-tagged proteins was performed using 300 μl of IgG-Sepharose 6 Fast Low resin (Amersham Biosciences). The protein was eluted from the beads by washing three times in TEV-cleavage buffer (25 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT) and rotating over night at 4° C in TEV-cleavage buffer containing 50 units AcTEV protease (Invitrogen). Supernatants from the TEV-cleavage reaction containing SIRT7 and interacting proteins were collected and substituted with CaCl₂ (final concentration 2 mM) and 3 volumes of calmodulin binding buffer (25 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazol, 2 mM CaCl₂, 10 mM β-
mercaptoethanol, 0.1% NP-40). The proteins were batch-purified with 200 μl of calmodulin beads (Stratagene) under rotation for 2 h at 4° C. The beads were washed three times in calmodulin binding buffer, transferred into a column and bound proteins eluted with calmodulin elution buffer (25 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazol, 20 mM EGTA, 10 mM β-mercaptoethanol, 0.02% NP-40).

**RNAi experiments**

To knock-down cellular SIRT7 by RNAi, dsRNAs were synthesized as a custom “Smart Pool” against SIRT7 and “non-specific control duplexes – XIII” (Dharmacon). To determine the effect of these siRNAs on pre-rRNA levels, the duplexes were transfected into U2OS cells using RNAiFect (Qiagen). After incubation for 48 h, cells were harvested and lysed with SDS loading buffer for Western blot analysis or with GITC for isolation of cellular RNA. Cellular RNA was reverse transcribed with random primers. The amount of pre-rRNA determined by semiquantitative PCR in the presence of α-32P-dCTP with primer 1 (forward) 5’-GCT GTC CTC TGG C-3’ and primer 2 (reverse) 5’-CGG CAG GCG GCT CAA G-3’ that amplify a fragment from +9 to +120 of the external transcribed spacer of the human rDNA repeat. Radiolabeled PCR fragments were analyzed on 8% polyacrylamide gels and quantified using a Phosphorimager. GAPDH-cDNA was amplified by PCR using the primers 5’-CCA TCA CCA TCT TCC AGG AG-3’ and 5’-CCT GCT TCA CCA CCT TCT TG-3’.

293T cells for ChIP assays were transfected with the above siRNAs using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. 48 hours post-transfection the cells were transfected a second time and 48 hours after the second transfection the cells were harvested for ChIP experiments as described above.
REFERENCES


Acknowledgements

The authors thank Marcia Haigis, and Gil Blander for thoughtful discussion and careful reading of the manuscript. LG and EF are supported by NIH grants.
FIGURES

Fig. 1. SIRT7 is a nucleolar protein. (A) SIRT7 is a nuclear component of mammalian cells. 20 mg cytoplasmic and nuclear extracts from NIH-3T3 and U2OS cells were subjected to Western blot analysis with anti-SIRT7 antibodies. (B) RNA was isolated from the indicated tissues (left panels) and embryos (right panels) in wild-type adult mice, resolved by electrophoresis, and subjected to Northern blotting. Blots were probed with $^{32}$P-labeled cDNA specific to SIRT7 (top panels) or actin (lower panels). Muscle actin found in heart and skeletal muscle samples migrated as a distinct band. (C) Protein extracts from the indicated wild-type mouse tissues were resolved by SDS-PAGE, and analyzed by Western blotting using antibodies specific to SIRT7 (top panel) or actin (lower panel). For each tissue type, samples from two different mice were included. (D) Nucleolar localization of GFP-SIRT7 in live human U2OS cells. (E) Endogenous SIRT7 and Pol I were visualized by immunofluorescence microscopy in U2OS cells using anti-SIRT7 (green) and anti-Pol I antibodies (red). The DNA was counter-stained with Hoechst 33342. (F) During mitosis SIRT7 is associated with condensed chromatin. SIRT7-GFP was visualized in live U2OS cells stained with Hoechst 33342.
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**Cell lysates**

- **SIRT7**
  - Lane 1: NIH 3T3
  - Lane 2: HeLa

- **Actin**
  - Lane 1: NIH 3T3
  - Lane 2: HeLa

**Figure 1**

- **B**
  - Heart, Brain, Spleen, Lung, Liver, Muscle, Kidney, Testis
  - Lanes: 1-8 and 9-13
  - mSIRT7
  - Actin

- **C**
  - Brain, Liver, Pancreas, Heart, Spleen, Kidney, Muscle, Testis
  - Lanes: 1-17
  - mSIRT7
  - Actin

- **D**
  - GFP-SIRT7
  - Phase

- **E**
  - α-SIRT7
  - α-Pol I
  - DAPI
  - Phase

- **F**
  - GFP-SIRT7
  - DAPI
  - Prometaphase
  - Anaphase

Figure 1
Fig. 2. SIRT7 is associated with the rDNA and RNA polymerase I.

(A) Diagram depicting the location of the primer pairs used: (H0) promoter, (H1) 5’ external transcribed spacer, (H13) end of 28S rRNA, and (H23/H27) intergenic region of the human rDNA gene. (B) Results of ChIP analysis showing the localization of SIRT7 and RNA pol I within transcribed region of the rDNA gene repeats. Chromatin from 293T cells was precipitated by the indicated antibodies and analyzed by PCR with the indicated primer pairs. (C) Coimmunoprecipitation of SIRT7 and RNA polymerase I (RPA116). Anti-FLAG immunoprecipitations were performed from whole cell extracts prepared from 293T cells harbouring empty vector (pCMV) or a plasmid expressing FLAG-tagged SIRT7 (pCMV-SIRT7-FLAG). Immunoprecipitates were analyzed by Western blot as indicated. (D) Pol I is a component of the SIRT7-TAP complex. U2OS cell lines harboring the empty vector or SIRT7-TAP were established. TAP-tagged complexes were purified on IgG and Calmodulin resins, and the eluted protein complexes tested for copurifying proteins by Western blot analysis. Lane 1, 50 μg of cell extract; lane 2, eluate from mock transfected cells; lane 3, eluate from a cell line expressing SIRT7-TAP. The Western blot was probed with an antibody specific for RPA116. (E) Endogenous SIRT7 interacts with RNA polymerase I. Partially purified human nuclear extract was immunoprecipitated with control non-specific human antibodies (lane 2) and anti-RNA polymerase I antibodies (lane 3). The precipitated proteins were analyzed by western blotting with anti-SIRT7 and anti-RPA-116 antibodies as indicated. (F) SIRT7 interacts with histones. Histones were isolated from butyric acid treated HeLa cells by ion exchange chromatography incubated with GST-SIRT7-Sepharose and GST-Sepharose beads. The proteins were eluted and separated by SDS-PAGE and visualized by staining with Coomassie.
Figure 2
Fig. 3. SIRT7 is an activator of Pol I transcription. (A) Overexpression of SIRT7 stimulates Pol I transcription from an rDNA minigene reporter. 293T cells were co-transfected with a Pol I reporter plasmid and increasing amounts of an expression plasmid encoding SIRT7-Flag. RNA was isolated from transfected cells. The amount of reporter transcripts was determined by Northern blot analysis and quantified using a PhosphorImager. Bottom panel: Western blot of the transfected cells probed with anti-Flag antibody. (B) Knock-down of SIRT7 by RNAi impairs rRNA synthesis. Left panel: Western blot of extracts from U2OS cells transfected with control-dsRNA (lane 1) or SIRT7-specific dsRNA (lane 2). Right panel: Pol I transcription was analyzed by RT-PCR. RNA was isolated from cells treated with the respective dsRNAs. Increasing amounts of cDNA were used for PCR with primers that amplify a region of the 5' external transcribed spacer of the human pre-rRNA. Results from two independent experiments (lanes 1,2,5,6 and 3,4,7,8, respectively) are shown. As a control, RT-PCR was performed to analyze expression of GAPDH. (C) Stimulation of cellular Pol I transcription by SIRT7 requires residues H188 and S112. Northern blot analysis of pre-rRNA transcripts from 293T cells transfected with different amounts of plasmids expressing wild-type SIRT7 or the point mutants H188Y and S112A (top panel). Expression levels of SIRT7-Flag, SIRT7/H188Y-Flag, and SIRT7/S112A-Flag were monitored on Western blots using anti-Flag antibodies (bottom panel). (D) The SIRT7 mutants H188Y and S112A were expressed as GFP-tagged proteins in U2OS cells and their cellular localization was examined in live cells. (E) Nicotinamide represses rRNA synthesis. NIH3T3 cells were cultured for 6h in medium containing 40 nM TSA or 5 mM nicotinamide, and pre-rRNA levels were measured by Northern blot analysis. The blot was subsequently reprobed for cytochrome c oxidase (cox1) mRNA. (F) Treatment with actinomycin D, but not with nicotinamide releases SIRT7-GFP from nucleoli. U2OS cells expressing SIRT7-GFP were cultured in the presence of either 50 mg/ml of actinomycin D for 2h to inhibit Pol I activity or 5 mM nicotinamide for 6h to inhibit NAD+-dependent deacetylase activity. Localization of SIRT7-GFP was examined in live cells.
Fig. 4. SIRT7 stimulates the association of RNA Polymerase I with the rDNA
(A) Western blot showing overexpression levels of SIRT7. (B) Enrichment of RNA pol I
at the transcribed region of the rDNA genes in the presence of ectopic SIRT7. Chromatin
was prepared from cells harbouring empty vector (pCMV) or plasmids expressing wild-
type SIRT7 (SIRT7-wt) and mutant SIRT7 (SIRT7-S112A and SIRT7 H188Y). The
chromatin was precipitated with anti-RPA116 antibodies and analyzed by PCR with the
primer pairs indicated on the right side of the figure. (C) Following siRNA transfections,
whole-cell lysates were analyzed by immunoblot for SIRT7 and β-actin. (D) ChIP
analysis of Pol I levels in cells transfected with siRNAs. Chromatin was prepared from
cells transfected with non-specific (ns) or anti-SIRT7 siRNA. The chromatin was
precipitated with anti-RPA116 antibodies and analyzed by PCR with the primer pairs
indicated on the right side of the figure
Figure 4
**SUPPORTING FIGURE**

**Fig. S1.** Sequence alignment of mouse sirtuin core domains. Conserved core domains of mouse SIRT1, SIRT4, SIRT5, and SIRT7 were aligned with the yeast Sir2 core using ClustalW. In the sirtuin phylogenetic tree, SIRT1 is class I, SIRT4 is class II, SIRT5 is class III, and SIRT7 is class IV. At each position of the alignment, identical residues are boxed and shaded in gray. Amino acids filled in black correspond to SIRT7 residues targeted for mutagenesis in this study.
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