Cell

Acetylation of Yeast AMPK Controls Intrinsic Aging Independently of Caloric Restriction

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DOI 10.1016/j.cell.2011.07.044

SUMMARY

Acetylation of histone and nonhistone proteins is an important posttranslational modification affecting many cellular processes. Here, we report that NuA4 acetylation of Sip2, a regulatory β subunit of the Snf1 complex (yeast AMP-activated protein kinase), decreases as cells age. Sip2 acetylation, controlled by antagonizing NuA4 acetyltransferase and Rpd3 deacetylase, enhances interaction with Snf1, the catalytic subunit of Snf1 complex. Sip2-Snf1 interaction inhibits Snf1 activity, thus decreasing phosphorylation of a downstream target, Sch9 (homolog of Akt/S6K), and ultimately leading to slower growth but extended replicative life span. Sip2 acetylation mimetics are more resistant to oxidative stress. We further demonstrate that the anti-aging effect of Sip2 acetylation is independent of extrinsic nutrient availability and TORC1 activity. We propose a protein acetylation-phosphorylation cascade that regulates Sch9 activity, controls intrinsic aging, and extends replicative life span in yeast.

INTRODUCTION

Reversible acetylation and deacetylation of histones are important in regulating chromatin structure and controlling transcription of genes that are crucial for maintenance of cell viability (Lin et al., 2008). Among the histone deacetylases, Sir2 (yeast homolog of mammalian SIRT1; Donmez and Guarente, 2010) and Rpd3 (yeast homolog of mammalian HDAC1; Willis-Martinez et al., 2010) are especially important in life span regulation in yeast (Chang and Min, 2002; Jiang et al., 2002). Sir2 extends replicative life span partially via deacetylating histone H4 lysine 16 that compromises transcriptional silencing (Dang et al., 2009; Imai et al., 2000; Sinclair and Guarente, 1997). However, the life span discrepancies between substrate histone mutants and the acetyltransferase/deacetylase mutants (Dang et al., 2009) suggest possible roles of nonhistone acetylation substrates in mediating life span regulation by these (de)acetylating enzymes.

In a previous study, we identified many nonhistone substrates of the NuA4 complex, of which the catalytic subunit, Esa1, is the only essential histone acetyltransferase in yeast (Lin et al., 2009). We show here that NuA4 catalytic mutants have replicative life span defects caused by impaired acetylation of Sip2, a known replicative life span regulator (Ashrafi et al., 2000). Sip2 is one of three β regulatory subunits of the Snf1 complex and the only β subunit implicated in yeast replicative aging (Ashrafi et al., 2000; Lin et al., 2003). The Snf1 complex contains (1) a catalytic α subunit, Snf1, which is an AMP-activated serine/threonine protein kinase, (2) a γ subunit, Snf4, (3) and one of the three β regulatory subunits—Sip1, Sip2, or Gal83—each with a distinctive substrate specificity (Schmidt and McCartney, 2000). In addition to being required for transcription of glucose-repressed genes and utilization of carbon sources other than glucose (Amodeo et al., 2007), Snf1 is also a key player in the response to cellular stress (Sanz, 2003). Importantly, Snf1 activity increases in aged cells even when ambient glucose is abundant (Ashrafi et al., 2000; Hedbacker and Carlson, 2008). Null mutations in *SIP2*, the *SNF1* repressor, decrease life span; these can be rescued by deletion of *SNF4*, the *SNF1* activator (Guarente and Kenyon, 2000).

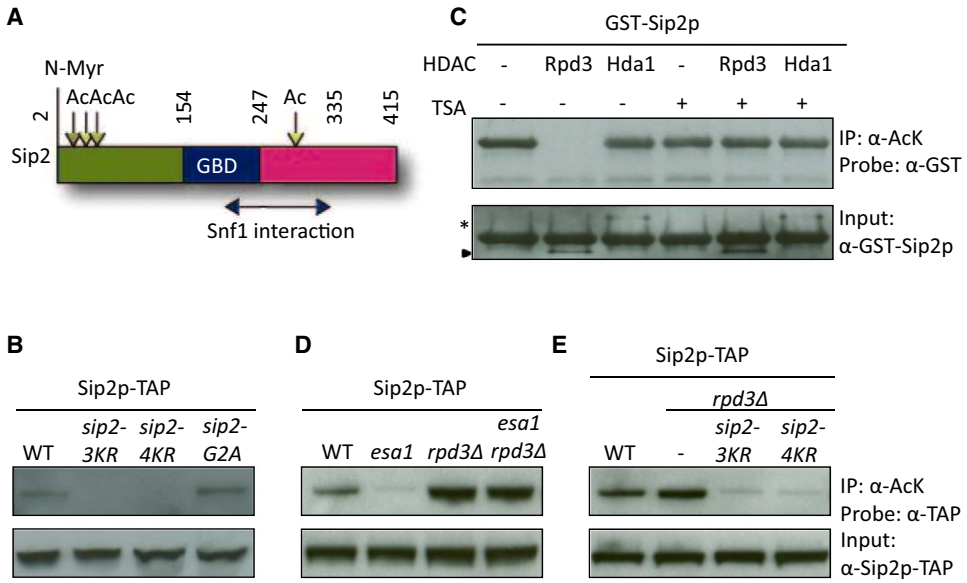

Figure 1. Sip2 Is Acetylated at Four Lysine Sites by Esa1, and Rpd3 Is the Counteracting Deacetylase

(A) Cartoon of Sip2 structural domains (adapted from Hedbacker and Carlson, 2008). Mass spectrometry identified four acetylated lysine residues of Sip2, K12, K16, K17, and K256. Numbers indicate amino acid residues. (Vertical straight line) Myristoylation site; (downward arrows) acetylation sites; (right-left arrow) regions mapped as sufficient for Snf1 interaction by deletion analysis (Amodeo et al., 2007). Ac, acetylation; N-Myr, N-myristoylation; GBD, glycogen-binding domain.

(B) Chromosomally integrated *sip2-3KR* and *sip2-4KR*, but not *sip2-G2A*, mutants are hypoacetylated in vivo. The *sip2-G2A* mutant blocks myristoylation. *sip2-3KR*, *sip2-K12/16/17R*; *sip2-4KR*, *sip2-K12/16/17/256R*.

(C) Rpd3, but not Hda1, removes Sip2 acetylation in vitro. Deacetylation reaction is inhibited by addition of trichostatin A (TSA). Asterisk indicates Hda1-TAP; arrowhead indicates Rpd3-TAP.

(D) Sip2 is hypoacetylated in strains carrying the Ts allele of *ESA1*, *esa1-531*, but is hyperacetylated in *rpd3Δ*; deletion of *RPD3* rescues the acetylation defect of *esa1-531*.

(E) Increased Sip2 acetylation in *rpd3Δ* is blocked when lysines are mutated to arginines.

See also Figure S1 and Figure S2.

Here, we report that Sip2 acetylation is controlled by the NuA4 and its counteracting deacetylase, Rpd3 (Chang and Pillus, 2009). We examined replicative life span in various *SIP2* acetylation mutants and found that Sip2 acetylation extends yeast life span; besides, constitutive Sip2 acetylation mimetics nearly totally rescue the life span-shortening phenotype of the NuA4 catalytic mutant, indicating the critical role of Sip2 acetylation in life span modulation. We further investigated glucose limitation, peroxide sensitivity, and age-associated changes in Sip2 acetylation, established the role of Sip2 acetylation in controlling Snf1 interactions and activities. Finally, we established that Sch9, the yeast homolog of Akt and S6K (Madia et al., 2009), was a common downstream target of two distinct replicative life span regulating pathways: the intrinsic aging defense pathway described here controlled by Snf1 kinase and the extrinsic nutrient-sensing pathway regulated by TORC1.

RESULTS

Sip2 Acetylation Is Controlled by NuA4 and Rpd3

To investigate the function of Sip2 acetylation, we identified four acetylated lysine residues (K12, K16, K17, and K256) (Figure 1A), using tandem mass spectrometry (Figures S1A–S1C available online). We then created unacetylable lysine-to-arginine mutant constructs at these four sites in various combinations by site-directed mutagenesis and introduced these mutant *SIP2* constructs into the endogenous chromosomal locus (Toulmay and Schneiter, 2006). Using a previously described reverse IP approach (Lin et al., 2009), we showed that Sip2 is hypoacetylated when the first three (K12, K16, and K17 [*3KR*]) or all four (K12, K16, K17, and K256 [*4KR*]) lysines are mutated (*Sip2-3KR* or *-4KR*; Figures S1D, Figure 1B). We also created a chromosomally integrated *SIP2* glycine 2-to-alanine mutant (*sip2-G2A*) to mimic the short-lived, non-N-myristoylated species of Sip2 (Ashrafi et al., 2000). We found that *sip2-G2A* did not have acetylation defects (Figure 1B). An in vitro deacetylation reaction carried out with purified Rpd3-TAP and Hda1-TAP (Figure S2A) revealed that Rpd3 treatment abolished the acetylation signal of Sip2 (Figure 1C). We demonstrated the activity of purified Hda1-TAP by showing that it deacetylated lysine 14 of FLAG-Htz1 purified from *hda1Δ* strain (Figure S2B) (Lin et al., 2008). As will be shown below, mutagenesis of the Sip2 acetylation sites affects replicative aging. The findings are consistent with a previous report that Rpd3, but not Hda1, is involved in replicative life span regulation (Kim et al., 1999). Previously we have shown that the acetylation signals of Sip2 are virtually completely dependent on NuA4/Esa1 both in vivo and in vitro (Lin et al., 2009). Importantly, simultaneous deletion

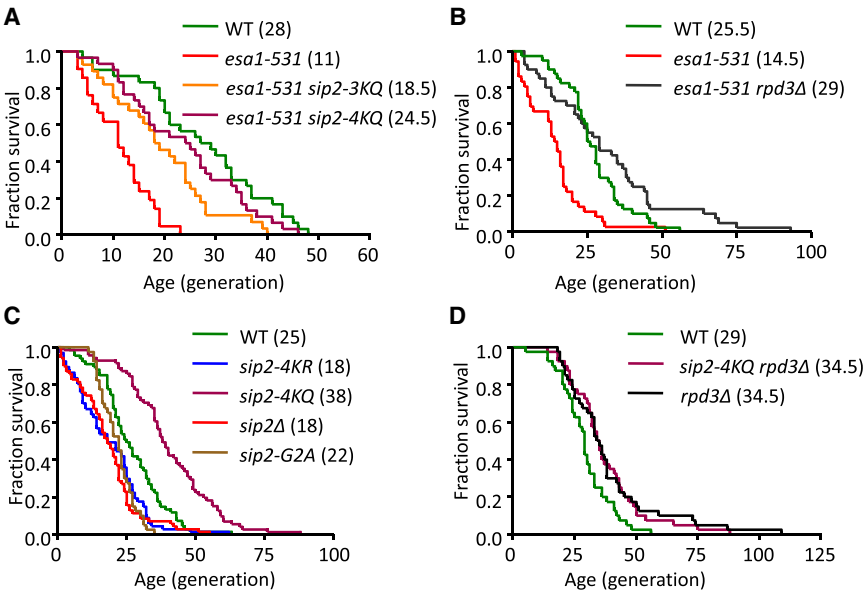

Figure 2. Sip2 Acetylation Increases the Cellular Replicative Life Span

(A) Survival curves of the indicated strains with each median life span in parentheses. The fractions of live cells are plotted as a function of age in generations. Strains carrying *esa1-531* exhibit life span shortening that is rescued by *SIP2* acetylation mimetics (*sip2-3KQ* and *sip2-4KQ*).

(B) Deletion of *RPD3* reverses the short life span of *esa1-531*.

(C) *sip2-4KR* mutants, as well as the *sip2-G2A* mutants and deletion of *SIP2*, decrease cellular life span, whereas *sip2-4KQ* mutants increase life span.

(D) Simultaneous *sip2-4KQ* mutants on the background of *rpd3Δ* fail to further increase life span of *rpd3Δ*. Statistical significance was determined by Mantel-Cox log-rank test and the details are presented in Table S1.

of *RPD3* reversed the hypoacetylation caused by NuA4 catalytic subunit temperature-sensitive (Ts) allele *esa1-531* when cells were shifted to the nonpermissive temperature (Figure 1D). Further, the increased acetylation in *rpd3Δ* was abolished by *sip2-3KR* and *-4KR* mutations (Figure 1E). These results support the simple hypothesis that K12, K16, K17, and K256 in Sip2 are acetylated by Esa1 and are deacetylated by Rpd3.

Sip2 Acetylation Mimetics Increase Replicative Life Span

We next examined replicative life span of the *SIP2* acetylation mutants (refer to Table S1 for detailed statistical results). We found that the *esa1-531* strain showed a shortened replicative life span when grown at a semipermissive temperature, 30°C. Importantly, in an *esa1-531* strain, the lysine-to-glutamine mutants of *SIP2* (*sip2-3KQ* and *sip2-4KQ*), mimicking acetylated Sip2, reversed the shortened life span of *esa1-531* (Figure 2A). Consistent with the above findings, deletion of *RPD3* also reversed the shortened life span of *esa1-531* (Figure 2B). We also found that *sip2-4KQ* significantly increased the life span, whereas *sip2-4KR* and *sip2Δ* strains decreased life span to a similar extent when compared to wild-type (WT) $SIP2^{+}$ strains (Figure 2C). Because the N-myristoylation on glycine 2 of Sip2 is essential for long life span (Ashrafi et al., 2000; Lin et al., 2003), the chromosomally integrated *sip2-G2A* mutant was used as a positive control for shortened life span (Figure 2C). Finally, we confirmed that simultaneous *sip2-4KQ* mutant did not further extend the life span of the null mutant of *RPD3* (Figure 2D). These results provide strong evidence supporting Sip2 as the critical downstream target of Esa1 and Rpd3 in regulating replicative life span in yeast.

Sip2 Acetylation and Calorie Restriction

Because carbon substitutions greatly influence longevity in yeast (Barker et al., 1999; Kaeberlein et al., 2005), we next asked whether different carbon sources affect Sip2 acetylation status. We monitored Sip2 acetylation (Ac-Sip2) levels after treating cells with 2% glucose, 2% galactose, 0.05% glucose, or 2% glycerol plus 3% ethanol. The only significant difference in acetylation states was observed when cells were treated with 0.05% glucose (low glucose, LG); although Sip2 protein abundance was upregulated, the acetylation signal decreased significantly (Figures S3A and S3B). This contradicts a simple model that calorie restriction extends replicative life span via Sip2 acetylation. Indeed, calorie restriction was shown to increase replicative life span through Sir2 (Lin et al., 2002) and other mechanisms (Kaeberlein et al., 2005). We therefore examined whether low glucose (LG) affected life span in the *SIP2* acetylation mutants and found that LG, when compared to 2% glucose (normal glucose, NG), increased life span by ~50% in the *sip2-4KR* and *sip2Δ* strains and, to somewhat a lesser extent, in WT (~30%) (Figures 3A–3C and Table S1). However, LG failed to significantly increase the life span of the *sip2-4KQ* mutant (Figure 3D and Table S1). Because glucose limitation significantly improved the replicative life span of *sip2-4KR* and *sip2Δ*, these findings imply that calorie restriction extends life span through a mechanism largely independent of Sip2 acetylation. The fact that calorie restriction failed to significantly extend the life span of *sip2-4KQ*, which was similar to that reported in *rpd3Δ* (Jiang et al., 2002), suggests the existence of a common effector(s) downstream of the Sip2 acetylation and calorie restriction modulating replicative life span in yeast.

Aging Decreases Sip2 Acetylation and Interaction with Snf1

To investigate the effect of replicative aging on Sip2 acetylation, we examined Ac-Sip2 in young and old cells by sorting biotin-labeled mother cells (Smeal et al., 1996). The sorting efficiency was confirmed by showing that old cells contained a mean of seven bud scars, whereas young cells had none (Figure S3C). We found that Ac-Sip2 levels decreased in old cells (Figure 4A). Coimmunoprecipitation of Sip2-TAP and Snf1-HA demonstrated

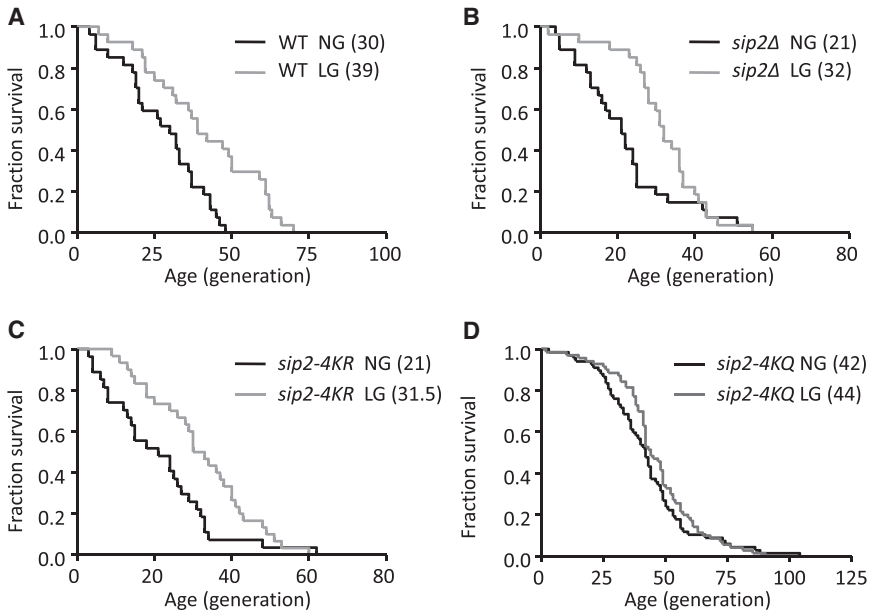

Figure 3. Glucose Limitation and Replicative Life Span in *SIP2* Acetylation Mutants

Survival curves of WT (A), *sip2Δ* (B), *sip2-4KR* (C), and *sip2-4KQ* (D) grown in normal (2%, NG) versus low glucose (0.05%, LG) media. The fractions of live cells are plotted as a function of age in generations. Median life span is shown in parentheses. Glucose limitation significantly increases life span in WT, *sip2Δ*, and *sip2-4KR*, but not in *sip2-4KQ*, as determined by Mantel-Cox log-rank test. The statistical results were summarized in Table S1. See also Figure S3.

that, although the interaction between Sip2 and Snf1 was readily seen in young cells, it became undetectable in old cells (Figure 4B).

To determine whether Sip2 acetylation dictates physical interaction between Sip2 and Snf1, we examined their association in *SIP2* acetylation mutants. Physical interaction between Sip2 and Snf1 significantly decreased in *esa1-531* but increased in *rpd3Δ* (Figure 4C). More importantly, whereas the *sip2-3KR* and *sip2-4KR* mutations almost abolished the interaction with Snf1, the *sip2-3KQ* and *sip2-4KQ* mutations markedly enhanced it (Figure 4D), supporting the idea Sip2 acetylation is required for Snf1 interaction and defining the N terminus of Sip2 as an enhancer of Snf1 interaction (Figure 1A). In contrast, the myristoylation null mutation *sip2-G2A* (Lin et al., 2003) did not affect the physical interaction between Sip2 and Snf1 (Figure 4D), suggesting that myristoylation regulates Snf1 activity via a different mechanism. Because Sip2 is a negative regulator of Snf1 (Ashrafi et al., 2000), these results at least partially explain why Snf1 activity, which is stimulated under carbon stress, increases with age and hence has detrimental effects on life span, even in the presence of abundant ambient glucose (Lin et al., 2003).

To establish the connection between aging and Sip2 acetylation status in cells, we measured trehalose levels, a general stress indicator in yeast, in young and old cells. This is based on the previous findings that, during the quiescent phase, trehalose is stored in favor of glycogen presumably to fulfill its numerous stress-protectant functions (Shi et al., 2010). Indeed, we found that young cells contained significantly less trehalose than old cells (Figure 4E). Glucose limitation, a form of nutrient stress, also significantly increased trehalose levels as reported before (Figure S3D) (Pluskal et al., 2011). Measurement of trehalose levels revealed that *esa1-531* contained elevated trehalose levels that were partially reversed by concomitant *RPD3* deletion (Figure S3E). *Sip2-4KR rpd3Δ* and *sip2Δ rpd3Δ* increased trehalose levels compared to *rpd3Δ* (Figure S3F). Also, *sip2-4KR*, *sip2-G2A*, and *sip2Δ* mutants had increased trehalose levels compared to WT, whereas *sip2-4KQ* had a lower level of trehalose (Figure 4F), supporting a role of Ac-Sip2 in counteracting stress. We further showed that *sip2-4KR* and *sip2Δ* mutants were much more sensitive to hydrogen peroxide (H_2O_2), a form of oxidative stress and a mediator of aging (Giorgio et al., 2007), when compared to WT (Figure 4G); on the contrary, *sip2-4KQ* was resistant to H_2O_2 (Figure 4G).

Consistent with previous findings that trehalose plays a key role in fueling cell-cycle progression during growth and division (Shi et al., 2010), we found that growth rates significantly decreased in the *rpd3Δ* strain (Figure S4A) and the *sip2-4KQ* strain on various genetic backgrounds (Figures S4B and S4C). Conversely, *sip2-4KR* and *sip2Δ* mutants partially rescued the *rpd3Δ* growth defect (Figure S4A). Cell volumes were similarly affected by the mutations; all of the slower-growing variants were smaller than normal cells (Figure S4D).

Sch9 Is the Downstream Target of Sip2-Snf1

Based on the above results and literature information, we proposed that Snf1 might have a downstream target that, when phosphorylated by Snf1, fuels rapid cell growth but results in shortened life span. In a previous phosphorylation proteome microarray study, 80 in vitro Snf1 substrates were identified (Ptacek et al., 2005). Among these, Sch9 (an Akt/S6K homolog) kinase was a promising candidate because it had already been shown to play an important role in yeast aging (Kaeberlein et al., 2005). In addition to Snf1, Sch9 is the in vitro substrate of two additional kinases (of 87 kinases tested): Tos3, the upstream kinase of Snf1 (Kim et al., 2005) and Pho85 (Ptacek et al., 2005). Sch9 is also a well-known in vivo kinase substrate of target of rapamycin complex 1 (TORC1). Similar to TORC1, it negatively regulates life span in response to nutrient availability (Kaeberlein et al., 2005; Urban et al., 2007). A kinase itself, Sch9 phosphorylates a critically important ribosomal protein, Rps6 (Urban et al., 2007); *rps6Δ* mutants grow slowly and are small, but they have increased replicative life span through general inhibition of translation machinery (Chiocchetti et al., 2007) and protein synthesis (Huber et al., 2009).

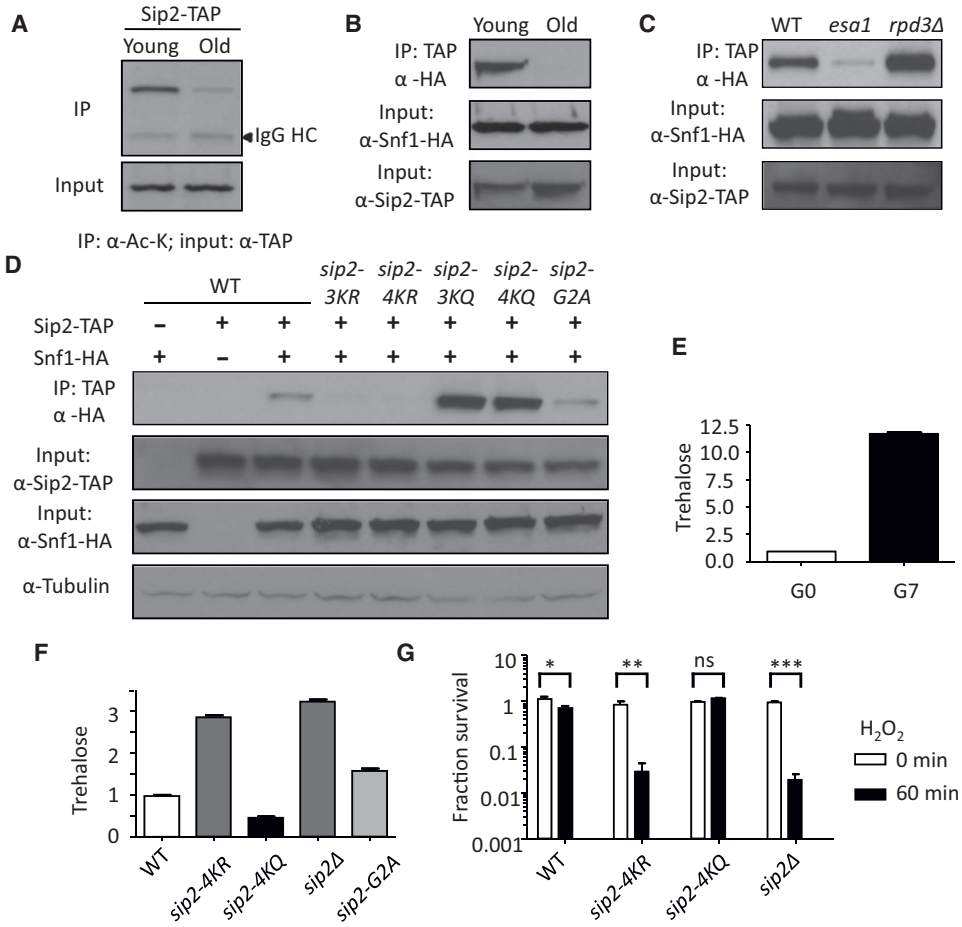

Figure 4. Sip2 Acetylation and Physical Interaction between Sip2 and Snf1 Decrease as Cells Age

(A) Sip2 acetylation is significantly decreased in old cells.

(B and C) Physical interaction between Sip2 and Snf1 assessed by coimmunoprecipitation is decreased in old cells (B) and *esa1-531* strain but increased in *rpd3Δ* strain (C).

(D) Interaction between Sip2 and Snf1 is decreased in *sip2Δ*, and deacetylation mimetics (*sip2-3KR* and *sip2-4KR*) but is not changed in *sip2-G2A* mutants. The interaction significantly increases in acetylation mimetics (*sip2-3KQ* and *sip2-4KQ* mutants).

(E) Cellular trehalose is significantly increased when cells age. Error bars show standard error of the mean. n = 3.

(F) Trehalose levels are significantly higher in *sip2-4KR*, *sip2-G2A*, and *sip2Δ* when compared to WT but are lower in *sip2-4KQ*. Error bars show standard error of the mean. n = 3.

(G) Fractions of survival of WT, *sip2-4KR*, *sip2-4KQ*, and *sip2Δ* before and after 1 hr of treatment with 1.5 mM H_2O_2, are plotted on a log scale. Error bars show standard error of the mean. n = 2. Statistical significance was assessed by two-way ANOVA with post-hoc test. ns, nonsignificant; *p < 0.05; **p < 0.01; ***p < 0.001.

See also Figure S3 and Figure S4.

We first showed that Sch9 is an in vitro and in vivo substrate of Snf1. We purified GST-Sch9 and assessed the phosphorylation signal by performing immunoblotting with antiphosphoserine and antiphosphothreonine antibodies (Jablonowski et al., 2004). As a proof of concept, the signal of phosphorylated GST-Sch9 nearly totally disappeared after treatment with calf intestine phosphatase, whereas sodium pervanadate (a phosphatase inhibitor) restored the phosphorylation signal (Figure S5A). We carried out an in vitro kinase assay with purified Snf1-TAP (Figure S2A) from a *sip2Δ* strain and showed serine and threonine phosphorylation of GST-Sch9 in vitro by Snf1 kinase (Figure 5A). We further confirmed that endogenous Sch9-HA phosphorylation markedly decreased in *snf1Δ* especially after treatment with rapamycin to block TORC1 activity (Figure 5B and whole gel images in Figure S5B). Moreover, a mutation that abolishes the kinase activity of Snf1 (*snf1-K84R*) fails to revert the decreased phosphorylation of endogenous Sch9-HA in a *snf1Δ* strain (Figure S5C), which suggests that Sch9 is a bona fide in vivo substrate of Snf1.

To detect Sch9 phosphorylation states in different *SIP2* mutants, we purified GST-Sch9 proteins from WT, *sip2-4KR*, *sip2-4KQ*, and *sip2Δ* strains and assessed their phosphorylation signals. Both serine and threonine phosphorylation increased slightly in *sip2-4KR* and *sip2Δ* compared to WT and *sip2-4KQ*

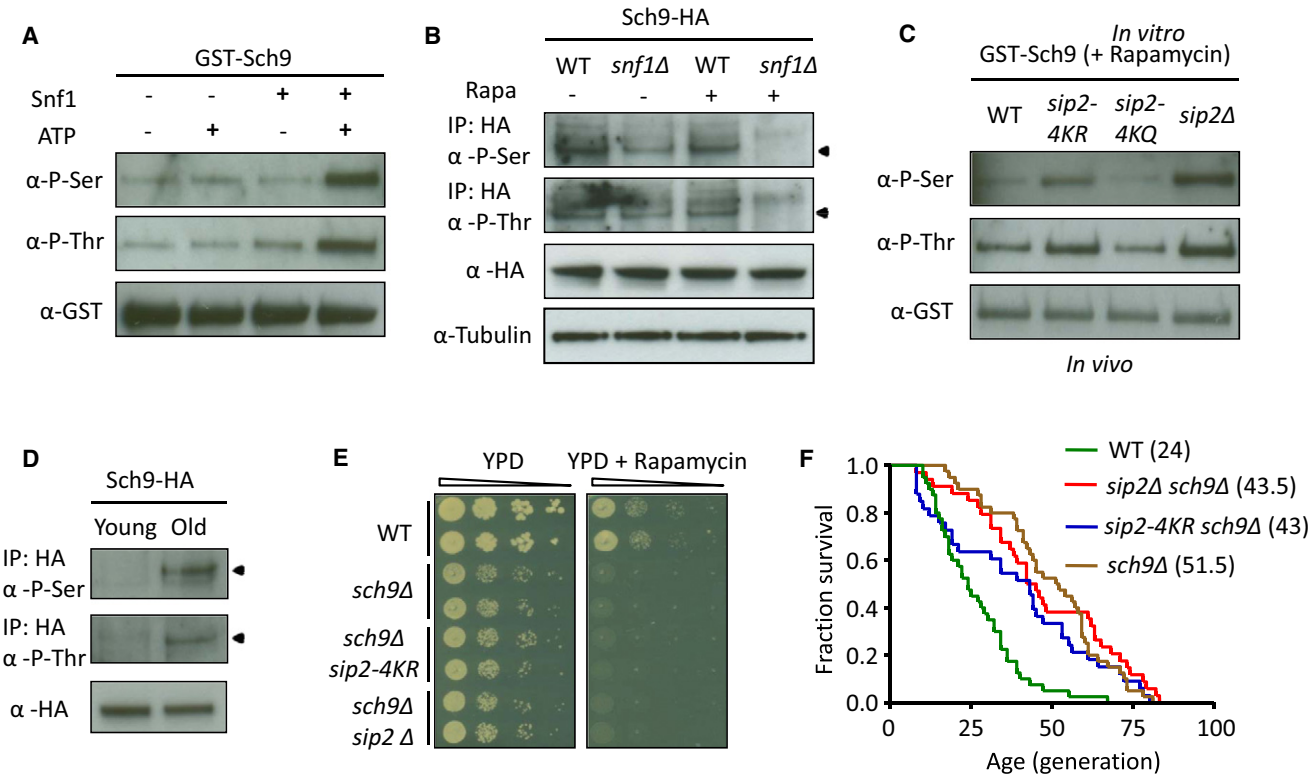

Figure 5. Acetylation of Sip2 Affects Snf1 Kinase Activity and Results in Hypophosphorylation of Sch9

(A) Snf1 phosphorylates GST-Sch9 in vitro at both serine and threonine sites. In vitro kinase assays were performed by incubating purified GST-Sch9 with or without Snf1 or ATP as indicated and analyzed by phosphoserine antibody (α-P-Ser) and phosphothreonine antibody (α-P-Thr) to detect Sch9p phosphorylation.

(B) Endogenous Sch9-HA phosphorylation decreases in *snf1Δ* mutants after rapamycin (200 ng/ml) treatment to suppress the TOR pathway. Arrowheads indicate the Sch9-HA band.

(C) GST-Sch9 phosphorylation increases in *sip2-4KR* and *sip2Δ* but decreases in *sip2-4KQ*, compared to WT after rapamycin (200 ng/ml) treatment.

(D) Endogenous Sch9-HA phosphorylation significantly increases in the old cells. Arrowheads indicate the Sch9-HA band.

(E) *SCH9* is epistatic and thus downstream to *SIP2* in regulating cellular growth. Ten-fold dilutions of the indicated strains were spotted and grown on YPD plates without (2 days, 30°C) or with rapamycin (25 ng/ml, 4 days, 30°C).

(F) Deletion of *SCH9* rescues the life span shortening of *sip2Δ* and *sip2-4KR*.

See also Figures S1 and Figure S5.

(Figure S5D); the differences were enhanced by inhibiting TORC1 with rapamycin (Figure 5C), with the lowest level of phosphorylation observed in the *sip2-4KQ* mutant. Finally, we confirmed that endogenous Sch9-HA phosphorylation markedly increased as cells aged (Figure 5D), which is consistent with aging-associated increase in Snf1 kinase activity.

To demonstrate that Sch9 is the critical downstream mediator of Sip2 in regulating growth and life span, we analyzed epistasis. The growth defects of *sch9Δ* cells were not rescued by *sip2-4KR* and *sip2Δ* (Figure S5E) as expected for a downstream effector. We showed that *sch9Δ* was hypersensitive to rapamycin as reported (Urban et al., 2007), which remained unaltered in the concomitant *SIP2* mutants (Figure 5E). We also observed that the phenotype of shortened life span in the *sip2Δ* and *sip2-4KR* mutant cells was nearly completely reversed by deleting *SCH9* (*sip2Δ sch9Δ* and *sip2-4KR sch9Δ* in Figure 5F). Consistent with these observations, *sch9Δ* cells also showed comparable life span extension to both double-mutant strains (Figure 5F). These data support Sch9 as the downstream target of Sip2-Snf1.

Sip2-Snf1 and TORC1 Function in Parallel Upstream of Sch9

By comparing growth patterns of Sip2 acetylation mutants in YPD and rapamycin, we further examined whether Sip2-Snf1 and TORC1 function in parallel upstream of Sch9 in regulating growth. Both *rpd3Δ* and *sip2-4KQ* showed growth defects in YPD, consistent with reduced Sch9 activity in these mutants. The growth differences among the mutants and WT were dampened by rapamycin treatment (Figures 6A–6C) through suppressing TORC1, an essential activator of Sch9; rapamycin did not further inhibit growth of the already Sch9-suppressed *rpd3Δ* and *sip2-4KQ*. Both *sip2-4KR* and *sip2Δ* partially rescued the growth defects of *rpd3Δ*, possibly through Snf1 activation of the downstream Sch9. Again, the growth differences disappeared when treated by rapamycin. In contrast, the growth defects of *rpd3Δ* were not rescued by *sip2-G2A*, another short-lived mutant (Figure S6A).

Sip2-Snf1 and TORC1 might similarly function in parallel upstream of Sch9 to regulate replicative life span. We first

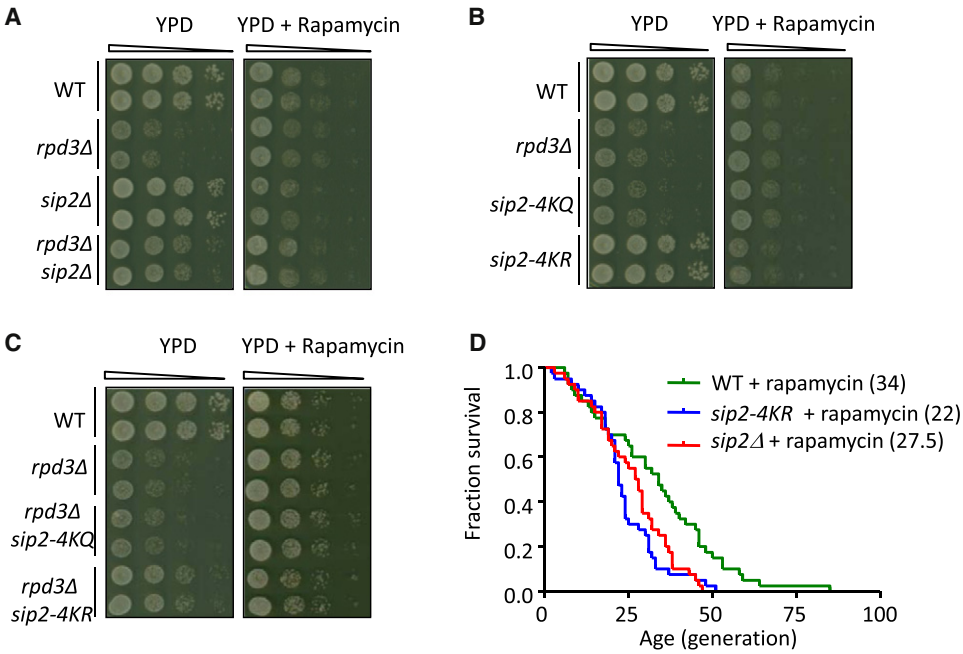

Figure 6. Sch9 Is a Parallel Downstream Target of TORC1 and Sip2-Snf1

(A–C) Comparison of growth in YPD with and without rapamycin to reveal genetic interactions between *RPD3* and *SIP2*. Ten-fold dilutions of the indicated strains were spotted and grown on YPD plates without (2 days, 30°C) or with rapamycin (25 ng/ml, 4 days, 30°C).

(D) The life span of *sip2-4KR* and *sip2Δ* in rapamycin is shorter than that of WT in rapamycin (25 ng/ml).

See also Figure S6.

confirmed that rapamycin increased life span of WT (Figure S6B) (Kaeberlein et al., 2005). We further showed that life span of *sip2-4KR* and *sip2Δ*, although mildly increased after rapamycin treatment (Figures S6C and S6D), remained significantly shorter than WT in rapamycin, suggesting the importance of Sip2 acetylation in controlling cellular life span independent of TORC1 activity (Figure 6D). Life span of *sip2-4KQ* and *rpd3Δ* were not extended by rapamycin treatment (Figure S6E), consistent with the fact that Sch9 serves as a common downstream effector. These results support a protein acetylation/phosphorylation signaling cascade distinct from the one mediated by TORC1 regulating cellular growth and life span through Sch9 as the common kinase effector.

Finally, we showed that *SCH9* mutants of seven known PDK (phosphoinositide-dependent protein kinase)-dependent and TORC1-dependent phosphorylation sites (Urban et al., 2007) still showed increased phosphorylation signal upon treatment with Snf1 kinase (Figure S6F), supporting the hypothesis that Snf1 catalyzes Sch9 phosphorylation at sites distinct from those used by PDK and TORC1. These results also corroborate our previous findings (Figure 3F) that glucose limitation and Sip2 acetylation seem to operate on the same effector protein in affecting replicative life span.

DISCUSSION

Yeast longevity studies have provided instructive models for understanding basic cellular processes in human aging (Bitterman et al., 2003). Among the conserved longevity pathways, acetyltransferase and deacetylases are well established as links between cell metabolism, growth, and aging processes (Chang and Min, 2002; Nakamura et al., 2010). Similar to their opposite effects on chromatin silencing (Rundlett et al., 1996; Zhou et al., 2009), mutations in deacetylases *RPD3* and *SIR2* showed disparities in extending replicative life span previously thought to be related to distinct histone tail targets (Chang and Min, 2002). However, although histone acetylation changes do occur in aging yeast cells, specific histone acetylation site mutants, such as those of H4K16 and H3K56, do not phenocopy the effects of the corresponding acetyltransferases and deacetylases on life span regulation (Dang et al., 2009; Ehrentraut et al., 2010). This suggests a role for critical nonhistone acetylation substrates in life span regulation (Close et al., 2010).

We report here an in-depth replicative life span analysis of a nonhistone substrate, Sip2, a β-regulatory subunit of yeast AMPK (Snf1 complex) that was previously identified in a large-scale proteomic screen of NuA4 acetyltransferase (Lin et al., 2009). Four functionally important acetylation sites were identified; one falls within the C-terminal half within a region of Sip2 that was previously cocrystallized in a heterotrimer with Snf1 and its γ subunit (Amodeo et al., 2007). The remaining three sites cluster in the N terminus in a region absent from the structure (and not phylogenetically conserved). Because mutation of these three lysines is sufficient to confer most of the interaction, life span, and other phenotypes observed in vivo, we suggest that Sip2 N-terminal acetylation might change Sip2 conformation, allowing binding to Snf1 and/or the γ subunit to stabilize the Sip2-Snf1 complex.

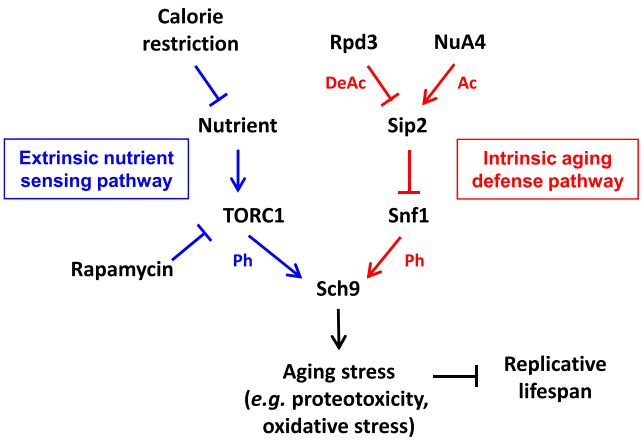

Figure 7. Model for the Effects of Sip2 Acetylation on Life Span Regulation

Red lines indicate the intrinsic aging defense pathway identified in this study. Blue lines indicate the extrinsic nutrient-sensing pathway regulating Sch9 activity. Not shown here for clarity is evidence of a connection between the two pathways in that we also observed a modest decrease in Sip2 acetylation in glucose limitation. Ac, lysine acetylation; DeAc, lysine deacetylation; Ph, phosphorylation of serine and threonine.

NuA4 acetyltransferase, along with the corresponding Sip2 deacetylase Rpd3, are directly involved in replicative life span regulation. Not only did Sip2 acetylation-mimic mutants live significantly longer, they nearly totally rescued the severely shortened life span of NuA4 catalytic mutants. This life span extension validates their importance as key substrates regulating aging. Furthermore, unacetylable Sip2 mutants, like a complete deletion, led to a shortened life span compared to WT, further supporting the critical role of Sip2 acetylation in Sip2 function. The fact that Sip2 (de)acetylation mimetics phenocopy the corresponding catalytic mutants of NuA4 and Rpd3 strongly supports the importance of Sip2 as the critical downstream nonhistone substrate of these enzymes in controlling replicative life span.

Sip2 is a regulatory subunit of the Snf1 complex that is homologous to the AMPK complex in higher organisms and plays a critical role in metabolism and other cellular processes in response to energy supply (Scott et al., 2009). As a key regulator of energy homeostasis controlled by AMP/ATP ratios and various upstream kinases (Lee et al., 2007; Sanz, 2008), we show here that Snf1 kinase activity can be independently controlled by acetylation of Sip2. These findings help interpret the fact that Snf1, required for transcription of glucose-repressed genes (Hedbacker and Carlson, 2008) and a key player in response to cellular stress (Sanz, 2003), exhibits increased activity with aging and detrimental effects on life span (Lin et al., 2003). Our results suggest that Sip2 acetylation enhances physical interaction with Snf1 and thereby antagonizes catalytic activity. An increase in the disaccharide trehalose is observed as a common response to glucose limitation and replicative aging, representing two distinct stressors in yeast (Shi et al., 2010; Wang et al., 2010). Decreased Sip2 acetylation observed in these distinct stress conditions helps explain increased Snf1 activity. The antagonistic effect of Sip2 acetylation on Snf1 activity explains how Sip2 suppresses the detrimental effects of Snf1 on replicative life span extension (Lin et al., 2003). Hydrogen peroxide (H_2O_2), yet another form of stress and aging mediator, accumulates as aging occurs (Giorgio et al., 2005; Migliaccio et al., 1999). We showed here that Sip2 acetylation was critical for H_2O_2 resistance, supporting the hypothesis that Sip2 acetylation protects yeast cells from oxidative stress.

Through in vitro and in vivo kinase assays and epistasis analyses, we confirmed that Sch9, the yeast homolog of mammalian Akt/S6K (Powers, 2007), is a critical downstream effector of both TORC1 (Kaeberlein et al., 2005) and the Snf1 complex. In addition to being boosted by sensing nutrient accessibility via TORC1, we observed that Sch9 can be independently phosphorylated and activated by the Snf1 kinase. This age-dependent regulation of Snf1 kinase activity is governed by progressive deacetylation of Sip2, which releases Snf1 from Sip2 inhibition in old cells. However, it is possible that this progression is only one dimension of this regulatory pathway and that there might be dynamic changes in Sip2 acetylation state in response to certain stresses in younger cells. The Snf1-mediated phosphorylation and activation of Sch9 associated with replicative aging processes might ultimately lead to dysregulated protein homeostasis, representing a type of "intrinsic aging." Intrinsic aging might represent exhaustion of a metabolite or regulatory factor or the unavoidable accumulation of damage (proteotoxicity, oxidative, metabolic, DNA damage, etc.) during aging (Cohen and Dillin, 2008; Haigis and Yankner, 2010; Silva and Conboy, 2008; Vijg and Campisi, 2008). This intrinsic aging is counteracted by a series of posttranslational modifications on several critical enzymes that works in parallel to an "extrinsic nutrient-sensing pathway" through common downstream machinery to coordinate vegetative growth and replicative life span in the yeast cells (Figure 7).

AMPK activation promotes longevity under calorie restriction in metazoans (Evans et al., 2011; Mair et al., 2011; Williams et al., 2009). The finding that AMPK in yeast seemingly plays an opposite role to that observed previously in metazoans is intriguing. However, AMPK activity has also been found to increase during senescence in various cellular models, including fibroblasts (Wang et al., 2003), skeletal muscle cells (Thomson et al., 2009), and aortic endothelial cells (Zu et al., 2010). Although transient activation of AMPK protects cells against various internal and external stresses (Narbonne and Roy, 2009), prolonged activation of AMPK is paradoxically correlated with irreversible senescence and has detrimental effects on normal physiological functions in mammalian cells (Jones et al., 2005; Mariño et al., 2008; Wang et al., 2011). Also, in a normal mouse brain, AMPK activity increased with age (Liu et al., 2011). These findings on senescent mammalian cells may be related to the "end-stage" phenotype observed in old yeast mother cells.

The acetylation-phosphorylation signaling cascade is largely independent of calorie restriction but could potentially integrate information of ambient nutrient availability and intracellular metabolic status through Sch9, a major downstream substrate of TORC1 kinase. Whether calorie restriction is the only means of life span extension remains a debated issue (Colman and Anderson, 2011). Previous reports suggest that calorie restriction

might lead to unwelcome health concerns, especially in elderly and nonobese subjects (Dirks and Leeuwenburgh, 2006). Thus modulators of the intrinsic aging might represent more attractive pharmacologic candidates than calorie restriction mimetics for life span extension. Although details might differ between yeast and metazoans, we note that a similar signaling cascade potentially exists in higher organisms because all of the major components are evolutionarily conserved.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

Yeast strains used in this study are derived from BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and listed in Table S2. Integrated site-directed mutagenesis was performed as previously described (Toulmay and Schneiter, 2006) and verified by sequencing. The primers used for site-directed mutagenesis and sequencing are listed in Table S3. Refer to the Extended Experimental Procedures for mass spectrometer, in vivo acetylation, enzyme purification, and in vitro deacetylation.

Replicative Life Span Determination and Statistical Analysis

Replicative life span was carried out as described (Kaeberlein et al., 1999). In brief, cells were freshly streaked to YPD (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) from glycerol stock and recovered at room temperature for 2 days and then lightly patched on a new YPD plate and grown overnight. An appropriate number of individual cells was randomly picked under a microscope and aligned in isolated areas with a micromanipulator. After about 2 hr of incubation at 30°C, virgin cells (newly budded cells) were separated and left at the original site, and the mother cells were removed to the grave yard. The life span of these cells was determined by counting the total number of daughter cells produced. The old mother cells were defined dead when the budding process stopped and the refractility was completely lost. For life span analyses in low glucose (LG), cells were grown on YEP plate (1% yeast extract, 2% peptone, 2% agar) containing 0.05% dextrose. For life span analyses in rapamycin, cells were grown on YPD containing 25 ng/ml rapamycin. The nonparametric Mantel-Cox log-rank test was performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA) to assess statistical significance of life span differences, as listed in Table S1.

Sorting Old Cells

Yeast old mother cells (generation 7, G7) were isolated from young cells (generation 0, G0) as described (Smeal et al., 1996). In brief, fresh cells were grown at 24°C in YPD (1% yeast extract, 2% peptone, and 2% dextrose) to an A_{600} of ~0.6, and then 10 A_{600} units of the cells was labeled with 7 mg freshly prepared biotin (EZ-link Sulfo-NHS-LC-LC; Pierce), cultured to 1 L YPD from A_{600} 0.01 to A_{600} 0.8 at 24°C in YPD, followed by affinity purification using Dynabeads MyOne and DynaMag-50 (Invitrogen) according to the manufacturer's suggestions. An aliquot of sorted cells was stained with 0.1 mg/ml Calcofluor White (Sigma-Aldrich, F3543) and observed using a Zeiss Axioskop fluorescent microscope equipped with a Cool Snap FX camera for bud scar counting.

Trehalose Assay

A trehalose assay was performed as previously described (Shi et al., 2010). In brief, 10 A_{600} units of cells grown in synthetic dropout media to exactly A_{600} 1.2 were pelleted and quickly washed with 1.2 ml ice-cold H_2O and then resuspended in 0.3 ml 0.25 M Na_2CO_3. The samples were boiled for 4 hr, and then 0.18 ml of 1 M acetic acid and 0.72 ml of 0.2 M sodium acetate was added to each sample. For controls, half (0.6 ml) of each sample was transferred to a microfuge tube, and the remaining half (0.6 ml) of the sample was incubated overnight with 0.025 U/ml trehalase (Sigma-Aldrich, T8778) at 37°C. Samples were then centrifuged at top speed for 3 min and assayed for glucose using a Glucose Assay Kit (Sigma-Aldrich, GAGO20).

H_2O_2 Stress Tolerance Test

The test was performed as previously described (Jamieson, 1992) with slight modification. Exponential phase cultures of WT, *sip2-4KR*, *sip2-4KQ*, and *sip2Δ* strains growing at 30°C in YPD (A_{600} 0.25, equals to 5.4×10^6 per ml) were divided into two aliquots (duplicated) and treated for 1 hr at 30°C with 1.5 mM H_2O_2. About 200 cells were placed on YPD plates before (0 min) and after (60 min) H_2O_2 treatment. Number of colonies formed was counted after overnight culture at 30°C. Fraction of survival was calculated by dividing the number of colonies formed after H_2O_2 treatment by the number before treatment, with the fraction of survival of each strain before treatment being set as 1. Statistical significance was assessed by two-way ANOVA plus post-hoc tests. Refer to Extended Experimental Procedures for cell size determination.

In Vitro Kinase Assay

Snf1-TAP in a *sip2Δ* strain was purified as described (Puig et al., 2001). Kinase reactions were done in kinase buffer (25 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM KCl, with or without 1 mM ATP) for 30 min at 30°C using ~1 μg substrate and ~100 ng Snf1-TAP. Reactions were stopped by boiling in 2× SDS sample buffer and then separated by SDS-PAGE. Phosphorylation signals were assessed by immunoblot blotting using phosphoserine antibody (QIAGEN, 37430) and phosphothreonine antibody (QIAGEN, 37420) following essentially the manufacturer's manual as described (Jablonowski et al., 2004). To verify that the antibodies are phosphorylation specific, GST-Sch9 purified from WT was treated with calf intestinal alkaline phosphatase (New England Biolabs, M0290S) following the manufacturer's protocol, with or without adding Na_3VO_4 (Sigma-Aldrich, S6508), a phosphatase inhibitor. Reactions were separated by SDS-PAGE and probed with phosphoserine and phosphothreonine antibodies as described above. In vitro kinase assay using GST-Sch9 mutated at four serine or three threonine phosphorylation sites previously identified as PDK and TORC1 targets (Urban et al., 2007) was performed to test whether these sites were Snf1 targets. Refer to Extended Experimental Procedures for in vivo Sch9 phosphorylation.

SUPPLEMENTAL INFORMATION

Supplemental information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2011.07.044.

ACKNOWLEDGMENTS

We thank Sheng-Ce Tao and Chien-Sheng Chen for their early contribution to this work. We are grateful to Dr. Brian K. Kennedy for providing *sch9Δ* strain, Dr. Robbie Loewith for providing *SCH9* phosphorylation mutants, and Dr. Marian B. Carlson for providing *snf1-K84R* construct. We thank Dr. Fang-Jen Lee for kindly sharing the laboratory space and reagents. We thank Department of Medical Science, National Taiwan University for technical help with sequencing. We thank Dr. Yi-Juang Chern for critical suggestions on this work. Work was supported by National Science Council (NSC 98-2314-B-002-031-MY3 to J.-Y.L.), National Taiwan University Hospital (099-001376, to J.-Y.L.), National Taiwan University (99C101-603 to J.-Y.L., Y.-Y.L., and L.-M.C.), Liver Disease Prevention & Treatment Research Foundation (Taiwan) (to J.-Y.L. and Y.-Y.L.), and NIH Common Fund grant (USA) (U54-RR020839 to H.Z. and J.D.B.).

Received: January 18, 2011
Revised: May 2, 2011
Accepted: July 29, 2011
Published online: September 8, 2011

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