Sip2, an N-Myristoylated β Subunit of Snf1 Kinase, Regulates Aging in *Saccharomyces cerevisiae* by Affecting Cellular Histone Kinase Activity, Recombination at rDNA Loci, and Silencing

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*Saccharomyces cerevisiae* has evolved a number of mechanisms for sensing glucose. In the present study we examine the mechanism by which one of these pathways, involving Snf1, regulates cellular aging. Snf1 is a heterotrimer composed of a catalytic α subunit (Snf1p) that phosphorylates target proteins at Ser/Thr residues, an activating γ subunit (Snf4p), and a β subunit (Sip1p, Sip2p, or Gal83). We previously showed that forced expression of Snf1p or loss of Sip2p, but not the other β subunits, causes accelerated aging, while removal of Snf4p extends life span (Ashrafi, K., Lin, S. S., Mancheser, J. K., and Gordon, J. I. (2000) Genes Dev. 14, 1872–1885). We now demonstrate that in wild type cells, there is an age-associated shift in Sip2p from the plasma membrane to the cytoplasm, a prominent redistribution of Snf4p from the plasma membrane to the nucleus, a modest increase in nuclear Snf1p, and a concomitant increase in cellular Snf1 histone H3 kinase activity. Covalent attachment of myristate to the N-terminal Gly of Sip2p is essential for normal cellular life span. When plasma membrane association of Sip2p is abolished by a mutation that blocks its N-myristoylation, Sip4p is shifted to the nucleus. Rapidly aging sip2Δ cells have higher levels of histone H3 kinase activity than their generation-matched isogenic wild type counterparts. Increased Snf1 activity is associated with augmented recombination at rDNA loci, plus desilencing at sites affected by Snf1-catalyzed Ser phosphorylation of histone H3 (the INO1 promoter plus targets of the transcription factor Adr1p). The rapidaging phenotype of sip2Δ cells is fully rescued by blocking recombination at rDNA loci with a fob1Δ allele; rescue is not accompanied by amelioration of an age-associated shift toward gluconeogenesis and glucose storage. Together, these findings suggest that Sip2p acts as a negative regulator of nuclear Snf1 activity in young cells by sequestering its activating γ subunit at the plasma membrane and that loss of Sip2p from the plasma membrane to the cytoplasm in aging cells facilitates Snf4p entry into the nucleus so that Snf1 can modify chromatin structure.

*Saccharomyces cerevisiae* is a unicellular model for studying the molecular mechanisms of aging. Mother yeast cells undergo replicative senescence, with different strains having characteristic mean and maximum life spans (1). Senescence is associated with a number of cellular and molecular phenotypes. Progressive sterility arises from the loss of silencing at HM loci (2). This loss of silencing is accompanied by a redistribution of Sir3p, a component of the Sir-silencing complex, from HM loci and telomeres to the nucleolus (3). Homologous recombination at rDNA loci liberates an extrachromosomal rDNA circle (ERC) that contains an autonomously replicating sequence (4). With each round of cell division, replicated ERCs segregate to the mother rather than to daughter, leading to an exponential increase in cellular ERC concentrations over successive generations.

ERC formation is thought to be a mediator as well as a marker of aging. Exponential increases in ERCs likely leads to titration of critical DNA repair/replication and transcription factors (5). Fob1p specifically acts at the rDNA locus, where it supports opening of the replication bubble (6) and binds to HOT1 sites, thereby promoting homologous recombination. fob1Δ alleles block rDNA formation and extend life span ≈ 20% beyond wild type (wt) (4).

Mutations, such as sir2Δ, that reduce rDNA silencing are associated with increased rates of recombination and accelerated aging (7, 8). The importance of the regulation of silencing in aging is also emphasized by the findings that Sir2p is a NAD+-dependent histone deacetylase (9) and that NAD$^+$ production leads to Sir2p-dependent life span extension (10). To catalyze deacetylation, Sir2p converts NAD$^+$ to nicotinamide and O-acetyl-ADP-ribose (11, 12). Yeast grown on medium containing nicotinamide, a noncompetitive Sir2p inhibitor, display Sir2p-dependent rDNA desilencing and a shortened life span (13).

The effects of NAD$^+$ on Sir2p provide a potential link between aging and cellular glucose/energy metabolism. They also raise the question of how aging affects upstream elements in glucose-sensing pathways and whether such pathways affect replicative life span.

*S. cerevisiae* has evolved elaborate and elegant mechanisms for sensing environmental glucose, its principal nutrient source. A major glucose-sensing pathway involves Snf1, the homolog of mammalian AMP-activated protein kinase (AMPK) that is involved in regulating cellular stress responses (14). Snf1 is a heterotrimeric complex composed of α, β, and γ subunits (15, 16). The complex contains one of three β subunits, Sip1p, Sip2p, or Gal83p, that binds to the catalytic α subunit, Snf1p (a serine/threonine kinase) (17). The γ subunit, Snf4p (18), binds to an autoinhibitory domain of Snf1p, releasing its activity.

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catalytic domain. Phosphorylation of Thr\textsuperscript{210} in the Snf1p C-terminal activation loop also promotes kinase activity (19), although the Snf1p kinase has yet to be identified.

The Snf1 complex responds to glucose starvation by catalyzing phosphorylation of a number of target proteins, including transcriptional regulators of genes involved in alternative carbon source utilization, gluconeogenesis, and respiration (20). For example, when glucose is limiting, Mig1p is hyperphosphorylated by Snf1p causing it to translocate from the nucleus to the cytoplasm and derepress gene expression (21).

We have reported that forced expression of Snf1p, or loss of Sip2p (sip2Δ), produces an accelerated aging phenotype (i.e. a shortened replicative life span accompanied by progressive sterility, redistribution of Sir3p from telomeres and HM loci to the nucleolus, and increased ERC accumulation) (22). The effect of Sip2p on aging is unique among Snf1p β-subunits: gal83Δ has no effect on life span and sip1Δ produces a modest (<20%) reduction in life span but without the other manifestations of accelerated aging. Loss of the γ subunit (snf4Δ) produces a 20% increase in life span (22). Together, these results implied that the effects of Snf1 activation on life span must extend beyond its phosphorylation of Mig1p since mig1Δ cells did not undergo rapid aging (22).

Follow-up DNA microarray analysis revealed that a number of Mig1p-repressed genes were derepressed in generation 7 – 8 wt and sip2 cells compared with their generation 0–1 counterparts (23). The analysis also identified a number of other genes with age-associated changes in their expression that are not regulated by Mig1p but are involved in various aspects of cellular energy metabolism. Direct biochemical analysis of cellular metabolism established that aging in wt cells is accompanied by a shift away from glycolysis and toward gluconeogenesis and energy storage (23). This shift is deferred in snf4Δ cells and accelerated in isogenic sip2Δ strains leading to a proposal that the shift toward glucose and energy storage may be a mediator as well as a marker of aging (23).

Studies of the promoter region of INO1 (inositol-1-phosphate synthase) showed that Snf1p phosphorylates histone H3 at Ser\textsuperscript{10}, which facilitates acetylation at Lys\textsuperscript{14} by Gcn5p (histone acetyltransferase) (24). These findings in young cells raise the possibility that Snf1 could promote aging by modifying chromatin structure at specific genomic loci. Therefore, in the current study, we have explored the mechanisms by which sip2Δ operates through Snf1 to regulate aging and chromatin structure. We show that covalent addition of myristate, a 14 carbon-saturated fatty acid, to the N-terminal Gly residue of Sip2p is essential for its localization to the plasma membrane and its contribution to a normal generational life span. As yeast cells age, Snf4p is redistributed from the plasma membrane to the nucleus. This redistribution, which is promoted by sip2Δ, is accompanied by increased cellular Snf1p histone H3 kinase activity, desilencing, and increased recombinational rDNA loci with increased ERC formation. The rapid-aging phenotype of sip2Δ cells is completely rescued by removing Fob1p, an essential mediator of ERC formation. Together, our results suggest that the glucose-sensing Snf1 pathway regulates aging by affecting chromatin structure and genomic stability.

**EXPERIMENTAL PROCEDURES**

**Strains**—The isogenic strains YB332 (wt; S288CMAT\textsuperscript{a}MAT\textsuperscript{α}ura3–52 his3–112 leu2–3,112 ade2–101 lys2–801 can1–100 RDN1::HIS3) and YB810 (sip2Δ HIS3) are described in Ref. 22. Additional strains were constructed using standard methods (25) and are listed in Table I.

**Life Span Determination—**Micromanipulation assays (26) were used to define the number of divisions that individual virgin mother yeast cells undergo on YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates at 24 °C (n = ≥40 mothers/genotype/experiment; n = two to three independent experiments/strain). Wt cells were used as reference controls in each experiment. The statistical significance of observed differences in life spans was evaluated using the nonparametric Wilcoxon signed rank test.

**Isolation of Young and Older Yeast Cell Populations**—When the surface proteins of a mother yeast cell are labeled with biotin, the daughter does not inherit the biotinylated proteins because its surface is generated de novo at the budding site (2). This allows mothers to be isolated from their progeny by streptavidin-magnetic bead sorting. The protocol used to obtain sorted populations of generation 0 – 1 and generation 7 – 8 or 9–10 cells is described in Ref. 2. Note that generation 0 – 1 refers to the population of cells that remains after old cells are removed by sorting; on average, 50% of this population will be composed of newly formed daughters, while 25% are mothers that have undergone a single cell division.

**Defining the Intracellular Localization of Snf1, Snf4, and Sip2**—Isogenic wt and sip2Δ cells were transformed with one of the following four CEN plasmids: pOV84 (Snf1p-green fluorescent protein (GFP fusion)), pOV76 (Snf4p-GFP), pTF9 (Sip2p-GFP) (27), (gifts from Marian Carlson, Columbia Univ.); or pBS507 (Sip2p-GFP) (2). Generation 0–1 and 7–8 cells were recovered by magnetic bead sorting from cultures grown in synthetic medium containing 2% glucose, suspended in a solution of 8 mM potassium phosphate buffer, pH 7.0, 4 mM MgSO\textsubscript{4}, 26 mM ammonium sulfate, and 2% glucose, incubated for 5 min at 24 °C.
washed twice in the same solution, and then examined under a Zeiss Axiovert microscope (2 and three independent experiments).

**Snf1p Histone H3 Kinase Assay**—Snf1p contains an N-terminal domain with 13 histidine residues (His<sub>11</sub>-His<sub>12</sub>-His<sub>13</sub>), facilitating its purification from cell lysates by Ni-NTA-agarose affinity chromatography (20).

Generation 0–1 and 7–8 isogenic wt and sip2Δ cells (25 × 10<sup>5</sup>; defined by hemocytometer counts) were recovered from a mid-log phase YPD culture by magnetic bead separation. DNA was purified from liquid N,<sub>2</sub>-frozen, and then incubated for 5 min at 1°C in 1 ml of extraction buffer (20 mM potassium phosphate buffer, pH 7.4, 0.02% bovine serum albumin, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 25% glycerol, 0.5% Triton X-100, and 50 mM potassium fluoride). The resulting cell lysate was added directly to 1 ml of Ni-NTA-agarose (Qiagen) pre-equilibrated with 5 ml of 50 mM HClO<sub>4</sub>, 0.5 M NaCl, 75 mM sodium phosphate buffer, pH 7.0, and 50 mM potassium fluoride). The mixture was incubated for 60 min at 4°C. The resin was then washed with buffer A (three cycles, 5 ml/cycle) and a reaction containing a synthetic phosphorylated histone H3 peptide derivative (1ARTKQTARKpSTGGKAPRKQLASKAARC) containing 0.5 mM of a previously published histone H3 peptide substrate for Snf1p (24) (ARTKQTARKGKPARKPLASKAAC), Biomolecules Midwest, Waterloo, IL). The 1-pH mixture was incubated for 60 min at 20°C. The reaction was stopped with 0.5 μl of 0.2 M HCl (10 min, 20°C), and a second reaction initiated by adding 0.5 μl of a solution containing 200 mM imidazole-HCl, pH 7.0, 80 mM NaOH, 0.08% bovine serum albumin, 20 mM EDTA, 0.2 mM NAD<sub>H</sub>, 80 μg/ml beef heart lactate dehydrogenase (specific activity = 500 units/mg, Sigma). The mixture was incubated for an additional 10 min at 20°C. 0.5 μl of 0.4 M HCl was then introduced to destroy any excess NADH.

To detect the NAD<sup>+</sup> product generated from the Snf1p histone H3 kinase reaction (Fig. 1A), the product had to be amplified through a series of coupled reactions (for details of this well established analytic method, see Refs. 23 and 28). Five thousand cycles of amplification were achieved by adding 0.5 μl of the reaction mixture to 0.1 ml of NAD cycling reagent (100 mM Tris-HCl, pH 8.1, 2 mM oxaloacetic acid, 2 mM β-mercaptoethanol, 0.3 μl ethanol, 0.02% bovine serum albumin, 12.5 μg/ml alcohol dehydrogenase (specific activity = 300 units/mg; Sigma), 1.2 μg/ml malic dehydrogenase (specific activity = 3000 units/mg; Sigma)) and incubating the solution for 60 min at 24°C. The reaction was terminated (100°C, 5 min), cooled to room temperature, and 1 ml of malate indicator reagent (50 mM amino-methylpropanol (pH 9.9), 5 mM l-glutamate (pH 9.9), 0.2 mM NAD<sup>+</sup>, 0.2 M HCl was then introduced to destroy any excess NADH.

Several types of reference standards and controls were run in parallel with the experimental reaction. NAD<sup>+</sup> standards were always added at the cycling step into cycling reagent alone. Known amounts of malate were included as another reference control. A minus peptide reaction, and a reaction containing a synthetic phospho[methylated histone H3 peptide (‘ARTKQTARKKpSTGGKAPRKQLASKAARC’, Biomolecules Midwest) were used to confirm Ser<sup>10</sup>-specific phosphorylation. (Note that we were unable to detect Snf1p-catalyzed phosphorylation of the H3 peptide using unfractionated whole cell lysates due to the high background signal, as defined with the histone H3 peptide and its phospho-Ser<sup>10</sup> derivative.)

**ERC Analysis**—DNA was prepared from sorted generation 0–1 and 7–8 cells according to Sinclair and Guarente (5) with one exception: no zymolase was used in the first step; instead, 0.5 ml of sorbitol solution (0.9% sorbitol, 0.1 mM Tris, pH 8.3, 0.1 mM EDTA) was added to freeze-dried cells. Purified DNA was extracted by electrophoresis through 0.7% agarose containing TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 7.5). Following capillary transfer to GeneScreen Plus (Invitrogen), blots were probed with a 32P-labeled, 2.8-kb EcoRI fragment containing cDNA sequences from pNL47 (5).

**Recombination and Silencing Assays**—Assays for recombination and silencing were performed on YPD media, strains containing Ade2p integrated into RDN1 or telomeric loci (Table I). To measure recombination rates, cells with a 2μ plasmid containing Snf1p under the control of its own promoter (21), or the empty YEP24 vector, were spread onto YPD/agar plates and incubated for 30°C for 3 days. Recombination frequency was defined as the number of half-sectored red/white colonies divided by the total number of colonies (n = >10<sup>4</sup> colony scored/strain; n = four independent experiments). To measure silencing, cells were grown on synthetic complete medium. Individual colonies were picked and transferred to 1 ml of PBS. Cells were serially diluted in PBS and plated onto synthetic medium minus adenine. Plates were incubated for 20 h at 30°C, and the number of viable colonies scored per dilution.

**Microanalytic Biochemical Assays**—Levels of fructose 1,6-bisphosphatase, glycogen, NAD<sup>+</sup>, ATP, and AMP were assayed in young and old isogenic wt, sip2Δ, fob1Δ, and sip2Δ/fob1Δ cells using pyridine nucleotide-based enzyme-cycling methods. These previously described, sensitive methods permit analysis of enzymes and metabolites in a small sample of 10<sup>3</sup> sorted cells (23, 28, 29).

**Gene Expression Profiles of Young and Old Cells**—Details of this analysis are provided in an earlier publication (23). Briefly, RNAs were prepared from sorted generation 0–1 and 7–8 wt and sip2Δ cells (5 × 10<sup>7</sup> cells/sort; n = three independent sorts from separate cultures). Equivalent amounts of RNA from generation-matched cells from each sort of a given strain were pooled. Two cDNA targets were independently prepared from each RNA pool, and each cRNA used to interrogate the yeast gene expression by the Affymetrix microarray platform.
phenotypes produced by the null alleles of the corresponding gene in log phase cells (27). This rescue suggests that the GFP fusions accurately represent the trafficking patterns of the wild type proteins.

The majority of Snf1p-GFP is present in the cytoplasm of young (generation 0–1) cells, with a small fraction appearing in the nucleus. By generation 9–10, there is a modest shift in the protein to the nucleus (Fig. 2A). Snf2p-GFP is largely associated with the plasma membrane in young cells. As cells age, there is increased representation in the cytoplasm of a subset of cells (Fig. 2B). Like Sip2p-GFP, Snf4p-GFP is plasma membrane-bound in young cells, although there is also cytoplasmic localization. With age, Snf4p-GFP shifts away from the plasma membrane to the cytoplasm and nucleus (Fig. 2C).

Our previous in vitro studies had established that Sip2p is substrate for myristoyl-CoA:protein N-myristoyltransferase (Nmt1p) (30). Nmt1p catalyzes the transfer of tetradecanoate (myristate; C14:0) from CoA to the N-terminal Gly residues of ~70 yeast proteins (30). This modification occurs co-translationally and appears to be irreversible. N-Myristoylation is known to promote protein-membrane and protein-protein interactions that typically involve components of signal transduction cascades (e.g. kinases, kinase substrates, protein phosphatases, and α subunits of heterotrimeric G proteins; reviewed in Ref. 31).

We examined the contribution of N-myristoylation to the plasma membrane targeting of Sip2p. Substituting the Gly1 of Nmt1p substrates with Ala is sufficient to completely block their N-myristoylation (31). Therefore, we mutated Gly2 of Sip2-GFP to Ala, yielding Sip2G1A-GFP. Studies of generation 0–1 wt cells containing a sip2G1A-GFP CEN episme revealed that loss of the myristoyl moiety changed the protein distribution from the plasma membrane to the cytoplasm and nucleus (Fig. 2D).

Comparison of isogenic generation 0–1 wt and sip2Δ cells containing SNA4-GFP or SNA1-GFP CEN episomes disclosed that in the absence of Sip2p, Snf4p was redistributed from the plasma membrane to the cytoplasm and nucleus (Fig. 2E). The distribution of Snf1p was not affected; it remained principally cytoplasmic with a small fraction in the nucleus (data not shown). Based on these findings, we conclude that N-myristoylation of Sip2p is essential for its plasma membrane localization, as well as the plasma membrane localization of Snf4p, and that loss of Sip2p from the plasma membrane allowed Snf4p to enter the nucleus.

We next assessed the impact of N-myristoylation of Sip2p on cellular life span. To do so, CEN episomes containing SIP2 or sip2Δ were introduced into sip2Δ cells. Wt cells as well as sip2Δ cells with an empty CEN episme served as controls. The SIP2 episme completely rescued the shortened generational life span of sip2Δ cells, while sip2G1A, or the vector alone, had no effect (Fig. 3). Thus, N-myristoylation of Sip2p is required for a normal cellular life span.

**Fig. 2. Localization of Snf1 complex subunits in young and aged cells.** A–D, Wt cells containing a CEN plasmid encoding either Snf1p-GFP, Sip2p-GFP, Snf4p-GFP, or a N-myristoylation-defective Sip2p (Gly1→Ala) mutant were grown in synthetic medium containing 2% glucose, and generation 0–1 and/or 9–10 cells isolated by magnetic bead sorting. Note that with increasing age, Snf4p is redistributed from the plasma membrane to the nucleus. Loss of the myristoyl moiety from Sip2p abrogates its ability to affiliate with the plasma membrane. E, studies of sip2Δ cells showing the effect of loss of myristoylSip2p on Snf4p localization in generation 0–1 cells. Comparison with C reveals that Sip2p is a key contributor to the plasma membrane association of Snf4p. Bars, 2.5 μm.

_Snip1p-directed Histone H3 Kinase Activity Increases in Aging Wt Cells—To further define the effects of the observed age-associated redistribution of Snf1 complex components to the nucleus, we developed a sensitive in vitro assay to measure Snf1p histone H3 kinase activity in small numbers (~3 × 10⁴) of sorted generation 0–1 and 7–8 wt and sip2Δ cells. Snf1p can catalyze phosphorylation of Ser10 in a 26-amino acid peptide acceptor representing Ala⁹–Arg²⁶ of S. cerevisiae histone H3 (ARTKQTARKSTGGKAPRKQQLASKAARC; Ref. 24). Our as-
say was linear with time, and with H3 peptide substrate and input protein concentration (Fig. 1B). The specificity of the Snf1 kinase for Ser\textsuperscript{10} was confirmed in control reactions that either contained no peptide, or the preformed phosphopeptide (\textsuperscript{1}ARTKQTARKpSTGKAPRKQALKARKpSTGGKAPRKQLASKAARC; e.g. Fig. 1B) The \( K_m \) for the H3 peptide (163 \( \mu \)M; Fig. 1C) is significantly better than the \( K_m \) of a peptide that contains the phosphorylation site of another known Snf1p target, acetyl-CoA carboxylase (HMR-SAMSGLHLVKRR; \( K_m = 1000 \mu \)M).

The in vitro assay disclosed that Snf1 histone H3 kinase activity was 2-fold higher in generation 7–8 compared generation 0–1 wt cells (\( p < 0.05; \) Fig. 4A). Moreover, the activity in young (generation 0–1) sip2Δ cells was up to 3-fold higher than young wt cells. Levels did not increase significantly as sip2Δ cells aged to generation 7–8 (Fig. 4A).

Like its mammalian homolog AMPK, Snf1 may be activated by the low ATP/AMP ratios that result from impaired glycolytic activity during starvation (20). However, the difference in histone H3 kinase levels observed between wt and sip2Δ cells was not attributable to differences in their ATP:AMP ratios (Fig. 4B).

These results establish that the redistribution of Snf1 complex components to the nucleus in aging wt cells and the shift in Snf1p/Snf4p to the nucleus in young cells lacking plasma membrane-associated N-myristoylated Sip2p are associated with increased cellular levels of histone H3 kinase activity. Together with the Snf1 subunit localization data presented above, our findings indicate that (i) the Sip2p \( \beta \) subunit functions as a negative regulator of nuclear Snf1 activity in young cells by sequestering its activating Snf4p \( \gamma \) subunit at the plasma membrane and (ii) this inhibition is overcome in aging cells through a shuttling of Sip2p from the plasma membrane to the cytoplasm and a concomitant rise in nuclear Snf4p/Snf1p.

Snf1p Promotes Recombination and Desilencing—We next examined whether the observed in vitro changes in Snf1p-catalyzed histone H3 kinase activity were accompanied by in vivo alterations in chromatin structure at rDNA loci. Fob1p regulates recombination at rDNA repeats (6), and \( fob1\Delta \) produces decreased ERC formation and increased replicative life span in wt strains (4). Therefore, we introduced a \( fob1\Delta \) allele into sip2Δ cells. Removal of Fob1p not only rescued the shortened generational life span of sip2Δ cells, but extended it beyond wt to levels observed with the isogenic \( fob1\Delta \) strain (125\% of wt; Fig. 5A). ERC levels in generation 7–8 sip2Δ/fob1Δ cells were also equivalent to that of \( fob1\Delta \) alone (Fig. 5B). Finally, the life span of the \( snf4\Delta /fob1\Delta \) strain did not extend beyond \( fob1\Delta \) alone (data not shown). These findings indicate the Snf1 pathway is a regulator of ERC formation and that recombina-

![Fig. 3. Genetic evidence that N-myristoylation of Sip2p is essential for a normal cellular life span. Life spans of sip2Δ cells containing SIP2 or sip2G1A CEN episomes or the empty vector.](http://www.jbc.org/)

![Fig. 4. Effects of aging on Snf1-catalyzed Ser\textsuperscript{10} phosphorylation of histone H3. A, histone H3 kinase assay of Snf1p recovered from generation 0–1 and 7–8 wt and sip2Δ cell lysates. n, number of determinations on independently sorted cell populations. Mean values ± S.D. are plotted. An asterisk indicates that kinase activity is significantly different (\( p < 0.05\), Students' \( t \) test) compared with generation 0–1 wt cells. B, ATP and AMP concentrations in generation 0–1 and 7–8 cells. Mean values ± S.E. are plotted (\( n = \) three to six independent determinations, each in duplicate).](http://www.jbc.org/)
Results obtained from a GeneChip analysis of generation 0–1 and 7–8 isogenic wt and sip2Δ cells provided a final set of observations supporting the notion that age-associated increases in Snf1p-mediated histone H3 phosphorylation/acetylation is accompanied by changes in chromatin structure and desilencing at sites other than rDNA loci. Levels of Ino1p mRNA are 23-fold higher in old versus young wt cells and 14-fold higher in old versus young sip2Δ cells (Table II). Previous studies of young cells (32) indicated that in the absence of glucose, Snf1p-mediated phosphorylation of histone H3 regulates binding of the transcriptional activator Adr1p to its target gene promoters, leading to induction of ADH2 (ADHII isozyme which catalyzes the first step in ethanol metabolism), ACS1 (acetetyl-CoA synthetase), and POT1 (3-oxoacyl-CoA thiolase) expression. Our GeneChip analysis disclosed that each of these mRNAs rises in aging wt and/or sip2Δ cells (Table II).

The Metabolic Shift Observed in sip2Δ Cells Also Occurs in sip2Δfob1Δ Cells—Snf1 promotes glycogen production by activating Gcp7p, a protein phosphatase that activates glycogen synthase (33). We found that the rapid-aging phenotype of sip2Δ cells is associated with a metabolic shift toward gluconeogenesis and glycogen storage (23). This shift is also a consequence of aging. Wt cells accumulate glycogen as they undergo replicative senescence; the increase is equivalent to that observed in sip2Δ cells that have traversed an equivalent percentage of their mean generational life span (Fig. 7A).

We had speculated that the shift toward gluconeogenesis, glucose storage, and energy conservation may be a mediator as well as a marker of aging since it was foretelled in longer-lived snf4Δ cells. The extended life span of the sip2Δfob1Δ strain allowed us to explore this hypothesis further.

Populations of sorted generation 0–1 and 7–8 cells were prepared from isogenic wt, sip2Δ, fob1Δ, and sip2Δfob1Δ strains grown in YPD medium. Well established microanalytic biochemical assays (23) revealed that introduction of fob1Δ does not ameliorate the marked age-associated increase in fructose 1,6-bisphosphatase and glycogen levels prompted by sip2Δ, nor does it significantly change total cellular NAD (phosphate) have a normal life span (data not shown). We concluded that the metabolic shift toward gluconeogenesis is a marker but not necessarily a mediator of aging.

**DISCUSSION**

Our studies have revealed a mechanism that links the Snf1 glucose-sensing pathway in *S. cerevisiae* to modification of chromatin structure and aging. An age-associated increase in Snf1 histone H3 kinase activity occurs coincident with a shift in its β subunit from the plasma membrane to the cytoplasm and its activating γ subunit (Snf4p) from the plasma membrane to the nucleus. These shifts in subunit localization are accompanied by age-associated increases in recombination at rDNA loci (generating ERCs) and augmented desilencing at rDNA loci, telomeres, as well as sites known in younger cells to be affected by Snf1p-catalyzed phosphorylation of histone H3. Several observations established that *N*-myristoylated Sip2p is a key regulator of Snf1 in aging cells. Among the three genes encod-
source-responsive elements present in genes encoding gluconeogenic enzymes (16, 34). Gal83p alone is required for phosphorylation of the same protein (16).

All β subunits have conserved C-terminal domains that mediate interactions with Snf1p and Snf4p (these domains are termed KIS and ASC, respectively) (35). The N termini of the three β subunits are more divergent than their C-terminal domains and contribute to their distinct intracellular locations and functions. Sip1p is found in the vacuole when cells are grown in glycerol (27). In contrast, Gal83p moves from the cytoplasm to the nucleus when cells are shifted from glucose to glycerol (27). The N-terminal 90 residues of Gal83p are sufficient to confer nuclear localization (27). Sip2p is the only Snf1 β subunit that is N-myristoylated and, as noted above, is affiliated with the plasma membrane. (The β subunit of the mammalian Snf1 homolog, AMPK, is also predicted to be a substrate for N-myristoyltransferase) (14).

The importance of N-myristoylation of Sip2p in modulating cellular life span is highlighted by two findings. First, the accelerated aging phenotype of sip2Δ cells can be fully rescued by a CEN episme containing SIP2 under the control of its own promoter but not by an episome containing a sip2 mutant with a Gly1 → Ala substitution that blocks N-myristoylation. Second, a strain containing a mutant nmt1 allele that produces global defects in protein N-myristoylation (nmt1–451D) has a rapid-aging phenotype (22). The mean life spans of isogenic nmt1–451D and nmt1–451Dsip2Δ strains are the same. In addition, introducing null alleles of genes encoding a number of prominent N-myristoylproteins into wt cells has no effect on life span (22). Together, these observations suggest that loss of myristoylsip2p is the principal contributor to the rapid aging of nmt1–451D cells.

We know that the trigger to Snf1 activation in aging cells is not a reduction in intracellular glucose levels; they rise 2- and 8-fold as wt and sip2Δ cells go from generation 0–1 to 7–8, respectively (23). Impaired glucose transport does not appear to be the trigger: transport rates rise modestly in aging cells as judged by the uptake of 2-deoxyglucose (22). Although one possible trigger could be an age-associated change in the level of N-myristoylation of Sip2p, to date we have no evidence to support such a notion. Our GeneChip analysis revealed that Nmt1p mRNA levels were not appreciably different in young and old wt and sip2Δ cells, nor were there detectable changes in expression of genes that regulate myristoyl-CoA pool size, e.g. FAA1–4 (fatty acyl-CoA synthetases) and FAS1,FAS2 (fatty acid synthetases) (data not shown). A sensitive assay for Nmt1p activity (36) could not be used to directly measure enzyme activity in unfractionated lysates prepared from young and old cells because of the high background. However, immuno blot analysis of total cellular proteins using previously characterized antibodies to Nmt (37) revealed no apparent differences in enzyme concentration between young and old wt or sip2Δ cells (data not shown).

It is important to note that for many N-myristoylproteins myristate is a key but not an exclusive regulator of membrane association. Protein N-myristoylation promotes weak and reversible protein-membrane interactions (38). Proteins such as myristoylated alanine-rich C kinase substrate use myristate plus electrostatic interactions between positively charged protein side chains and negatively charged phospholipids to stabilize their association with the plasma membrane (39). Phosphorylation of these side chains functions is used to sever the association (39). Other N-myristoylproteins such as ADP-ribosylation factors or recoverin use ligand binding to produce a conformational change that exposes or sequesters their myristoyl chain ("myristoyl-conformational switch") (40, 41). Thus,
the age-associated redistribution of Sip2p from the plasma membrane to the cytoplasm could reflect changes in its levels of N-myristoylation or reversible modifications that affect electrostatic interactions with membrane phospholipids and/or a conformation change triggered by acquisition of a ligand that affects presentation of its myristoyl group. Distinguishing among these possibilities should provide further mechanistic details about how Snf1p is activated in aging cells and how such activation can be overcome.

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Sip2, an N-Myristoylated β Subunit of Snf1 Kinase, Regulates Aging in *Saccharomyces cerevisiae* by Affecting Cellular Histone Kinase Activity, Recombination at rDNA Loci, and Silencing

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