Identification and characterization of rapidly accumulating \textit{sch9}Δ suppressor mutations in \textit{Saccharomyces cerevisiae}

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Abstract

Nutrient sensing is important for cell growth, aging, and longevity. In \textit{Saccharomyces cerevisiae}, Sch9, an AGC-family protein kinase, is a major nutrient sensing kinase homologous to mammalian Akt and S6 kinase. Sch9 integrates environmental cues with cell growth by functioning downstream of TORC1 and in parallel with the Ras/PKA pathway. Mutations in \textit{SCH9} lead to reduced cell growth in dextrose medium; however, reports on the ability of \textit{sch9}Δ mutants to utilize non-fermentable carbon sources are inconsistent. Here, we show that \textit{sch9}Δ mutant strains cannot grow on non-fermentable carbon sources and rapidly accumulate suppressor mutations, which reverse growth defects of \textit{sch9}Δ mutants. \textit{sch9}Δ induces gene expression of three transcription factors required for utilization of non-fermentable carbon sources, Cat8, Adr1, and Hap4, while \textit{sch9}Δ suppressor mutations, termed \textit{sns}1 and \textit{sns}2, strongly decrease the gene expression of those transcription factors. Despite the genetic suppression interactions, both \textit{sch9}Δ and \textit{sns}1 (or \textit{sns}2) homozygous mutants have severe defects in meiosis. By screening mutants defective in sporulation, we identified additional \textit{sch9}Δ suppressor mutants with mutations in \textit{GPB1}, \textit{GPB2}, and \textit{MCK1}. Using library complementation and genetic analysis, we identified \textit{SNS1} and \textit{SNS2} to be \textit{IRA2} and \textit{IRA1}, respectively. Furthermore, we discovered that lifespan extension in \textit{sch9}Δ mutants is dependent on \textit{IRA2} and that PKA inactivation greatly increases basal expression of \textit{CAT8}, \textit{ADR1}, and \textit{HAP4}. Our results demonstrate that \textit{sch9}Δ leads to complete loss of growth on non-fermentable carbon sources and mutations in \textit{MCK1} or genes encoding negative regulators of the Ras/PKA pathway reverse \textit{sch9}Δ mutant phenotypes.

Keywords: Sch9; Ira1/2; Mck1; Gpb1/2; respiratory metabolism

Introduction

In eukaryotic cells, physiological adaptations to changes in the internal and external environments are regulated by dynamic signal transduction networks that are generally conserved throughout evolution. For \textit{Saccharomyces cerevisiae}, nutrient availability is the major environmental factor driving cell growth and survival (reviewed in Schuller 2003; Zaman et al. 2008; Turcotte et al. 2010; Loewith and Hall 2011; Broach 2012). Yeast cells utilize interconnected signaling pathways and transcriptional regulatory networks to respond to the external supply of nutrients and adjust their growth and metabolism. Carbon and nitrogen nutrients in particular serve as both substrates to support cell growth and as signaling molecules to guide cellular processes necessary for survival. An important mediator of this coordinated response is the protein kinase Sch9, which functions in both glucose and nitrogen sensing pathways.

Sch9 is a member of the AGC family of serine/threonine protein kinases and is a homolog of mammalian Akt/Protein Kinase B and ribosomal protein (RP) S6 kinase (S6K). Several AGC-family protein kinases are phosphorylated and activated by the evolutionary conserved Target of Rapamycin (TOR) kinase, a major regulator of cell growth and metabolism in response to environmental signals (Wullschleger et al. 2006). Akt is directly phosphorylated in its hydrophobic motifs by mammalian TOR complex 2 (mTORC2) in response to hormones and other growth factors. Another well-characterized AGC-family kinase is S6K, which is phosphorylated in its hydrophobic motifs and activated by mTORC1 (Burnett et al. 1998) to stimulate ribosomal biogenesis (Ribi) and protein synthesis. In yeast, Sch9 was identified as a major downstream effector of TORC1 signaling and functional ortholog of S6K or PKB/Akt1 (Geyskens et al. 2001; Sobko 2006, Urban et al. 2007). Sch9 is directly phosphorylated in its C-terminal hydrophobic motifs and an adjacent site by TORC1 and in its activation loop by the yeast PDK1 orthologs Pkh1 and Pkh2. When nitrogen is abundant or preferred nitrogen sources are not limiting, TORC1 is activated and promotes mass accumulation and cell growth by inducing ribosome biosynthesis, which is achieved partly through the activation of Sch9 (reviewed in Loewith and Hall 2011). Sch9 positively regulates the expression of genes in Ribi and RP regulons by phosphorylating repressors Dot6, Tof6, and Sth3 and thereby inhibiting the recruitment of the RPD3L histone deacetylase complex to target gene promoters (Urban et al. 2007; Huber et al. 2009; Lee et al. 2009; Lippman and Broach 2009; Huber et al. 2011). Sch9 also positively regulates RNA polymerase III-dependent transcription of 5S ribosomal RNA and tRNA genes by directly phosphorylating and inhibiting Maf1, as well as RNA polymerase I-dependent expression of 35S pre-ribosomal RNA (Huber et al. 2009; Lee et al. 2009; Wei and Zheng 2009).
In addition to its functions in the TORC1-mediated nitrogen sensing branch of nutrient signaling, Sch9 may also play a role in the regulation of cell growth in response to the quality of carbon sources. Glucose-grown cells exhibit an increase in both protein level and phosphorylation of Sch9 (Jorgensen et al. 2004; Urban et al. 2007). There has been much evidence to suggest that Sch9 functions in a pathway parallel to cAMP-dependent protein kinase PKA and that the two kinases exhibit some functional redundancy (reviewed in Loewith and Hall 2011; Broach 2012). Sch9 was originally identified as a multicopy suppressor of growth defects associated with loss of Ras or PKA activity and, conversely, hyperactivation of PKA due to TPK1 overexpression, bcy1Δ, or expression of a constitutive activating mutant of RAS2, RAS2^{G17V}, suppresses the slow growth defects of sch9Δ strain (Toda et al. 1988; Prusty and Kell 2004). The activity of PKA is regulated by cAMP, whose synthesis depends on the activity of adenylate cyclase (reviewed in Thevelein and de Winde 1999; Broach 2012). PKA is a tetramer of two identical regulatory subunits encoded by Bcy1 and two catalytic subunits encoded by the three functionally redundant genes, TPK1, TPK2, and TPK3. cAMP activates PKA by binding to Bcy1, relieving its inhibition of the catalytic subunits. Activation of PKA is positively regulated by the G protein-coupled receptor Gpr1 and its associated Gα protein, Gap2 (Kraakaan et al. 1999; Lorenz et al. 2000). Adenylate cyclase activity is also mediated by Ras proteins Ras1 and Ras2, which are regulated positively by Cdc25, the guanine nucleotide exchange factor of Ras, and negatively by Ira1 and Ira2, the GTPase-activating proteins of Ras. Two other related proteins, Gpb1 and Gpb2, also negatively regulate the activity of the Ras/PKA signaling pathway (Harashima and Heitman 2002; Harashima et al. 2006; Budhwar et al. 2011). Gpb1 promotes ubiquitin-dependent proteolysis of Ira2 and Gpb2 inhibits Ras activity through direct interactions with Ira1 and Ira2 (Harashima et al. 2006; Phan et al. 2010). PKA stimulates cell growth in part by inhibiting transcription factors Msn2/4 and Gis1, which regulate the expression of stress-responsive element- and post-diauxic shift-driven genes, respectively, and Rim15, a protein kinase that induces progression into stationary phase and meiosis (reviewed in Roosen et al. 2005; Broach 2012). Despite the suggested functional redundancy with PKA, the role of Sch9 in glucose sensing is still unclear. On one hand, 90% of the glucose-induced changes in gene expression can be recapitulated by PKA activation or Sch9 overexpression in glycerol-grown cells. On the other hand, Sch9 inactivation does not interfere with the glucose response, while PKA inactivation does (Zaman et al. 2009). The common substrates of Sch9 and PKA include Dot6, Tod6, and Maf1, which are important factors for the regulation of ribosome synthesis and the expression of tRNA genes (Huber et al. 2009; Lee et al. 2009; Lippman and Broach 2009; Soulard et al. 2010; Huber et al. 2011). Sch9 and PKA have similar phosphorylation site motifs and share overlapping target substrates (Plank et al. 2020), which helps to explain their close relationship in mediating cell growth and survival in response to nutrient signals.

Consistent with its role as an important kinase sensing nutrient and stress conditions, Sch9 has been implicated in many cellular processes. Sch9 is involved in the regulation of stress-responsive genes (Pedruzzi et al. 2003; Roosen et al. 2005; Lavoie and Whiteway 2008). Inhibition of both Sch9 and PKA is known to induce autophagy (Yorimitsu et al. 2007). Sch9 negatively regulates the expression of genes in the nitrogen discrimination pathway (Smetts et al. 2008), which are controlled by several GATA transcription factors including Gin3 and Gat1 (Cooper 2002; Smetts et al. 2008). Interestingly, a gnt3 gat1 double mutation confers resistance to rapamycin treatment and partially suppresses the growth defects of sch9Δ mutant cells (Schmelzl et al. 2004; Urban et al. 2007). Sch9 and PKA converge on Rim15 to regulate transcription of genes involved in the stress response and during the post diauxic shift (Pedruzzi et al. 2003; Roosen et al. 2005). Sch9 regulates both chronological and replicative life span (Morano and Thiele 1999; Fabrizio et al. 2001, 2003; Kaebelerin et al. 2005), which is partly mediated by Rim15 (Fabrizio et al. 2001; Wei et al. 2008; Burtnet et al. 2009). Sch9 has also been shown to regulate cell size, osmotic stress response, sphingolipid metabolism, ceramide biosynthesis, V-ATPase assembly, and pH homeostasis (Jorgensen et al. 2002; Pascual-Abuir and Profi 2007; Swinnen et al. 2014; Wilms et al. 2017). Together, the evidence suggests that Sch9 participates in a dynamic signaling network to regulate cell growth and survival in response to nutrient and stress conditions.

Sch9 and its eukaryotic homologs are broadly implicated in integrating extracellular and intracellular signals to promote cell growth and survival. It is compelling to understand the functions and regulatory mechanisms of Sch9. There are currently discrepancies in the literature with regards to how sch9 mutations impact cells’ ability to utilize non-fermentable carbon sources. Sch9 mutations have been reported to cause a range of severe to no growth defects on non-fermentable carbon sources (Crauwels et al. 1997; Jorgensen et al. 2004; Roosen et al. 2005; Lavoie and Whiteway 2008; Zhang et al. 2011; Teixeira et al. 2014). There are also conflicting reports about the effects that sch9 mutations have on the expression of genes encoding mitochondrial proteins, the HAP4 gene, which encodes the regulatory subunit of the Hao2/3/4/5 transcription factor complex, and stress-responsive genes such as HSP12 and HSP26 (Fabrizio et al. 2003; Roosen et al. 2005; Lavoie and Whiteway 2008; Smets et al. 2008; Pan and Shadel 2009; Pan et al. 2011; Teixeira et al. 2014). These discrepancies could be due to fast accumulation of sch9Δ suppressor mutations, originally reported by Huber et al. (2009) who used analog-sensitive sch9 alleles instead of sch9Δ to minimize complications that may be caused by sch9Δ mutant strains.

Nevertheless, many published results were generated using sch9Δ mutant strains.

Here, we report the isolation and characterization of spontaneous and rapidly accumulating suppressor mutations that occur in slow-growing sch9Δ mutant cells. We found that sch9Δ cells are unable to grow on non-fermentable carbon sources in two commonly used strain backgrounds, W303-1A and W303-1B as well as BY4741 and BY4742. The suppressor mutations rescue the growth defects of sch9Δ mutants on both dextrose medium and non-fermentable carbon sources. We found that mutations in IRA1 and IRA2 account for all of the spontaneous, recessive sch9Δ suppressor mutations that were isolated. We further found that mutations in GBP1, GBP2, and MCK1 can suppress the growth defects of sch9Δ mutant cells. We also show that PKA negatively regulates the expression of CAT8, ADR1, and HAP4. Altogether, we have identified genes responsible for spontaneous sch9Δ suppressor mutations that may complicate the analysis of the cellular functions of Sch9.

Materials and methods
Strains, plasmids, growth media, growth conditions, and yeast transformation

Yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Yeast strains were grown at 30°C in YPD (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose),
| Strain | Genotype | Source | Application |
|--------|----------|--------|-------------|
| BY4741 | MATa his3Δ1 leu2Δ0 met17Δ0 ura3Δ0 | Lab stock | Figures 2B, 3, 4, 5, 6C, and 7 |
| BY4742 | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Lab stock | Figure 2A |
| W303-1A | MATα ura3-1 ade2-1 leu2-3,112 trp1-1 his3-11,15 | Lab stock | Figures 1 and 3D |
| W303-1B | MATα ade2-1, his3-11,15, ura3-1, leu2-3, 112, trp1-1, can1-100 | Lab stock | Figure 3D |
| PPY666 | BY4741 sch9Δ:kanMX4 sn1 | This study | Figures 2A and 3B |
| ZLY6669 | BY4741 sch9Δ:kanMX4 | This study | Figures 2B, 4D, 5B, and 5C |
| ZLY6660 | BY4741 sch9Δ:kanMX4 | This study | Figures 4C, 4E, 5C, and 8D |
| PPY730 | BY4741 sn1 | This study | Figures 2B and 3B |
| PPY733 | BY4741 sch9Δ:kanMX4 sn1 | This study | Figures 2B and 5D |
| PPY632 | BY4741 sch9Δ:kanMX4 | This study | Figures 3B, 4F, and 5E |
| PPY633 | BY4742 sch9Δ:kanMX4 | This study | For constructing PPY745 |
| PPY731 | BY4742 sn1 | This study | For constructing PPY737 |
| PPY734 | BY4742 sch9Δ:kanMX4 sn1 | This study | Figures 2C, 3, 4A, 5, and 8D |
| BY4743 | BY4741 × BY4742 | This study | Figures 2C, 3, 4A, 5, and 8D |
| PPY745 | PPY632 × PPY633 | This study | Figures 2C, 3, 4A, 5A, 5D, and 8D |
| PPY737 | PPY730 × PPY731 | This study | Figures 2C, 3, 4A, and 8D |
| PPY741 | PPY733 × PPY734 | This study | Figures 2C, 3, 4A, 5A, and 8D |
| PPY624 | W303-1B MATα sch9Δ:kanMX4 | This study | Figures 1 and 3D |
| PPY673 | W303-1A MATα sch9Δ:kanMX4 sn1 | This study | Figure 1 |
| PPY622 | W303-1A MATα sch9Δ:kanMX4 | This study | Figure 3D |
| ZLY6614 | BY4741 sn2 | This study | Figures 2B, 3C, and 4A |
| ZLY6619 | BY4741 sch9Δ:kanMX4 sn2 | This study | Figures 2, A, B, 3C, 4A, and 5D |
| ZLY6626 | BY4742 sch9Δ:kanMX4 sn2 | This study | Figures 4A and 5D |
| ZLY6581 | BY4742 sn2 | This study | Figure 4A |
| ZLY6519 | BY4741 iss1A:kanMX4 | This study | Figure 4B |
| ZLY6606 | BY4741 mck1Δ:kanMX4 | This study | Figure 4F–P |
| ZLY6609 | BY4741 ira2Δ:kanMX4 | This study | Figure 5, B and E |
| ZLY6629 | BY4741 get2A:kanMX4 | This study | Figure 4B |
| ZLY6634 | BY4742 vma6Δ:kanMX4 | This study | Figure 4B |
| ZLY6633 | BY4742 paf1Δ:kanMX4 | This study | Figure 4B |
| ZLY6637 | BY4741 atg15A:kanMX4 | This study | Figure 4B |
| ZLY6640 | MATα his3Δ1 leu2Δ0 ura3Δ0 met17Δ0 def1Δ:kanMX4 | This study | Figure 4B |
| ZLY6642 | BY4742 tps2A:kanMX4 | This study | Figure 4B |
| ZLY6644 | BY4742 dep1Δ:kanMX4 | This study | Figure 4B |
| ZLY6645 | BY4742 sem1Δ:kanMX4 | This study | Figure 4B |
| ZLY6647 | BY4741 ira1A:kanMX4 | This study | Figure 5E |
| ZLY6662 | BY4741 vac8Δ:kanMX4 | This study | Figure 4B |
| ZLY6665 | BY4742 erv14A:kanMX4 | This study | Figure 4B |
| ZLY6669 | BY4741 vma2Δ:kanMX4 | This study | Figure 4B |
| ZLY6670 | BY4741 gpb2Δ:kanMX4 | This study | Figures 4B, 4C, E, and F |
| ZLY6660 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met17Δ0 sch9A:kanMX4 ira2Δ:kanMX4 | This study | Figures 4, B, C, E, and F |
| ZLY6661 | BY4741 sch9Δ:kanMX4 ira2A:kanMX4 | This study | Figure 5, B and D |
| ZLY6601 | BY4742 ira2A:kanMX4 | This study | Figure 5B |
| ZLY6648 | BY4742 ira1A:kanMX4 | This study | Figure 5C |
| ZLY6651 | BY4742 sch9A:kanMX4 | This study | Figure 4C |
| ZLY6652 | BY4742 gpb2:kanMX4 | This study | Figure 4C |
| ZLY6659 | BY4741 mck1Δ:kanMX4 | This study | Figures 4, D and F |
| PPY754 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met17Δ0 sch9A:kanMX4 ira1A:kanMX4 | This study | Figure 5C |
| PPY755 | BY4742 sch9A:kanMX4 ira1A:kanMX4 | This study | Figures 5, D and E |
| PPY734 | BY4742 sch9A:kanMX4 sn1 | This study | Figure 5D |
| PPY777 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met17Δ0 sch9A:kanMX4 gpb1Δ:kanMX4 | This study | Figure 4F |
| PPY757 | BY4741 gpb1Δ:kanMX4 | This study | Figures 4, C, E, and F |
| ZLY6657 | BY4742 sch9A:kanMX4 gpb2Δ:kanMX4 | This study | Figures 4, C, E, and F |
| PPY763 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gpb1Δ:kanMX4 gpb2Δ:kanMX4 | This study | Figures 4, E and F |
| PPY767 | BY4742 sch9A:kanMX4 gpb1Δ:kanMX4 gpb2Δ:kanMX4 | This study | Figures 4, E and F |
| ZLY4313 | BY4741 yak1A:kanMX4 | This study | Figures 6A, B, and 7A |
| ZLY5023 | BY4741 yak1A:His3Δ tpk1Δ:kanMX4 tpk2Δ:kanMX4 tpk3Δ:kanMX4 | This study | Figure 6C and 7B |
| ZLY6715 | BY4741 bcy1A:kanMX4 | This study | Figure 6C |
| PPY776 | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pde1Δ:kanMX4 pde2Δ:kanMX4 | This study | Figure 7A |
| ZLY6578 | BY4743 ira2A/IRA2 | Yeast genome | Figure 5B |
| ZLY6656 | BY4743 ira1A/IRA1 | deletion project | Figure 5C |
| ZLY6707 | BY4741 sch9A:kanMX4 yak1A:kanMX4 | This study | Figure 7A |
| ZLY4368 | BY4742 pde2A:kanMX4 | This study | Figure 7B |
| PPY758 | BY4742 sch9A:kanMX4 pde2A:kanMX4 | This study | Figure 7B |

YPL [1% Bacto-yeast extract, 2% Bacto-peptone and 3.7% DL-lactic acid (w/w, 85%), adjusted to pH 5.3 using NaOH], YPEG (1% Bacto-yeast extract, 2% Bacto-peptone, 2% ethanol, and 3% glycerol), minimal dextrose medium (SD) (0.67% yeast nitrogen base and 2% glucose), YPR (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% raffinose), sporulation media (0.1% Bacto-yeast extract, 1% potassium acetate, and 0.02% raffinose), YNBcasD or YNBcasSD (0.67% yeast nitrogen base, 0.25% casamino acids, 2% or 5% dextrose), YNBcasR (0.67% yeast nitrogen base, 0.25% casamino acids, and 2% raffinose), or YNBcasL (0.67% yeast nitrogen base, 0.25% casamino acids, and 2% raffinose).
Yeast cell extract preparation, Ponceau S staining, and Western blotting

Total cellular proteins were prepared by treating yeast cells with a freshly made solution of 7.5% β-mercaptoethanol and 1.85 N NaOH and precipitated with trichloroacetic acid as described previously (Yaffe and Schatz 1984). Protein samples were resuspended in SDS-PAGE sample buffer with 100 mM dithiothreitol and boiled for three minutes before being separated by SDS-PAGE. Pre-stained protein ladder (broad range, 10–230 kDa, P7710S, New England Biolabs) was used in protein gels. Proteins were transferred to a nitrocellulose membrane for immunoblotting with monoclonal anti-HA antibody, clone 3F10. HRP-conjugated secondary antibody from Jackson ImmunoResearch Laboratories (West Grove, PA) was used to probe the primary antibody. Chemiluminescence images of Western blots were captured using the Bio-Rad ChemiDoc MP imaging system and processed using Bio-Rad Image Lab software. Protein bands on Western blots were quantified using the same software.

β-Galactosidase assays

Yeast strains were grown at 30° to mid-log phase for at least six generations to reach an optical density at 600 nm (OD₆₀₀) of 0.6–0.8. Cells were collected by centrifugation and β-galactosidase activity assays were conducted as described previously (Amberg et al. 2005). Assays were carried out in duplicate for each sample from three independent cultures. β-galactosidase activities were given as the mean ± SD, n = 3.

Chronological lifespan assay

Yeast cultures (two replicates per sample) were grown in YPR media with a starting OD₆₀₀ of 0.01 and allowed to grow until cells reached stationary phase (two days for wild type, sns1, and sch9A sns1 strains; four days for sch9A mutant strain), which was set to be day 0 for the lifespan assay. Cultures were removed from the flask every five days starting from day 0 and live cells were quantified by colony forming assay. For colony forming assay, 500 cells from each culture were plated on YPD plates and colony forming units (CFUs) were counted and averaged after 3 days’ growth at 30°. The percentage of viable cells relative to day 0 (100% survival) was calculated and plotted. The values are the mean ± SD of two independent experiments.

Sporulation efficiency assay

Diploid strains were grown on SD plate for 2 days and then transferred into 1 ml sporulation media with starting OD₆₀₀ of 0.3. After eight days of incubation with shaking at 220 rpm at 30°, total cells were counted for each independent strain and the percentage of cells that had formed dyads and tetrads was recorded. The data were generated from the results of three replicate samples per strain.

Data availability

The authors affirm that all data necessary to draw conclusions in this article are present within the texts, figures, and tables. Strains, plasmids, and other noncommercial research reagents are available upon request.

Results

sns1 and sns2 mutations reverse the growth defects of sch9Δ mutants on both dextrose and non-fermentable carbon sources

sch9Δ mutations have been reported to cause a range of severe to no growth defects in cells grown on non-fermentable carbon sources. To understand the discrepancies reported in the literature, we generated a heterozygous sch9Δ mutation in the W303 strain background and obtained sch9Δ mutants via tetrad analysis. Figure 1A shows that each tetrad yielded two big wild type and two small sch9Δ mutant colonies on dextrose medium, consistent with published results (Toda et al. 1988; Lorenz et al. 2000). W303 background strains have an ade2 mutation, leading to a red colony phenotype on solid dextrose medium. The smaller sch9Δ mutant colonies were white, suggesting sch9Δ may lead to defects in respiratory metabolism. When sch9Δ mutant cells were streaked onto a dextrose medium plate, fast-growing red colonies appeared among the small white sch9Δ mutant colonies (Figure 1B). These large red sch9Δ mutant colonies were suspected to contain suppressor mutations. Upon further propagation on plates, the number of fast-growing suppressor mutant colonies quickly surpassed that of slow-growing sch9Δ mutant colonies. To quantify the growth phenotypes of sch9Δ mutants and the suppressor mutants, we performed a serial dilution growth assay of a wild type strain, an sch9Δ single mutant, and an sch9Δ sns1 (sch9 suppressor) double mutant on both a fermentable carbon source, dextrose, and non-fermentable carbon sources, lactate, and ethanol/glycerol. Figure 1C shows sch9Δ mutants have growth defects on dextrose and show no growth on the lactate and ethanol/glycerol plates except for putative

| TABLE 2 Plasmids used in this study |
|----------------------------------|
| **Plasmid** | **Description** | **Reference** | **Application** |
| pDC115 | pHA4-lacZ, expressing lacZ under the control of a 1.8kbp HAP4 promoter. | This study | Figures 3, A–D, 4, B, F, 5E, and 6 |
| pZL2140 | pCAT8-lacZ, expressing lacZ under the control of an 866 bp CAT8 promoter. | This study | Figures 3, A–D, and 6 |
| pZL2108 | pADR1-lacZ, expressing lacZ under the control of a 1249 bp ADR1 promoter. | This study | Figures 3, A–D, and 6 |
| pZL3929 | pRS416-CAT8-HA, encoding Cat8 with a C-terminal 3xHA tag under the control of the endogenous promoter. | This study | Figure 3, F and G |
| pZL1324 | pRS416-HAP4-HA, encoding Hap4 with a C-terminal 3xHA tag under the control of the endogenous promoter. | This study | Figure 3, F and G |
| pZL2416 | YCp50 | Lab. stock | Figure 5A |
| pPP322 | YCp50-lib., a sns1-complementing library plasmid encoding IRA2 and REX4. | This study | Figure 5A |
suppressor mutant colonies. However, the sns1 mutation partially suppresses the growth defects of sch9A mutant cells on both dextrose and non-fermentable carbon sources. Our data indicate that the growth defects of sch9A mutant cells from the W303 background can be reversed by a high frequency of spontaneous suppressor mutations.

BY4741 and closely related strains are derived from the S288c background, which were used in the yeast genome deletion project and are commonly utilized in yeast research labs. We wanted to determine whether sch9A suppressor mutations would also be easily and spontaneously obtained in this strain background. We generated sch9A mutants in S288c background strains by direct transformation with an sch9A::kanMX4 disruption cassette and tetrad analysis. Similar results were obtained: sch9A results in a slow growth phenotype on dextrose medium and spontaneous suppressor mutations arise at a high frequency. A high frequency of sch9A suppressor mutations could skew some of the reported phenotypes of sch9A mutants and mask the cellular functions of Sch9. As such, it was difficult for us to maintain sch9A mutant cells free of suppressor mutants in both W303 and S288c background strains. Therefore, it is important to know the identity of genes whose spontaneous mutation suppresses sch9A.

To that end, we isolated 21 sch9A suppressor mutants from both W303 and S288c backgrounds. These sch9A sns double mutants were crossed to sch9A mutant cells and the resultant diploid strains were analyzed for growth defects on both dextrose and glycerol medium. Based on their growth phenotypes, three of the 21 mutant strains were found to carry dominant sch9A suppressor mutations and were not analyzed further. We conducted a complementation group analysis on the remaining 18 mutant strains carrying recessive sch9A suppressor mutations. Accordingly, sch9A suppressor mutant strains of opposite mating types were crossed to generate diploids and analyzed for their ability to grow on glycerol plates. All resultant diploid strains were homozygous for sch9A and would display a sch9A suppressor mutant phenotype if the two haploid strains carried mutations in the same gene. From our analysis, we placed the 18 recessive sch9A suppressor mutations into two complementation groups, which were designated sns1 and sns2.

To further characterize the sch9A suppressor mutations, we performed tetrad analysis on wild type × sch9A sns1 and wild type × sch9A sns2 diploid strains (Figure 2A). As in the W303 background strains, sch9A mutant colonies from the S288c background were slow-growing on dextrose medium. sns1 and sns2 single mutant colonies on the dissection plate exhibited a slight growth defect compared with the wild type and had a rough edge or “papillae” phenotype. In the sch9A sns1 and sch9A sns2 double mutant colonies, sns1 and sns2 suppressed the slow growth of sch9A and yielded colonies of a similar size to wild type. Interestingly, the double mutant colonies appeared to have a relatively smooth edge like wild type, suggesting that sch9A suppresses the papillae phenotype of sns1 and sns2 mutants.

We performed serial dilution growth assays to quantify the suppression of sch9A by sns1 and sns2 (Figure 2B). On both dextrose medium and non-fermentable carbon sources, lactate and ethanol/glycerol, sns1 and sns2 single mutants grew like wild type. On dextrose medium, both sns1 and sns2 mutations fully suppressed the slow growth phenotype of sch9A mutant cells. On non-fermentable carbon sources, sch9A mutant cells did not exhibit any growth, except for a few presumed suppressor mutant colonies, while the sns1 and sns2 mutations completely reversed the growth defect of sch9A mutant cells. It often took several trials to get serial dilution growth assay results from sch9A mutant strains with no or a small number of suppressor mutants. Since most of the suppressor mutants carried recessive mutations, we decided to test the growth phenotype in diploid strains with the expectation that spontaneous suppressor mutants would be less likely to arise in the diploid setting. To that end, we generated an sch9A × sch9A homozygous diploid strain and found they showed a lower frequency of suppressors. sch9A suppression by sns1 was also observed in serial dilutions of the diploid strains (Figure 2C). When cells were left on dextrose medium for seven days, wild type colonies developed a tan color while sch9A mutant cells were white. Yeast cells without mitochondrial DNA, also known as rho0 petites, are unable to grow on non-fermentable carbon sources and form completely white colonies on plate. The white colony phenotype of sch9A mutant cells is consistent with their inability to grow on non-fermentable carbon sources. DAPI staining of sch9A cells showed that mitochondrial DNA maintenance did not seem to be affected. Furthermore, diploid cells formed between the crossing of sch9A mutant cells and a rho0 strain without mitochondrial DNA were able to grow on non-fermentable carbon sources, indicating that sch9A mutant cells have functional mitochondrial DNA and thus are not mitochondrial petites. Since the identities of SNS1 and SNS2 were not known at this stage of our study, we looked for other unique phenotypes of sns1 and sns2 mutants. Interestingly, washing of the plate surface with water after seven days of cell growth on dextrose medium showed that the sns1/sns1 and sch9A/sch9A sns1/sns1 diploid strains displayed increased agar adhesion (Figure 2C). The same phenotype was also observed for haploid sns1/sns2, sch9A/sns1, and sch9A sns2 mutant strains. Together, these results indicate that the growth defects of sch9A mutants on both dextrose and non-fermentable carbon sources are reversed by spontaneously
arising sns1 and sns2 mutations and that sns1 and sns2 mutants exhibit similar phenotypes.

**sch9Δ and sns1 mutations have opposing effects on the expression of genes encoding transcription factors Cat8, Adr1, and Hap4**

Utilization of non-fermentable carbon sources requires transcriptional activators that regulate the expression of genes involved in both respiratory metabolism and gluconeogenesis. Cat8 and Adr1 are involved in the derepression of genes required for growth on non-fermentative carbon sources, while the Hap2/3/4/5 complex is a master regulator of mitochondrial biogenesis and respiratory metabolism (reviewed in Turcotte et al. 2010). It has been reported that Sch9 is a positive regulator of ADH2 expression, which requires Adr1 (Denis and Audino 1991). Reports on the effects of sns1 mutations on HAP4 expression were inconsistent (Roosen et al. 2005; Lavoie and Whiteway 2008). Discrepancy on the growth phenotype of sch9Δ mutant cells on non-fermentable carbon sources in the published studies and ours prompted us to examine the role of Sch9 in the regulation of Hap4, Cat8, and Adr1.

Accordingly, we generated lacZ reporter genes under the control of the CAT8, ADR1, and HAP4 promoters and examined their expression in wild type and sch9Δ mutant cells grown in dextrose as well as in raffinose medium. Raffinose was chosen because it allows sch9Δ mutant cells to grow and is a glucose-limiting carbon source, which is expected to lead to similar changes in gene expression to those in cells grown in non-fermentable carbon source media. Studies on the regulation of the retrograde signaling pathway in which rho0 cells without mitochondrial DNA were grown in raffinose medium to study gene expression under glucose derepression conditions have been successful (Liu and Butow 1999). The expression of HAP4, ADR1, and CAT8 are subject to glucose repression (Forsburg and Guarente 1989; Hedges et al. 1995; Dombek and Young 1997; Randez-Gil et al. 1997).

Consistently, Figure 3A shows that all three reporter genes exhibited higher activity in raffinose-grown wild type cells compared with dextrose. In S288c, background strains grown in dextrose and raffinose media, both the haploid and homozygous diploid sch9Δ mutant strains showed an increase in all three reporter genes compared with the wild type haploid and diploid strains,
Figure 3 sch9A and sns1 mutations have opposing effects on the expression of CAT8, ADR1, and HAP4. (A) β-galactosidase activities of CAT8-lacZ, ADR1-lacZ, and HAP4-lacZ reporter genes in the BY4741 wild type strain grown in YNBcas5D (Dextrose) and YNBcasR (Raffinose) medium. β-galactosidase activity assays were carried out as described in Materials and Methods. (B) β-galactosidase activities of CAT8-lacZ, ADR1-lacZ, and HAP4-lacZ reporter genes in haploid wild type (WT, BY4741), sch9A (PPY632), sns1 (PPY730), and sch9A sns1 (PPY566) mutant strains and corresponding diploid strains (WT × WT, PPY745, PPY737, PPY741) grown in YNBcas5D and YNBcasR medium. The reporter gene activity in wild type haploid and diploid strains grown in dextrose and raffinose medium was set as 1, respectively. (C) β-galactosidase activities of CAT8-lacZ, ADR1-lacZ, and HAP4-lacZ reporter genes in haploid wild type (WT, BY4741), sns2 (ZLY6614), and sch9A sns2 (ZLY6619) mutant strains grown in YNBcas5D and YNBcasR medium. (D) β-galactosidase activities of CAT8-lacZ, ADR1-lacZ, and HAP4-lacZ in the W303 background strains. Wild type haploid (W303-1B), sch9A mutant haploid (PPY622), wild type diploid (W303-1A × W303-1B), and sch9A homozygous mutant diploid cells (PPY622 × PPY624) were grown in YNBcas5D or YNBcasR. (E and F) Western blots of Cat8-HA and Hap4-HA in wild type and mutant diploid strains as indicated. Wild type (BY4741 × BY4742), sch9A × sch9A (PPY745), sns1 × sns1 (PPY737), and sch9A sns1 × sch9A sns1 (PPY741) mutant strains carrying a plasmid encoding CAT8-HA (pZL3929) (panel E) or HAP4-HA (pZL1324) (panel F) were grown in YNBcas5D and YNBcasR medium. The left and right panels of Cat8-HA were from the same membrane, but the Western blot from dextrose-grown cells required a longer exposure time due to weak signals. The arrowhead indicates the position of Cat8-HA bands. Ponceau S staining was used as the loading control. (G and H) Quantification of the ratios of Cat8-HA (panel G) and Hap4-HA (panel H) protein amount to the loading control by Ponceau S staining. The ratio of HA-tagged protein/loading control was set as 1 in wild type cells. The data are presented as the mean ± standard deviation, n = 2.
respectively (Figure 3B). The sns1 mutation had an opposite effect on the expression of CAT8, ADR1, and HAP4 and exhibited a significant decrease in β-galactosidase activity compared with the wild type. sch9A sns1 double mutant strains, like sns1 single mutants, also showed reduced expression of these three reporter genes compared with the wild type in both dextrose- and raffinose-grown cells (Figure 3B). The effect of sns2 on the expression of these three reporter genes was similar to that of sns1 (Figure 3C). To address whether the difference in reporter gene activity was strain dependent, we also performed β-galactosidase assays on these three reporter genes in wild type and isogenic sch9A strains in the W303 background and found that sch9A increased the expression of the three reporter genes in cells grown in both dextrose and raffinose media (Figure 3D). In this strain background in dextrose media, sch9A leads to an even more significant 3.6 to 4.8-fold increase in CAT8, ADR1, and HAP4 expression in the haploid strains and a 2.1- to 3.6-fold increase in the diploid strains. In raffinose-grown W303 background strains, an increase in reporter gene activity due to sch9A was comparable with what was observed for the S288c background strains.

To address whether changes in gene expression are reflected in changes in the protein level, plasmids encoding functional C-terminal HA-tagged Hap4 and Cat8 were generated and transformed into wild type, sch9A, sns1, and sch9A sns1 diploid strains of the S288c background and HA-tagged proteins were analyzed by Western blotting. Figure 3, E and G shows that sch9A increased the expression of Cat8-HA in both dextrose- and raffinose-grown cells and that sns1 and sch9A sns1 mutants exhibited reduced expression of Cat8-HA compared with wild type. sch9A and sns1 increased and decreased the protein level of Hap4-HA in dextrose-grown cells, respectively (Figure 3, F and H). Their effect on Hap4-HA expression is largely absent in raffinose-grown cells, which is somewhat consistent with the smaller effect of sch9A and sns1 on HAP4-lacZ expression compared with their effect on CAT8-lacZ expression in diploid cells (Figure 3B, right panel). It is possible that Hap4-HA protein expression was subject to a saturation effect due to its much higher expression level in raffinose-grown cells than CAT8 (Figure 3A).

**Mutations in MCK1 and GPB1/GPB2 partially suppress sch9A**

Since the identities of SNS1 and SNS2 were thus far unknown, we looked for sns1 and sns2 mutant phenotypes that might be useful in cloning SNS1 and SNS2. sns1 and sns2 mutants displayed other phenotypes, including the formation of papillae and tanner-colored colonies, increased adhesive growth, prominent vacuolar structures, and larger cell size (Figure 2, A and C, and data not shown). Phenotypes that are helpful in library complementation such as growth defects at high or low temperatures, or sensitivity to drugs such as caffeine, were not found for sns1 mutants.

During our genetic analysis of sch9A mutants, we found that an sch9A homozygous diploid mutant showed reduced tetrad formation by 7.7-fold compared with wild type (Figure 4A). Since sns1 and sns2 mutations reverse the growth defects of sch9A mutant cells, we asked whether they would also suppress sporation defects. Instead, however, the introduction of sns1 and sns2 mutations further reduced efficiency of tetrad formation, suggesting that mutations in SNS1 and SNS2 may have their own sporation defects. Indeed, both sns1 and sns2 homozygous mutant strains showed almost no tetrad formation. Two large-scale studies have been conducted to identify genes required for sporation (Deutschbauer et al. 2002; Enyenihi and Saunders 2003). We decided to use the sporation phenotype to identify SNS1 and SNS2. Accordingly, we chose 85 genes from the study by Enyenihi and Saunders (2003) and seven genes that showed both a genetic or physical interaction with SCH9 and sporation defects when mutated (curated data from the Saccharomyces genome database). Since HAP4-lacZ expression was greatly reduced in both sns1 and sns2 single mutant cells, we hypothesized that HAP4-lacZ reporter gene expression could be used to narrow down the list of 92 genes by looking for genes that showed reduced expression of HAP4-lacZ when mutated. Accordingly, we transformed a HAP4-lacZ reporter gene into these 92 haploid mutant strains obtained from the yeast genome deletion project and the resulting transformants were analyzed for β-galactosidase activity. We found mutations in ATG15, DEF1, DEF1, ERV14, GBP2, GET2, ISC1, MCK1, PAF1, SEM1, TPS2, VAC8, VMA2, and VMA6 reduced HAP4-lacZ expression by at least threefold. We then generated these 14 mutant strains via tetrad analysis from respective heterozygous mutant strains obtained from the yeast genome deletion project. Re-examination of HAP4-lacZ expression confirmed that HAP4-lacZ expression was reduced by at least twofold in 10 of the 14 mutants (Figure 4B). We crossed each of the 14 mutant strains with an sch9A mutant and obtained respective double mutant strains via tetrad analysis. Analysis of colony size on the dissection plates revealed that mutations in MCK1 and GBP2 partially suppressed the growth defects due to sch9A, which was confirmed by serial dilution growth assays (Figure 4, C, D, and E). Interestingly, mutations in MCK1 and GBP2 also caused the largest decreases in HAP4-lacZ expression among the 14 mutants (Figure 4B). A large decrease in HAP4-lacZ expression was observed in sns1 and sns2 mutant cells (Figure 3, B and C). Together, our data suggest that reduced HAP4 expression correlates with sch9A suppression.

Mck1 is a protein kinase of the glycogen synthase kinase-3 family, which has three other members in budding yeast, Msds1, Ygk3, and Mrk1 (Bianchi et al. 1993; Puzis et al. 1994; Hardy et al. 1995; Hirata et al. 2003). Mck1 and Ygk3 are paralogs, sharing 43% sequence identity and 64% sequence similarity. We generated an sch9A ygk3A double mutant via tetrad analysis and found that ygk3A did not suppress the growth defects of sch9A. We then generated sch9A mck1A ygk3A triple mutant strains and compared their growth phenotype with sch9A mck1A double mutants. We found their growth was indistinguishable. These results suggest that sch9A suppression by mck1A is not shared by ygk3A.

Gpb2 is a negative regulator of the Ras/PKA signaling pathway and has a paralog Gpb1 (Harashima and Heitman 2002; Broach 2012). We crossed a gpb1A mutant with an sch9A mutant and obtained sch9A gpb1A double mutant strains via tetrad analysis. We found gpb1A marginally suppressed the growth defect of sch9A mutant cells (Figure 4C, lower left panel). Nevertheless, tetrad analysis revealed the growth defects of sch9A mutant cells on dextrose medium are almost completely suppressed by a gpb1A/Δ double mutation (Figure 4C, lower right panel). We performed a serial dilution growth assay on sch9A gpb1A double mutant strains and found they accumulated suppressor mutations to a greater extent than sch9A mutant cells. This prevented us from drawing a definitive conclusion on whether there was weak suppression of sch9A by gpb1A. However, Figure 4E shows that a gpb1/Δ double mutation suppresses the growth defects of sch9A mutants better than a gpb2A single mutation on both dextrose medium and non-fermentable carbon sources. Together, these results indicate that GBP1 and GBP2 play a redundant role in inhibiting cell growth in sch9A mutant cells, with GBP2 playing a bigger role than GBP1.
in dextrose medium. We found that increased expression of HAP4-lacZ due to sch9Δ is not affected by mck1Δ, is partially reduced by gpb2Δ, and is almost completely reversed by a gpb1/2Δ double mutation (Figure 4F). gpb1/2Δ single and gpb2Δ single mutations reduced HAP4-lacZ expression by 2.5-fold and fourfold, respectively, while a gpb1/2Δ double mutation reduced HAP4-lacZ expression by 5.3-fold. The large error bar of HAP4-lacZ activity in sch9Δ gpb1/2Δ likely reflects fast accumulation of other suppressor mutations, which could reduce HAP4-lacZ expression as observed in sch9Δ sns1 and sch9Δ sns2 mutant cells. Taken together, there appears to be a correlation between suppression of sch9Δ mutant growth defects and the extent of reduction in HAP4-lacZ expression due to gpb1Δ, mck1Δ, gpb2Δ, gpb1/2Δ, sns1, and sns2 mutations. mck1Δ, gpb1Δ, gpb2Δ, and gpb1/2Δ mutant colonies did not have a clear papillae phenotype like sns1 and sns2 mutants. Therefore, SNS1 and SNS2 are unlikely to be MCK1, GPB1, or GPB2.

**IRA1 and IRA2 play a largely non-redundant role in sch9Δ suppression**

Alongside our genetic screening of mutant genes that lead to sporulation defects, we sought to clone the SNS1 gene by direct complementation using a yeast genomic DNA library. Our first attempt was to use the color phenotype of W303 background strains, as sch9Δ cells with an ade2 mutation lead to small white colonies, while sch9Δ sns1 double mutant cells form red colonies (Figure 1). We transformed sch9Δ sns1 double mutant cells with a yeast genomic DNA library and looked for transformants that were white, which would indicate complementation of sns1. We conducted several rounds of transformation and recovered many white colonies, none of which contained a sns1 complementing plasmid. We hypothesized that spontaneous mutations might complicate library plasmid complementation of sch9Δ in haploid strains. Accordingly, we used sch9Δ/sch9Δ sns1/sns1 diploid mutant cells in the S288c background and transformed them with a yeast genomic DNA library. In this strain background, we expected that sch9Δ sns1 transformants containing a complementing SNS1 plasmid would display sch9Δ mutant phenotypes of slow growth on dextrose medium and no growth on non-fermentable carbon sources. Of the approximate 12,000 transformants on dextrose medium, dozens of colonies that were slow-growing were chosen for further analysis. Transformant colonies were then examined for severe growth defects on glycerol medium. We found one transformant that showed plasmid-dependent growth defects. We recovered the plasmid from the
transformant, re-transformed it into the sch9Δ/sch9Δ sns1/sns1 mutant cells, and found that the library plasmid could complement the sns1 mutation (Figure 5A). Sequencing of the sns1-complementing plasmid found that it carried a ~15kb chromosomal DNA fragment, which included complete coding sequences of IRA2 and REX4, and partial coding sequences of ATG19 and AVO1.

ira2 mutant cells have been reported to exhibit increased adverse growth and sporulation defects (Tanaka et al. 1999b; Barrett et al. 2012). Our data showed that sns1 mutants also had sporulation defects and increased agar adhesion (Figures 2C and 4A), suggesting that IRA2, not REX4, complements the sns1 mutation. During dissection of tetrads from sporulated IRA2/ira2 cells, we observed that ira2Δ mutant colonies grew slower than wild type and formed papillae, of tetrads from sporulated IRA2/ira2 cells, we observed that ira2Δ mutant colonies grew slower than wild type and formed papillae, rough-edged colonies (Figure 5B, left panel), similar to sns1 colonies (Figure 2A, left panel), suggesting IRA2 is sns1. We then crossed an ira2Δ mutant with an sch9Δ mutant and obtained sch9Δ ira2Δ double mutant strains via tetrad analysis (Figure 5B, middle panel). A serial dilution growth assay showed that ira2Δ completely suppressed the growth defects of sch9Δ mutant cells on both dextrose and non-fermentable carbon sources (Figure 5B, right panel). IRA2 has a paralog, IRA1. We conducted similar genetic analyses and found that the phenotypes of ira1Δ mutants are similar to those of ira2Δ mutants and that ira1Δ also completely suppressed the growth defects of sch9Δ mutant cells (Figure 5C).

We next sought to determine whether IRA1 is sns2. We crossed mutant strains among sch9Δ sns1, sch9Δ sns2, sch9Δ ira1Δ, and sch9Δ ira2Δ mutants and analyzed cell growth of the resultant diploid strains via a serial dilution growth assay. Figure 5D shows that sch9Δ ira1Δ × sch9Δ sns2 and sch9Δ ira2Δ × sch9Δ sns1 diploid strains exhibited similar growth to wild type on both dextrose and lactate medium, suggesting that IRA1 is sns2 and IRA2 is sns1. sch9Δ ira1Δ × sch9Δ ira2Δ, sch9Δ ira1Δ × sch9Δ sns1, and sch9Δ ira2Δ × sch9Δ sns2 diploid strains grew slightly better than sch9Δ/sch9Δ homozygous mutant, but still exhibited clear growth defects. This result is somewhat surprising. Ira1 and Ira2 are homologous proteins and play a partially redundant role in inhibiting the activity of Ras1 and Ras2. On the one hand, loss of both copies of IRA1 or IRA2 can restore growth of sch9Δ/sch9Δ mutant cells to wild type level. On the other hand, loss of one copy each of IRA1 and IRA2 only partially suppresses the growth defects of sch9Δ/sch9Δ mutant cells. This result suggests that IRA1 and IRA2 play partially redundant but distinct roles in regulating cell growth of sch9Δ/sch9Δ mutant cells. It has been reported previously that IRA1 and IRA2 have similar but not the same functions (Tanaka et al. 1999b). Our results resemble those of ira1 and ira2 mutations in suppressing the slow growth phenotype of reg1 mutant cells (Barrett et al. 2012). fst1 and fst2 are two of the complementation groups of mutants that suppress the reg1 mutation. fst1 does not complement ira1Δ, and fst2 does not complement ira2Δ for reduced glycogen accumulation and increased agar adhesion. However, fst1 complements fst2 in the said phenotypes. The authors concluded that fst1 is IRA1 and fst2 is IRA2.

We also analyzed the expression of the HAP4-lacZ reporter gene in ira1Δ, ira2Δ, sch9Δ ira1Δ, and sch9Δ ira2Δ mutant cells grown in dextrose medium (Figure 5E). We found that HAP4-lacZ expression was greatly reduced in these mutants, to the same extent as what was observed in sns1, sns2, sch9Δ sns1, and sch9Δ sns2 mutant cells. Taken together, our data suggest that IRA1 and IRA2 play a largely non-redundant role in inhibiting cell growth in sch9Δ mutant cells. To confirm SNS1 is IRA2, we sequenced the IRA2 gene from a sns1 mutant and found a frameshift mutation in the codon for serine residue 1870 (Figure 5F). The deletion of the nucleotide C from codon TCT to TT leads to a stop codon almost right after the mutation. Ira2 is a GTPase-activating protein for Ras1 and Ras2, with its Ras GTPase-activating domain being comprised of amino acid residues 1651–1989. This mutation in IRA2 truncates this domain and helps to explain that sns1 acts like a total-loss-of-function allele of IRA2 (Figures 2, 3, and 5).

Mutations in genes encoding components of the PKA signaling pathway affect the expression of lacZ reporter genes under the control of ADR1, CAT8, and/or HAP4 promoters

Ira1 and Ira2 are GTPase-activating proteins of Ras1 and Ras2 and function as negative regulators of the Ras/PKA signaling pathway (Tanaka et al. 1999a, 1991). Gpb1 and Gpb2 interact with Ira1/2 and indirectly inhibit Ras1/2 by promoting the stability of Ira1/2 (Harashima et al. 2006). Our data show a clear correlation between the degree of suppression of sch9Δ mutant growth defects and reduced expression of HAP4-lacZ reporter genes caused by mutations in Gpb1, Gpb2, IRA1, IRA2, and MCK1. Activation of PKA signaling is known to suppress the growth defects of sch9Δ mutant cells (Toda et al. 1988). These results raise the possibility that PKA may inhibit the expression of HAP4, CAT8, and ADR1. The effect of sns1 and sns2 mutations on ADR1 expression is consistent with the reported finding that the expression of an ADR1-lacZ reporter gene in dextrose-grown cells is reduced by 9.9-fold due to bcy1Δ, which constitutively activates PKA (Dombek and Young 1997).

We decided to determine the effect of a PKA mutation on the expression of lacZ reporter genes under the control of these three gene promoters. PKA has three functionally redundant catalytic subunits, Tpk1, Tpk2, and Tpk3 (Toda et al. 1987a). A triple deletion in TFK1/2/3 is lethal, which can be suppressed by a yak1Δ mutation (Garrett and Broach 1989). We generated a tpk1/2/3Δ yak1Δ quadruple mutant and conducted a β-galactosidase assay in cells grown in dextrose as well as raffinose medium. A yak1Δ mutant strain was included as the control and was found to have little to no effect on the expression of these three lacZ reporter genes. In dextrose medium, tpk1/2/3Δ had a dramatic effect on the expression of the CAT8, ADR1, and HAP4 reporter genes and increased their expression by 54-, 6.5-, and 28-fold, respectively (Figure 6). In raffinose medium, tpk1/2/3Δ increased HAP4-lacZ expression by 3.6-fold, while it had little effect on the expression of CAT8/lacZ and ADR1-lacZ reporter genes. In contrast, constitutive activation of PKA activity due to a bcy1Δ mutation or a pde1Δ pde2Δ double mutation resulted in reduced expression of the HAP4-lacZ reporter gene in both dextrose and raffinose-grown cells. bcy1Δ and pde1Δ pde2Δ mutant cells are known to have growth defects on non-fermentable carbon sources (Nikawa et al. 1987; Toda et al. 1987b), which may be explained by a stronger reduction in HAP4 expression, and possibly CAT8 and ADR1 well, than what is observed in ira1 and ira2 single mutant cells. Together, these data indicate that PKA is a strong negative regulator of the expression of ADR1, CAT8, and HAP4 under glucose repression conditions.

Mutations in YAK1 and PDE2 partially suppress the growth defects of sch9Δ mutant cells

IRA1, IRA2, GPB1, and GFB2 are negative regulators of Ras1 and Ras2. Mutations in RAS2 lead to growth defects on nonfermentable carbon sources (Tatchell et al. 1985; Fasano et al. 1988). It has been suggested that Ras2 can regulate mitochondrial biogenesis and function via both PKA-dependent and independent mechanisms (Hlavata et al. 2003; Hlavata and Nystrom 2003). We sought
Figure 5 SNS1 and SNS2 are IRA2 and IRA1, respectively. (A) A sns1 mutation is complemented by a genomic DNA library plasmid. Wild type (BY4741 × BY4742), sch9Δ × sch9Δ (PPY745), and sch9Δ sns1 × sch9Δ sns1 (PPY741) mutant strains were transformed with control vector YCp50 or the sns1-complementing library plasmid, YCp50-lib. Transformants were serially diluted and spotted on YNBcasD (Dextrose) and YNBcasL (Lactate) plates. (B) Tetrad analysis of IRA2/ira2Δ (ZLY6578, left panel) and ira2Δ (ZLY6601) × sch9Δ (ZLY6649) (middle panel) on YPD plates. Wild type (BY4741) and isogenic sch9Δ (ZLY6649), ira2Δ (ZLY6609), and sch9Δ ira2Δ (ZLY6661) mutant strains were serially diluted and spotted on YPD, YPL, and YPEG plates (right panel). (C) Tetrad analysis of IRA1/ira1Δ (ZLY6666, left panel) and sch9Δ (ZLY6649) × ira1Δ (ZLY6648) (middle panel) on YPD plates. Wild type (BY4741) and isogenic sch9Δ (ZLY6650), ira1Δ (ZLY6648), and sch9Δ ira1Δ (PPY754) mutant strains were serially diluted and spotted on YPD, YPL, and YPEG plates (right panel). (D) Diploid strains obtained from the crossing among sch9Δ ira1Δ (PPY755), sch9Δ ira2Δ (ZLY6661), sch9Δ sns1 (PPY733, PPY1734), and sch9Δ sns2 (ZLY6619, ZLY6626) mutant strains as indicated were serially diluted and spotted on YPD and YPL plates. BY4741 × BY4742 and sch9Δ × sch9Δ (PPY745) strains were included as controls. (E) β-galactosidase activities of a HAP4-lacZ reporter gene in wild type (BY4741), ira1Δ (ZLY6647), and ira2Δ (ZLY6609) mutant strains with or without an sch9Δ deletion mutation grown in YNBcas5D medium. The HAP4-lacZ reporter gene activity in the wild type strain is set as 1. (F) IRA2 from sns1 mutant cells has a frameshift mutation. The chromatogram shows the sequence of IRA2 containing the frameshift mutation from a sns1 mutant. The nucleotide C missing in the IRA2 gene from the sns1 mutant was highlighted in red in the wild type IRA2 sequence.
to determine whether the suppression of an sch9Δ mutant's growth defects on non-fermentable carbon source is mediated through PKA-dependent or independent Ras2 functions. To that end, we analyzed the effect of mutations in genes functioning in the PKA pathway downstream of Ras1 and Ras2. Since bcy1Δ and pde1Δ pde2Δ lead to growth defects on non-fermentable carbon sources and may complicate the analysis of sch9Δ suppression, we determined the effect of single gene mutations of YAK1, PDE1, and PDE2 on sch9Δ suppression by generating double mutant strains via tetrad analysis. We found that yak1Δ and pde2Δ, but not pde1Δ, partially suppressed the growth defect of sch9Δ mutant cells on dextrose dissection plate. A cell growth assay via serial dilution confirmed that yak1Δ and pde2Δ single mutations partially suppressed the growth defects of sch9Δ mutant cells on both dextrose and lactate medium (Figure 7, A and B). Our results suggest that the suppression of growth defects of sch9Δ mutant cells on dextrose medium and non-fermentable carbon sources is mediated at least through the activation of the PKA pathway. We cannot exclude the possibility that activation of PKA-independent Ras2 function(s) may also contribute to sch9Δ suppression in some of the suppressor mutants.

Chronological lifespan extension due to sch9Δ is reversed by an ira2 mutation

sch9Δ mutant cells have been previously shown to extend chronological lifespan (Fabrizio et al. 2001; Longo 2003; Wei et al. 2008).
Mutations in IRA2 reverse the growth defects of sch9A mutant cells. We wanted to determine the effect of an ira2 mutation on chronological lifespan in sch9A mutant cells. To that end, we generated a survival curve of wild type, sch9A, ira2, and sch9A ira2 diploid strains grown in raffinose media (Figure 8). Cell growth in raffinose medium mimics glucose limitation conditions, which accounts for the relatively long chronological life span in wild type cells. As expected, sch9A mutant cells had better survival than wild type. Both ira2 and sch9A ira2 mutant strains showed a significant decrease in cell survival. Mutations in genes in the PKA signaling pathway that inhibit or activate PKA lead to a longer or shorter chronological lifespan, respectively (Fabrizio et al. 2001, 2003; Longo 2003). Reductions in Sch9 activity and PKA pathway signaling have been reported to extend life span in an additive manner (Wei et al. 2008); ira2 mutants are known to have increased Ras/PKA pathway activity (Tanaka et al. 1990b). Together, these data suggest that activation of PKA signaling due to an ira2 mutation antagonizes lifespan extension in sch9A mutant cells.

Discussion

Deletion of SCH9 has been previously reported to cause a slow growth phenotype on dextrose medium, but the growth phenotypes demonstrated on non-fermentable carbon sources are inconsistent. In this report, we propose that the rapid accumulation of suppressor mutations in sch9A mutant cells could account for the discrepancies in the published results. All 18 of the isolated spontaneously arising, recessive mutations that suppressed the growth defects of sch9A mutant cells were proposed to be in either IRA1 or IRA2. Using genetic techniques, we also found that mutations in GPB1, GPB2, and MCK1 suppress the growth defects of sch9A mutant cells in both dextrose medium and non-fermentable carbon sources. Many studies on the cellular functions of Sch9 employed strains of the W303 and S288c backgrounds, which we also used in this report. The discrepancies in the published literature regarding changes in the expression of HAP4, genes encoding mitochondrial proteins, stress-responsive genes, and oxygen consumption due to sch9 mutations may be attributed to the presence or absence of sch9 suppressor mutations (Crauwels et al. 1997; Fabrizio et al. 2003; Pedruzzi et al. 2003; Jorgensen et al. 2004; Roosen et al. 2005; Lavoie and Whiteway 2008; Smets et al. 2008; Pan and Shadel 2009; Pan et al. 2011; Teixeira et al. 2014). It is also possible that the use of other strain backgrounds, growth conditions, and growth phase could account for the discrepancies. By removing the complication of sch9A suppressor mutations, future studies will help reconcile the reported conflicting functions of Sch9 and may uncover novel functions for Sch9.

Fast accumulation of suppressor mutations in sch9A mutant cells was so prevalent that it was difficult for us to maintain sch9A mutant strains. It is not clear why there are frequent spontaneous mutations in IRA1 and IRA2 loci, which were calculated to arise at a frequency of $1.1 \times 10^{-3}$ (Halme et al. 2004). Halme et al. further showed that missense mutations and single base-pair deletions or insertions are behind the high-frequency genetic events in IRA1 and IRA2 genes. During the review of this manuscript, we were brought to the attention of a report by van Leeuwen et al., showing that IRA2 is a candidate suppressor gene in a spontaneous sch9A suppressor mutant (2016). sch9A cells are slow-growing on dextrose media, and the suppressor mutants grow considerably faster and quickly take over the population. We found it was necessary to purify sch9A strains right before they were used in each experiment. When purified sch9A mutant strains are grown in liquid cultures, it is also important to save small aliquots of cultured cells and examine their growth on dextrose plate medium to determine the extent of “contamination” by spontaneous suppressor mutants. Since most suppressor mutations we isolated were recessive, we propose that sch9A homozygous diploid mutant strains should be used for studies on the cellular functions of Sch9. The other alternative is to use analog-sensitive sch9 alleles, but the accumulation of suppressor mutations is still expected to take place. Given that all 18 recessive suppressor mutations we isolated were either IRA1 or IRA2, another option is to use sch9A haploid mutant strains carrying an extra copy of IRA1 and IRA2. However, extra copies of IRA1 and IRA2 may reduce the activity of the Ras/PKA signaling pathway, which could skew conclusions on the cellular functions for Sch9.

Suppression of the growth defects of sch9A mutants by IRA1, IRA2, GPB1, and GPB2 is consistent with the notion that PKA and Sch9 play overlapping roles in regulating cell growth in response to nutrient conditions. It has been reported that activation of PKA can suppress the mutant phenotype of sch9A and vice versa. IRA1 and IRA2 are GTPase-activating proteins of Ras1/2 and function as negative regulators of the Ras/PKA signaling pathway. Gpb1/2 are negative regulators of PKA signaling by inhibiting Ras, directly regulating PKA, and/or by other means (reviewed in Broach 2012). Therefore, the suppression of growth defects of sch9A mutant cells due to mutations in IRA1/2 and GPB1/2 can be explained by increased activity of Ras/PKA signaling. The mechanism behind mck1A suppression of sch9A is less clear. Mck1 was originally characterized for its role in meiosis and chromosome segregation (Neigeborn and Mitchell 1991; Shero and Hieter 1991). It has also been shown that Mck1 may be required for the activity of Msn2/4 (Hirata et al. 2003; Sadeh et al. 2011; Gütin et al. 2015, 2019). Mutations in IRA2, MCK1, MSN2/4, YAK1, and RIM15 lead to a similar transcriptional response of a HSP12 reporter, while sch9A has an opposite effect (Gütin et al. 2015, 2019). Mutations in YAK1, RIM15, and MSN2/4 have also been reported to suppress cell
It is conceivable that prk1Δ may suppress the growth defects of sch9Δ through the Ras/PKA pathway. A potential role of Mck1 in PKA signaling or in another pathway will be examined in future studies.

The effect of sch9Δ, ira1Δ, ira2Δ, gpb1/2Δ, and mck1Δ on the expression of HAP4, ADR1, and CAT8 presents an interesting paradox between cell growth and gene expression. Hap4, ADR1, and Cat8 are global transcriptional activators that are important for the expression of genes involved in gluconeogenesis, the glyoxylate cycle, mitochondrial biogenesis, and/or oxidative metabolism. sch9Δ mutants are unable to utilize non-fermentable carbon sources for growth, which suggests a decrease in the expression of genes involved in respiratory metabolism and gluconeogenesis. Instead, however, sch9Δ increases the expression of genes encoding key transcriptional activators that are required for the utilization of non-fermentable carbon sources. On the other hand, sch9Δ ira1Δ and sch9Δ ira2Δ mutant strains can grow robustly on non-fermentable carbon sources, but they exhibit reduced promoter activity of these three transcriptional activators. One possibility may have to do with greatly increased expression of HAP4, CAT8, and ADR1 genes under glucose limitation conditions (Figure 3A). Due to high levels of transcripts, a large reduction in the promoter activity in sch9Δ ira1Δ and sch9Δ ira2Δ mutant cells may not translate into reduced protein levels to the same degree. Functional redundancy may also help explain the lack of growth defects in sch9Δ ira1Δ and sch9Δ ira2Δ strains on non-fermentable carbon sources. For example, in raffinose-grown cells, CAT8 expression is reduced in these two mutant cells compared with wild type. However, the function of Cat8 is redundant with other transcriptional activators and its downregulation in these mutants may not prevent cells from utilizing non-fermentable carbon sources (reviewed in Schuller 2003; Turcotte et al. 2010). It is also possible that increased expression of HAP4, CAT8, and ADR1 due to sch9Δ may be an adaptative response to cell growth defects. It is important to note that a large difference in transcriptional response determined by the lacZ reporter genes is not translated to the same degree of change in HA-tagged Cat8 or Hap4 determined by Western blotting (Figure 3). This may suggest that translation of CAT8 and HAP4 transcripts may be different from that of lacZ. It is also possible that Cat8 and Hap4 may be subjected to post-translational controls that are not available to the lacZ protein.

The transcriptional response mediated by PKA has been proposed to be conducted mainly through Msn2/4, Gis1, and Rim15 (Roosen et al. 2005; Broach 2012; Pfanzagl et al. 2018). Our data show that PKA is a negative regulator of HAP4, CAT8, and ADR1 (Figure 6). The expression of ADR1 is under negative control by PKA signaling (Dombek and Young 1997). Downregulation of the expression of these three genes in ira1, ira2, and gpb1/2 mutants is likely to be caused by the activation of PKA signaling (Figures 3–5). How does PKA regulate the expression of HAP4, ADR1, and CAT8? Both HAP4 and CAT8 are subject to Mig1-dependent downregulation under glucose repression conditions (reviewed in Schuller 2003; Turcotte et al. 2010; Broach 2012). However, it is unknown whether the effect is direct or indirect. Expression of HAP4 is dependent on Hap1 and is subject to auto-regulatory control (Zhang et al. 2017). Increased expression of CAT8 is dependent on Hap2/3/4/5 under glucose limitation conditions (Rahner et al. 1996). Apart from the findings from this study and others that PKA negatively regulates ADR1 expression, transcriptional regulation of ADR1 is largely unknown. Future studies will be needed to understand the molecular mechanisms underlying the regulation of HAP4, ADR1, and CAT8 by PKA.

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Conflicts of interest
Authors have no conflict of interest to declare.

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