The protein kinase Sch9 is a key regulator of sphingolipid metabolism in Saccharomyces cerevisiae

Erwin Swinnen*a, Tobias Wilmsa, Jolanta Idkowski-Baldysb, Bart Smetsa, Pepijn De Snijdera, Sabina Accardoa, Ruben Ghilleberta, Karin Thevissenac, Jordi de Casteleynb, Dirk De Vosc, Jacek Bielawskic, Yusuf A. Hannunb, and Joris Winderickx a

ABSTRACT The Saccharomyces cerevisiae protein kinase Sch9 is an in vitro and in vivo effector of sphingolipid signaling. This study examines the link between Sch9 and sphingolipid metabolism in S. cerevisiae in vivo based on the observation that the sch9Δ mutant displays altered sensitivity to different inhibitors of sphingolipid metabolism, namely myriocin and aureobasidin A. Sphingolipid profiling indicates that sch9Δ cells have increased levels of long-chain bases and long-chain base-1 phosphates, decreased levels of several species of (phyto)ceramides, and altered ratios of complex sphingolipids. We show that the target of rapamycin complex 1–Sch9 signaling pathway functions to repress the expression of the ceramidase genes YDC1 and YPC1, thereby revealing, for the first time in yeast, a nutrient-dependent transcriptional mechanism involved in the regulation of sphingolipid metabolism. In addition, we establish that Sch9 affects the activity of the inositol phosphosphingolipid phospholipase C, Isc1, which is required for ceramide production by hydrolysis of complex sphingolipids. Given that sphingolipid metabolites play a crucial role in the regulation of stress tolerance and longevity of yeast cells, our data provide a model in which Sch9 regulates the latter phenotypes by acting not only as an effector but also as a regulator of sphingolipid metabolism.

INTRODUCTION

Sphingolipids, together with sterols and glycerophospholipids, are essential components of all eukaryotic membranes. Much of the progress in the understanding of sphingolipid metabolism is due to research performed in the budding yeast Saccharomyces cerevisiae, in which most, if not all, of the enzymes involved in sphingolipid metabolism have been identified (Figure 1; Funato et al., 2002; Sims et al., 2004; Dickson et al., 2006). Apart from their structural function, sphingolipids also play important regulatory roles. More specifically, in yeast, sphingolipids are involved in the regulation of cellular processes as diverse as cell growth, endocytosis, actin cytoskeleton organization, protein trafficking, cell wall integrity, nutrient uptake, and longevity (Dickson, 2010). In these processes, signaling functions are ascribed not only to the complex sphingolipid species, but also to the intermediate metabolites, that is, the long-chain bases (LCBs), the long-chain base 1-phosphates (LCBPs), and ceramide species. Accumulating evidence in yeast and mammalian model systems strongly suggests that the dynamic balance between the different sphingolipid metabolites, often referred to as the LCBP/ceramide rheostat or sphingolipid rheostat, is an important factor determining their regulatory action (Kobayashi and Nagiec, 2003; Kihara et al., 2007; Dickson, 2008; Matmati and Hannun, 2008; Breslow and Weissman, 2010; Bikman and Summers, 2011; Van Brocklyn and Williams, 2012). As such, proper regulation of this...
balance is key to cellular survival, and recent reports have shed some light on the molecular mechanisms by which yeast controls this balance. For instance, the yeast Orm1 and Orm2 proteins are crucial regulators of sphingolipid homeostasis by controlling the activity of the serine palmitoyl transferase (SPT) enzyme, which catalyzes the first and rate-limiting step in de novo synthesis of sphingolipids. Orm1/2 are members of the conserved ORMDL family of endoplasmic reticulum (ER) membrane proteins (ORMDL 1/2/3 genes in humans) and physically interact with SPT, inhibiting the activity of the enzyme (Breslow et al., 2010; Han et al., 2010). This inhibitory action is abrogated through Orm1/2 phosphorylation by the protein kinase Ypk1 (Roelants et al., 2011). When sphingolipid levels drop—for example, when cells are treated with the SPT inhibitor myriocin—the target of rapamycin complex 2 (TORC2) kinase activates Ypk1, boosting de novo sphingolipid synthesis to counteract dropping sphingolipid levels. In addition, the increase in LCB accumulation on heat shock is also dependent on Ypk1’s action, downstream of the Pkh1/2 kinases (Sun et al., 2012).

Interestingly, as yeast cells approach stationary phase, they accumulate LCBs and LCBPs as well (Lester et al., 2013). This increase may also be mediated in part by an increase in SPT activity, although additional mechanisms, such as a decrease in ceramide synthase activity as cells enter the stationary phase, were also demonstrated. Although phosphorylation of the Orm proteins depends on the rapamycin-sensitive, nutrient-responsive TOR1 kinase, recent evidence shows that these phosphorylation sites are distinct from those regulated by myriocin treatment, indicating that TORC1 and TORC2 have distinct roles in the control of the Orm proteins (Liu et al., 2012; Shimobayashi et al., 2013). Indeed, rapamycin-induced phosphorylation of Orm1/2 did not seem to affect SPT activity. Instead, a stimulation of complex sphingolipid synthesis was observed, through as-yet-unknown mechanisms. Additional evidence for a nutrient-dependent regulation of complex sphingolipid levels comes from a recent lipidomic approach, in which it was shown that relative levels of different complex sphingolipid species change as yeast cells enter the stationary phase (Klose et al., 2012).

To help coordinate cellular responses to changing nutrient levels, yeast depends on Sch9, a protein kinase with a central role in the carbon and nitrogen source–dependent signaling pathway that controls cell growth, longevity, and stress resistance (Toda et al., 1988; Crauwels et al., 1997; Fabrizio et al., 2001; Roosen et al., 2005; Smets et al., 2008, 2010). Sch9 performs this function, in part, by acting as an effector of the TORC1 complex (Urban et al., 2007), although several studies indicated that Sch9 also acts independently of TORC1 (Yorimitsu et al., 2007; Smets et al., 2008). Like Ypk1/2, Sch9 can be phosphorylated by Pkh1 and Pkh2 at its PDK1 site in its activation loop, and this phosphorylation is indispensable for its function (Roelants et al., 2004; Urban et al., 2007; Voordeckers et al.,

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**FIGURE 1:** Schematic representation of sphingolipid biosynthesis (adapted from Dickson, 2008). A schematic representation of the de novo biosynthesis pathway for sphingolipids, with the genes encoding for the involved enzymes in italic and both the complex sphingolipids and the different intermediates of sphingolipid biosynthesis in bold. Used inhibitors of sphingolipid biosynthesis are shown. Changes in sphingolipid profile for the sch9Δ strain, compared with the WT strain, are indicated with closed arrows for LCB and ceramide species (see Figure 3 for results) and open arrows for complex sphingolipids (see Figure 7 for results).
RESULTS
Deletion of SCH9 confers resistance to myriocin and increases sensitivity to Aureobasin A and phytosphingosine

To investigate the potential relationship between Sch9 and sphingolipid metabolism in more detail. Our data demonstrate that Sch9 has a regulatory role in de novo ceramide biosynthesis and ceramide production by hydrolysis of complex sphingolipids. We provide evidence for a transcriptional regulatory mechanism in which Sch9 is required to repress ceramidase activity during growth by acting downstream of the TORC1 kinase. In addition, for hydrolytic ceramide production, Sch9 is essential for the proper translocation of the inositol phosphosphingolipid phospholipase C, Isc1, from the ER to mitochondria during the diauxic shift. As such, Sch9 appears to be the gatekeeper of a monitoring system required to balance biosynthetic and hydrolytic ceramide production. We illustrate and discuss some of the phenotypic repercussions associated with this role of Sch9, focusing on mitochondrial activity, oxidative stress, aging, and longevity. Given the function of Sch9 in nutrient signaling and the fact that sphingolipids mediate Pkh-dependent phosphorylation and activation of Sch9, one obvious conclusion is that Sch9 is part of a feedback system balancing the sphingolipid rheostat in response to both internal sphingolipid signals and nutrient availability.

Deletion of SCH9 alters the LCB(P)/ceramide balance

To evaluate the hypothesis that cells lacking Sch9 have higher levels of intermedary sphingolipid metabolites and thus that Sch9 may function as modulator of sphingolipid metabolism in vivo, we determined the levels of PHS and dihydrophosphingosine (DHS), their phosphates, phytosphingosine-1-phosphate (PHS-P) and dihydrophosphingosine-1-phosphate (DHS-P), and the levels of (phyto)ceramides in the membranes of the WT and the sch9Δ strain. Compared to the WT strain, sch9Δ cells were characterized by increased levels of DHS, PHS, and PHS-P, whereas levels of DHS-P remained below the detection limit (Figure 3A). Conversely, the concentrations of the ceramides C18-dihydroceramide, C20-dihydroceramide, and C24-phytoceramide were decreased in sch9Δ cells (Figure 3, B and C), as was the amount of α-hydroxylated phytoceramide (αOH-Phyto-Cer-C24, Figure 3D). Thus, these data suggest that the Sch9 protein kinase fulfills a regulatory role to maintain de novo ceramide synthesis.

An increase of LCB(P) in sch9Δ cells could be the result of an increase in SPT activity, perhaps through phosphorylation of the Orm proteins. However, in this case we would expect that also ceramide levels would have increased, as shown before for the orm1Δ orm2Δ strain (Breslow et al., 2010; Shimobayashi et al., 2013). Alternatively, Sch9 could also be involved in very long-chain fatty acid formation, which serves as a substrate in the ceramide production (Figure 1). As such, the LCB(P)s may accumulate due to the inability to react with very long-chain fatty acids to form ceramides, as is the case in sur4Δ cells (Breslow et al., 2010). However, we present genetic evidence that SCH9 and SUR4 work in parallel pathways, making this possibility unlikely (Supplemental Figure S1, B and C). Therefore a more obvious explanation is that Sch9 plays a role at the step of ceramide production or breakdown. This is supported by our genetic data showing that deletion of either gene encoding ceramidase (YDC1 or YPC1) abrogates myriocin resistance of the sch9Δ strain (Supplemental Figure S1A), as does overexpression of the ceramide synthase LAG1 or LAC1 (Supplemental Figure S1D). To have a closer look at the enzymes involved in ceramide production and breakdown, we constructed WT and sch9Δ strains expressing chromosomally encoded, Myc-tagged versions of Lag1, Lac1, Ydc1, and Ypc1 and analyzed their protein levels. We included the addition of rapamycin in these experiments, as this compound is well known for its ability to inactivate TOR1, a major nutrient-dependent activator of Sch9 (Urban et al., 2007). Results in Figure 4 indicate that either deleting SCH9 or treating WT cells with rapamycin increases the presence of the ceramidases Ydc1 and Ypc1.
Because this effect of rapamycin addition is abrogated in sch9Δ cells, this suggests that Sch9 functions to repress ceramidase levels downstream of TORC1. Concerning the levels of the ceramide synthases, we also observed an increase in the levels of Lag1 and Lac1 in the sch9Δ strain, although the effects on Lac1 were not statistically significant. Of importance, this increase in Lag1/Lac1 levels is not seen when treating WT or sch9Δ cells with rapamycin. One possible explanation is that Sch9 might specifically regulate ceramide synthase levels, independent of TORC1. Note, however, that the rapamycin treatment represents a short-term response to TORC1 inactivation, whereas conditions for the sch9Δ strain represent a steady state of this strain during growth. Therefore, an alternative explanation is that during steady-state growth under nutrient-rich conditions, the increased abundance of ceramidase enzymes in the sch9Δ strain may trigger additional (TORC1-independent) homeostatic mechanisms, which increase Lag1/Lac1 levels to try to compensate the imbalanced sphingolipid rheostat. Because elevated levels of phosphorylated LC8s act as inhibitory to growth (Kim et al., 2000), this compensatory response could function to reduce LCB(P) levels to some extent to avoid complete growth inhibition.

Sch9 controls de novo ceramide synthesis via transcriptional regulation mechanisms

To investigate whether the changes in protein levels under our experimental conditions reflect a transcriptional response, we constructed lacZ reporter fusions with the promoters of LAG1, LAC1, YDC1, and YPC1 in the multicopy plasmid Yep357. First, Figure 5A shows that deletion of SCH9 indeed results in a significant induction in reporter activity for both the ceramide synthase promoters, LAG1 and LAC1, as for the ceramidase promoters YDC1 and YPC1. This induction in the sch9Δ strain was higher for the ceramidase promoters compared with the induction of the promoters of the ceramide synthase genes, and as such correlates with our observation that the increase in protein levels for Ydc1-Myc and Ypc1-Myc is larger than the increases in Lag1-Myc and Lac1-Myc levels in the sch9Δ strain (Table 1). Second, treatment of the WT strain with rapamycin resulted in significant transcriptional induction in ceramidase promoter activity for both the YDC1 and YPC1 reporters, whereas such an induction was absent for both the LAG1 and LAC1 reporters (Figure 5B). The fact that derepression of the ceramidase enzymes is higher in sch9Δ cells than in WT cells treated with rapamycin might be due to the fact that our rapamycin treatment does not result in a complete inactivation of Sch9, especially in the BY4741 strain, which is more resistant to rapamycin treatment than most other backgrounds. Finally, the rapamycin-induced effect on the YDC1 and YPC1 reporters was abrogated in the sch9Δ strain (Figure 5C). As such, the effects on the Myc-tagged protein levels shown in Figure 4 are recapitulated on a transcriptional level by our lacZ results shown in Figure 5. Thus, Sch9 seems, indeed, to be the downstream effector of the TORC1 kinase in the transcriptional control of ceramidase expression. However, one may argue that the absence of rapamycin-induced expression of the YDC1 and YPC1 reporters in the sch9Δ strain may be due to an already complete derepression of promoter activity. Furthermore, induction of ceramidase promoter activity by rapamycin treatment in the WT strain might be due to Sch9-dependent and -independent effects, for instance through the Tap42 signaling branch of TORC1 (Smets et al., 2010). To further demonstrate that Sch9 is the principal mediator of the observed rapamycin effects, we used SCH9 constructs in which specifically the five TORC1 phosphorylation sites were mutated (Urban et al., 2007). Mutation of these sites to alanine (SCH9A) results in the expression of an inactive Sch9 protein that cannot be phosphorylated by TORC1. On the other hand, mutation of these sites to aspartic (D) or glutamic (E) acid (SCH9D/E) results in the expression of a variant of Sch9 that mimics a constitutively phosphorylated, and therefore active, Sch9, which cannot be inactivated by rapamycin treatment (Urban et al., 2007). We subcloned these SCH9 variants, along with the wild-type SCH9 gene, into the pRS415 vector to allow for coexpression with our YDC1- and YPC1-lacZ constructs in the sch9Δ strain. As shown in Figure 6, coexpression of the ceramidase lacZ constructs with wild-type SCH9 in the sch9Δ strain can complement the high basal lacZ activity observed with the empty vector (pRS415), as well as restore rapamycin induction of promoter activity. Furthermore, expressing the inactive SCH9A variant mimics the empty vector situation, demonstrating that phosphorylation of Sch9 by TORC1 is required for keeping basal promoter activities in check. Further induction by rapamycin is also abolished in cells expressing this SCH9A construct. Expressing the phospho-mimic SCH9D/E variant, on the other hand, results in basal promoter activities, comparable to those observed when expressing the wild-type SCH9 gene (Figure 6). They are somewhat elevated compared with the wild-type gene, likely due to the slight differences in vivo conformation between the phospho-mimic and truly phosphorylated Sch9. Still, comparing basal promoter activities of cells expressing SCH9A and SCH9D/E clearly demonstrates the importance of phosphorylation of the TORC1-dependent residues of Sch9 in regulating ceramidase promoter activity. Intriguingly, rapamycin-mediated induction of YDC1- and YPC1-lacZ activity is completely lost in cells expressing the SCH9D/E variant, indicating that this induction by rapamycin requires a loss in phosphorylation of Sch9 by TORC1. Also, these results argue against additional effectors besides Sch9 in mediating the rapamycin effect.

In conclusion, due to the tight correlation between the effects observed in the lacZ experiments and the determination of protein levels (summarized in Table 1), our results point to the existence of a nutrient-dependent transcriptional response in which the TORC1–Sch9 signaling pathway acts to repress the expression of the ceramidase enzymes (YDC1 and YPC1). In addition, TORC1-independent regulation mechanisms exist that affect the transcriptional regulation of the genes encoding the ceramide synthases (LAG1 and LAC1), thereby constituting an independent homeostatic mechanism controlling sphingolipid balance. Whether the latter mechanism is also independent of Sch9 remains to be established. Indeed, despite being a major downstream effector of TORC1, Sch9 also performs functions independent of this upstream regulator (Pascual-Ahuir and Proft, 2007; Smets et al., 2008).

Sch9 has a regulatory role in the metabolism of complex sphingolipids

Because Sch9 proved to have a significant effect on the LCB(P)/ceramide balance, we set out to test how the complex sphingolipid profile was affected by deletion of SCH9. To this end, we measured the levels of the three major inositol phosphosphingolipid classes, namely IPC, mannosylinositolphosphoryl ceramide (MIPC), and mannosylsphingolipid phosphorylceramide (MIP(2)C), in membranes of WT and sch9Δ cells. This revealed that the sch9Δ mutant combined relative reduced levels of IPC with increased levels of MIPC and MIP(2)C (Figure 7). Intriguingly, a recent lipidomic approach indicated that yeast cells go into the stationary phase, IPC levels drop, whereas MIPC levels increase and MIP(2)C levels remain constant (Klose et al., 2012). The net result is the same as we observe upon deletion of SCH9; that is, a relative decrease in IPC levels combined with a relative increase in MIPC and MIP(2)C levels. Thus, the complex sphingolipid profile of a sch9Δ strain reflects that of a WT
FIGURE 2: Deletion of SCH9 confers resistance to myriocin and increases sensitivity to phytosphingosine and aureobasidin A. (A) Serial dilutions of exponentially growing wild-type and sch9Δ cells of two different genetic backgrounds (BY4741 and W303-1A) and with different markers were spotted on YPD and on YPD containing 0.5 μg/ml myriocin. (B) Serial dilutions of sch9Δ cells (BY4741 background, JW 01 306), transformed with YEpLac195 (ev) or
complex sphingolipids may also contribute to myriocin resistance (Supplemental Figure S1A). Specifically interesting to us was the observation that additional deletion of \textit{ISC1} resulted in an increase in myriocin resistance of the \textit{sch9}Δ strain (Figure 1SB). This result indicates a functional link between Sch9 and Isc1, the inositol phosphosphingolipid phospholipase C, which hydrolyses IPC, MIPC, and M(IP)\textsubscript{2}C back into (phyto)ceramides (Sawai \textit{et al.}, 2000). We first examined whether Sch9 might influence Isc1 protein levels, similar to the situation seen for the enzymes involved in de novo ceramide synthesis. To this end, we constructed WT and \textit{sch9}Δ strains expressing a chromosomally encoded, Myc-tagged version of Isc1. Determination of the levels of Isc1-Myc in exponentially growing cells demonstrated that deletion of \textit{SCH9} or treatment of yeast strain in stationary phase, consistent with the model that Sch9 activity signals the presence of nutrients (Urban \textit{et al.}, 2007). The reduction of IPC could be explained by the reduced content of (phyto)ceramides, which serve as precursors. However, the increment in the levels of mannosylated sphingolipids suggests an additional regulatory role for Sch9 in the biosynthesis or turnover of mannosylated sphingolipids.

\textbf{Sch9 affects the mitochondrial translocation of \textit{Isc1}}

To further decipher the connection between Sch9 and complex sphingolipid synthesis, we performed a genetic analysis on myriocin resistance of an \textit{sch9}Δ strain lacking additional genes involved in the synthesis of complex sphingolipids. Results indicated that complex sphingolipids may also contribute to myriocin resistance (Supplemental Figure S1A). Specifically interesting to us was the observation that additional deletion of \textit{ISC1} resulted in an increase in myriocin resistance of the \textit{sch9}Δ strain (Figure 1SB). This result indicates a functional link between Sch9 and Isc1, the inositol phosphosphingolipid phospholipase C, which hydrolyses IPC, MIPC, and M(IP)\textsubscript{2}C back into (phyto)ceramides (Sawai \textit{et al.}, 2000). We first examined whether Sch9 might influence Isc1 protein levels, similar to the situation seen for the enzymes involved in de novo ceramide synthesis. To this end, we constructed WT and \textit{sch9}Δ strains expressing a chromosomally encoded, Myc-tagged version of Isc1. Determination of the levels of Isc1-Myc in exponentially growing cells demonstrated that deletion of \textit{SCH9} or treatment of
Consistent with Sch9 acting as a regulator of Isc1, several studies documented that Isc1 plays a role in well-known Sch9-dependent phenotypes, including diauxic shift reprogramming and respiration, oxidative stress resistance, and the determination of longevity (Almeida et al., 2001; Wei et al., 2009), loss of Isc1 is associated with a higher oxidative state, suggesting that Sch9 impairs mitochondrial translocation of Isc1 in postdiauxic-phase cells in both glucose- and galactose-grown cells.

Implications of the functional interaction between Sch9 and Isc1 for yeast longevity

Consistent with Sch9 acting as a regulator of Isc1, several studies documented that Isc1 plays a role in well-known Sch9-dependent phenotypes, including diauxic shift reprogramming and respiration, oxidative stress resistance, and the determination of longevity (Almeida et al., 2001; Kitagaki et al., 2009). Intriguingly, although loss of Sch9 is known to trigger increased resistance to oxidants and increased chronological life span (Fabrizio et al., 2001; Wei et al., 2009), loss of Isc1 is associated with a higher oxidative state, suggesting that Sch9 impairs mitochondrial translocation of Isc1 in postdiauxic-phase cells in both glucose- and galactose-grown cells.

In agreement with previous work, we observed Isc1-GFP localization at the ER in WT and sch9Δ cells during exponential growth (Vaena de Avalos et al., 2004), with almost no sign of mitochondrial colocalization (Supplemental Figure S3). In the postdiauxic growth phase, Isc1-GFP mainly colocalized with mitochondria in WT cells, whereas in sch9Δ cells, Isc1-GFP remained mostly localized at the ER (Supplemental Figure S3), indicating involvement of Sch9 in mitochondrial translocation of Isc1 on galactose-containing medium. We were curious as to whether Isc1 also translocates to the mitochondria in the postdiauxic phase of cells grown on glucose-containing medium and whether deletion of SCH9 would have a similar effect under these conditions. Furthermore, because we determined our sphingolipid profiles for cells growing on glucose as the carbon source, studying the intracellular localization of Isc1 in cells grown on glucose could be directly related to our observed sphingolipid profiles. To this end, we subcloned the ISC1-GFP construct behind its own promoter. In addition, we made two parallel analyses of Isc1-GFP localization: in a first series of experiments, we scored colocalization of Isc1 with an ER-specific mCherry marker (Figure 8A), whereas colocalization with mitochondria was analyzed in a second series of experiments using a mCherry marker targeted to the mitochondria (Figure 8B). For both the WT and sch9Δ strains, we saw that in cells growing exponentially on glucose, Isc1 localized almost exclusively in the perinuclear and cortical ER (Figure 8A), without any significant colocalization with mitochondria (Figure 8B). When scoring colocalization of Isc1 with the ER in cells in the postdiauxic phase, we noticed a higher retention of Isc1 in the ER in sch9Δ cells than in WT cells (Figure 8A). Consistent with this observation, postdiauxic-phase WT cells showed more colocalization of Isc1 with the mitochondrial marker than sch9Δ cells (Figure 8B). Therefore we conclude that deletion of SCH9 impairs mitochondrial translocation of Isc1 in postdiauxic-phase cells in both glucose- and galactose-grown cells.
oxidative stress sensitivity and reduced chronological life span (Almeida et al., 2008). To further elaborate on the functional interaction between Sch9 and Isc1, we determined the chronological life span of mutants lacking SCH9 and/or ISC1 (Figure 9A). In agreement with previous published results, we observed an increase in life span in the sch9Δ mutant as compared with the WT strain (Fabrizio et al., 2001; Wei et al., 2009), whereas the isc1Δ mutant displayed reduced viability (Kitagaki et al., 2009; Barbosa et al., 2011). In parallel with viability, we assessed the levels of cellular reactive oxygen species (ROS) under the same conditions, as an increase in oxidative stress plays a major role in determining viability in stationary phase. As compared with WT cells, the level of ROS was already elevated in exponential isc1Δ cells, and this difference became more pronounced when the cells enter the stationary phase (Figure 9, B and C). In sch9Δ cells, the level of ROS also increased during growth, but here the amount of ROS was always significantly lower than in WT cells. Of interest, the additional deletion of SCH9 in the isc1Δ mutant restored the ROS production to levels comparable to those of WT cells (Figure 9, B and C). In line with these reductions in ROS levels, survival of the sch9Δ isc1Δ double mutant increased compared with the isc1Δ strain (Figure 9A). Finally, the decreased chronological life span of cells lacking Isc1 was shown to be due to increased apoptotic cell death (Almeida et al., 2008). We confirmed the involvement of apoptosis in mediating the accelerated cell death of isc1Δ cells by staining yeast cells with the cell death markers of apoptosis and necrosis (annexin/P1 costaining. Supplemental Figure S4). We also showed that deletion of SCH9 decreases apoptotic cell death, independent of the presence or absence of ISC1, correlating with the decreased oxidative stress observed in these strains. Linking these viability data with our observation that Sch9 is required for proper mitochondrial translocation of Isc1 in postdiauxic-phase cells is a delicate matter. Because Isc1-mediated sphingolipid metabolism in mitochondria is required for proper mitochondrial function and viability in stationary phase (Almeida et al., 2008; Kitagaki et al., 2009), this would indicate a prosurvival function of Sch9 in stimulating Isc1 activity, which contradicts the increased life span of cells deleted for SCH9 (Figure 9A). On the other hand, decreased mitochondrial translocation of Isc1 in the sch9Δ mutant implies a reduction of mitochondrial ceramide species produced by Isc1 in the postdiauxic phase of sch9Δ cells (Vaena de Avalos et al., 2005; Kitagaki et al., 2007). Several studies showed that ceramides can interact directly with mitochondria, thereby increasing mitochondrial membrane permeability (Birbes et al., 2001; Siskind et al., 2002, 2005; Chipuk et al., 2012; Rego et al., 2012) and disrupting electron transport, resulting in elevated ROS levels (Garcia-Ruiz et al., 1997; Gudz et al., 1997; Di Paola et al., 2000). As such, impaired Isc1 translocation might be responsible for part of the increased longevity observed in a sch9Δ strain, although this antisuress function of Isc1 does not fit with the loss of viability upon deleting ISC1 in the WT or sch9Δ strain (Figure 9A). Clearly, more work is needed to further characterize the molecular connections between Sch9 and Isc1 functions. For instance, although it was proven that Isc1 activity increases upon translocation to the mitochondria (Vaena de Avalos et al., 2004, 2005), it may also possess (basal) enzymatic activity at the ER. Future studies may test the effect of Sch9 specifically on the enzymatic activity of Isc1 at these different cellular locations. Furthermore, due to the plethora of ceramide species, it is conceivable that specific ceramide species, possibly generated at different intracellular locations, mediate different cellular responses, thereby regulating either survival or death, depending on the cellular context (Hannun and Obeid, 2011; Matmati et al., 2013). Of interest, the complex sphingolipids, whose profile is also altered by deletion of SCH9, were recently demonstrated to influence yeast viability, independent of their ceramide precursors (Kajiwara et al., 2012). As such, these species should not be overlooked when building a model for the cell death/survival mechanisms operating in yeast cells.

**DISCUSSION**

**Sch9 is a regulator of sphingolipid metabolism**

Regulation of the sphingolipid rheostat is essential for yeast cells to ensure proper growth and survival properties (Dickson, 2010).
obtained by short-term rapamycin treatment, steady-state levels of complex sphingolipids may thus be regulated by TORC1 through both the Sit4-Npr1 effector branch and the Sch9 effector branch, the latter being supported by this work.

Concerning the LCB(P)/ceramide rheostat, we demonstrate a novel mechanism in which Sch9 controls the presence of the Ydc1 and Ypc1 ceramidase enzymes. Using promoter-lacZ reporter constructs, we demonstrate that Sch9 performs this function by acting at the transcriptional level, with deletion of SCH9 resulting in a significant derepression of YDC1 and YPC1 expression. Treating WT cells with rapamycin, which inhibits the nutrient-dependent kinase TORC1, also results in derepression of YDC1 and YPC1, and we show that this effect is mediated by Sch9, which is known to act as a downstream effector of TORC1 (Urban et al., 2007). Therefore we conclude that signaling through the TORC1–Sch9 pathway during nutrient-rich conditions serves to repress transcription of the YDC1

Therefore it is not surprising that this sphingolipid balance changes when nutrient conditions alter. In this study, we observed that, during growth, the sch9Δ strain displays sphingolipid profiles described for cells in stationary phase. Indeed, compared with the WT strain, sch9Δ cells accumulate LCB(P)s and have decreased (phyto)ceramide levels and an altered profile of complex sphingolipids, which is similar to cells entering into stationary phase (Klose et al., 2012; Lester et al., 2013). The specific increase of mannosylated sphingolipid species compared with IPC levels indicates that Sch9 has additional inputs into complex sphingolipid synthesis, apart from its role in maintaining the LCB(P)/ceramide balance. Interestingly, TORC1 inhibition has been reported to stimulate complex sphingolipid synthesis through Npr1-dependent phosphorylation of the Orm1/2 proteins (Shimobayashi et al., 2013). The molecular mechanism behind this phenotype is unknown, although it was shown to be independent of SPT activity. Given that these results were obtained by short-term rapamycin treatment, steady-state levels of complex sphingolipids may thus be regulated by TORC1 through both the Sit4-Npr1 effector branch and the Sch9 effector branch, the latter being supported by this work.

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FIGURE 6: The rapamycin-induced effects on YDC1 and YPC1 lacZ activity are mediated by Sch9. LacZ activities of the YDC1 (left) and YPC1 (right) promoters was assessed in the sch9Δ strain (JW 03 038) coexpressing the empty vector (EV, pRS415), the wild-type SCH9 gene, or one of the SCH9 mutant genes in which its TORC1 phosphorylation sites are mutated to either alanine (SCH95A) or glutamic/aspartic acid residues (SCH92D3E). The WT strain (BY4741) coexpressing the EV was taken as an additional control. Precultures were grown overnight in SD-URA-LEU, inoculated into YPD medium, and grown for 6–7 h, after which part of the culture was treated with 200 nM rapamycin. After 1 h, samples of treated and untreated cells were pelleted at 4°C and stored at −80°C. Protein extracts were prepared, and specific activities were determined. Results are expressed as mean of four independent transformants, with SD noted as error bars. Top: all results in one graph. Bottom: for clarity, selective omission of results of conditions that mimic the condition of the sch9Δ strain with the EV. Results for both promoters are equivalent, in that the SCH95A construct mimics the absence of SCH9 in its high basal lacZ activity and no induction by rapamycin. The SCH92D3E construct, on the other hand, gives basal lacZ activities compared with the SCH9 control strain, although induction by rapamycin is lost due to the inability of the SCH92D3E protein to be inhibited by loss of TORC1 activity. Statistical significance of rapamycin addition for each construct was assessed by a paired t test (**p < 0.01, ***p < 0.001).
and YPC1 ceramidase-encoding genes. We also observed TORC1-independent effects on the expression of the ceramide synthase genes LAG1 and LAC1 in the sch9Δ strain. However, we cannot discriminate between whether this represents a true Sch9-dependent, TORC1-independent, signaling event or is due to additional, Sch9-independent homeostatic mechanisms that counteract the significant increased ceramidase presence in the sch9Δ strain. Identification of the transcription factors involved in these processes, which is currently being pursued, will help to shed light on this issue.

It is intriguing that, besides TORC1, multiple in vitro and in vivo studies show that Sch9 activity is also regulated independently by the LCB (PHS)-dependent Pkh1/2 kinases (Roelants et al., 2004; Liu et al., 2005; Urban et al., 2007; Voordecker et al., 2011; Huang et al., 2012). According to this model, if PHS drops (e.g., by myriocin treatment), Sch9 activity will also drop due to reduced phosphorylation by Pkh1/2. This would lead to increased ceramidase action, helping to restore internal LCB pools. Therefore Sch9 comprises a major gatekeeper of sphingolipid homeostasis, optimizing ceramidase activity by integrating information on internal LCB (PHS) levels through Pkh1/2, as well as information on nutritional status via TORC1, thereby adjusting sphingolipid metabolite levels to cellular needs. Given that Orm1/2-mediated regulation of sphingolipid metabolism was shown to be mainly regulated via (rapid) Orm1/2 phosphorylation, the Sch9-mediated transcriptional response may comprise a complementary mechanism, operating somewhat more slowly, as it is independent on transcription, although probably essential to generate major long-term changes in the sphingolipid rheostat.

Altered sphingolipid levels and longevity in sch9Δ cells

A well-established phenotype of the sch9Δ strain is its increased survival during stationary phase (Fabrizio et al., 2001; Wei et al., 2009), and much research has been performed to elucidate the mechanisms behind this phenotype. Sphingolipids are also crucial regulators of cell viability, and it has been shown that in higher eukaryotes, the balance in intermediary sphingolipid signaling metabolites, the so-called sphingolipid rheostat, has an important function in cell survival, disease and senescence (Olivera et al., 1999; Levade et al., 2002; Halt et al., 2006; Saddoughi et al., 2008; Breslow and Weissman, 2010; Dickson, 2010; Bikman and Summers, 2011; Van Brocklyn and Williams, 2012). Therefore it is not surprising that the altered sphingolipid profile we describe for sch9Δ cells may have important contributions to the increased life span of this strain.

The increased survival of the sch9Δ strain is reported to be partly due to increased mitochondrial function and respiration during growth (Lavoie and Whiteway, 2008; Pan and Shadel, 2009; Pan et al., 2011). Of interest, the elevated PHS-P levels of sch9Δ cells we demonstrated in this study might be crucial for this process, as cellular PHS-P have been implicated in the control of the HAP complex transcription factor (Cowart et al., 2010), which regulates mitochondrial respiration. As such, our results would provide the missing mechanistic link between Sch9 and the Hap4 transcription factor, which was shown to be essential for the increased cellular respiration in sch9Δ cells (Lavoie and Whiteway, 2008).

The decreased ceramide levels may also contribute to the increased life span of the sch9Δ strain. Indeed, evidence indicates that ceramides can induce a mitochondrial cell death pathway in yeast, which seems to involve increasing mitochondrial membrane permeability (Carmona-Gutierrez et al., 2011; Rego et al., 2012). In addition, ceramides exert proapoptotic effects via ceramide-activated protein phosphatases, such as Slt4 in yeast (Nickels and Broach, 1996; McCourt et al., 2009; Barbosa et al., 2011). In addition to the aforementioned effect of Sch9 on the de novo synthesis of ceramides, we demonstrate that Sch9 is required for the proper mitochondrial localization of Isc1 in postdiauxic cells, which, to our knowledge, is the first report of a protein required for Isc1 translocation (Vaena de Avalos et al., 2004, 2005; Kitagaki et al., 2007). Given that this translocation of Isc1 is blocked in sch9Δ cells, this may abrogate the production of a specific mitochondrial ceramide pool.

<table>
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<th>lacZ construct</th>
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Statistical significance was determined using an unpaired t test for the effects of sch9Δ vs. WT strain, and paired t tests for the effects of rapamycin addition. Statistical significance: *p < 0.1 (only used for protein levels), **p < 0.01, and ***p < 0.001.

*Analysis of results shown in Figure 4.
**Analysis of results shown in Figure 5.

TABLE 1: Effects of SCH9 deletion and rapamycin addition on protein levels and lacZ promoter constructs.
which could contribute to apoptotic cell death, as demonstrated in both yeast and mammals (Birbes et al., 2001; Chipuk et al., 2012; Rego et al., 2012). Still, we and others have shown that loss of ISC1 impairs survival during stationary phase (Almeida et al., 2008; Kitagaki et al., 2009; Barbosa et al., 2011), indicating that Isc1-mediated ceramide production can elicit prosurvival and antisurvival programs, depending on specific cellular conditions. Finally, since Sch9 activity is proposed to decline upon nutrient depletion, this indicates that Sch9 affects the translocation of Isc1 indirectly. Given that sch9Δ cells were shown to have altered mitochondrial properties, such as reduced superoxide production, in stationary phase (Pan and Shadel, 2009; Pan et al., 2011), our experimental observations support a model in which mitochondrial ROS production may provide the cellular signal for Isc1 translocation.

In conclusion, we show that the TORC1–Sch9 signaling branch controls sphingolipid homeostasis by controlling the expression of the ceramidase enzymes. Given that transcriptional regulation mechanisms of sphingolipid metabolism are still largely elusive (Breslow and Weissman, 2010), it will be of interest to see whether the mechanisms described here are conserved in higher eukaryotes. Furthermore, the altered sphingolipid profile exhibited by the sch9Δ strain provides additional molecular links that help explain the extended life span of this strain. As such, it appears that changing the sphingolipid balance in yeast by tipping it in favor of the LCBs and LCBPs, concomitant with decreased ceramide levels, results in lower apoptosis and increased survival, which in many ways is similar to what is seen for mammalian cells (Olivera et al., 1999; Levade et al., 2002; Hait et al., 2006; Saddoughi et al., 2008; Bikman and Summers, 2011; Van Brocklyn and Williams, 2012). Given this conserved nature of the role of sphingolipid signaling in eukaryotic cells, yeast may continue to serve as an important model system for higher eukaryotes.

MATERIALS AND METHODS

Materials, yeast strains, plasmids, and growth media
S. cerevisiae strains and plasmids used in this study are listed in Supplemental Tables S1 and S2, respectively. Deletion strains were made using PCR-derived deletion cassettes (Brachmann et al.,
**FIGURE 8**: Fluorescence microscopic analysis of the intracellular localization of Isc1. Intracellular localization of Isc1-GFP expressed from its own promoter (plasmid FBp708) in wild-type (BY4741) and sch9Δ (JW 03 038) cells was examined by assessing colocalization with a mCherry marker targeted to the ER or the mitochondria. Cells precultured on SD-URA-LEU were inoculated in fresh YPD medium (2% glucose) at an initial OD600 of 0.5. Pictures were taken after 6–8 h (exponential) and after 28 h (postdiauxic). Representative pictures are shown for each condition, showing (from left to right) bright field, green channel (GFP), red channel (mCherry), and the corresponding merged picture.

(A) Colocalization with the ER. During exponential growth, nearly all cells displayed colocalization of Isc1-GFP with the ER-mCherry marker (plasmid FBp709). In postdiauxic-phase cells, two distinct populations are observed, showing either an overlap or no overlap with the ER. (B) Colocalization with mitochondria. During exponential growth, virtually no cells displayed colocalization of Isc1-GFP with the mitochondria-mCherry marker (plasmid FBp459). In postdiauxic phase cells, two distinct populations are observed, showing either an overlap or no overlap with the mitochondrial marker. For each condition, overlap was assessed in 100–150 cells of three independent transformants. Average results of overlap are noted with SDs, and unpaired t tests were performed to designate statistical difference of each type of colocalization between the WT strain and the sch9Δ strain at the postdiauxic phase (*p < 0.05, **p < 0.001).
Drug sensitivity assays

For analysis of sensitivity to myriocin and aureobasidin A, overnight yeast cultures in YPD were diluted to an OD_{600} of 0.5 in YPD and further incubated at 30°C for 4–6 h. Sensitivity of the yeast cultures to myriocin (Sigma-Aldrich, St. Louis, MO) was assayed by spotting 5-μl samples of 10-fold serial dilution of these yeast cultures on YPD plates containing 0.5 μg/ml myriocin. Growth was assessed after 48 h of incubation at 30°C. To determine sensitivity of the yeast cultures to aureobasidin A, 0.5 ml of these cultures was used to inoculate an YPD agar plate on which Whatman paper disks were placed containing 5 μl of 1 mg/ml aureobasidin A (Takara Bio, Shiga, Japan) in water. After 3 d of incubation at 30°C the size of the halo was measured. To assess sensitivity of yeast cultures to phytosphingosine (Sigma-Aldrich), cells were grown to stationary phase (>5 d) in YPD and subsequently diluted to an OD_{600} of 0.05 in YPD containing 5 or 15 μM phytosphingosine and 0.0015% Nonidet P-40. The OD_{600} of the different cultures was measured at regular time points.
points. For PI staining, yeast cells growing exponentially inYPD medium were treated for 2 h with 0.5 μg/ml myriocin. Next cells were collected, washed in phosphate-buffered saline, and stained with PI using the Annexin-V-Fluos Staining Kit (Roche Diagnostics, Vilvoorde, Belgium) according to the provided protocol.

Northern blot analysis
Cells were grown to an OD_{600} of 1.5 for a period of at least 24 h by repeated dilution. Samples were taken at the indicated time points, before and after the addition of 0.5 μg/ml myriocin. RNA extraction, probe preparation, and Northern blotting were performed as described previously (Swinnen et al., 2005). Hybridized blots were exposed to phosphorimager screens, and images were scanned with a Bas 1000 Phosphorlager (Fujix, Tokyo, Japan).

Sphingolipid analysis
Sphingolipid labeling and sphingolipidomics were performed as described previously (Kitagaki et al., 2007; Thevissen et al., 2010). In short, yeast cells growing exponentially in YPD medium were frozen in a methanol–dry ice bath and stored at −80°C. Electrospray tandem mass spectrometry (ESI-MS/MS) analysis of endogenous LCBs, their phosphates, and nonhydroxylated phytoceramide and dihydroceramide species was performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA). Sphingolipids were expressed in pmol, normalized for the level of phospholipids (Pi, in nmol) present in the Bligh-Dyer lipid extract: pmol/nmol of Pi (Bligh and Dyer, 1959). For the analysis of complex inositophosphoryl-containing sphingolipids, extracts were made from yeast cells grown to an OD_{600} of 5 in YPD medium using a procedure described previously (Stock et al., 2000). Sphingolipid analysis of the extracts was performed using ESI-MS on a Finnigan LCQ Deca mass spectrometer (Thermo Scientific) in the negative mode. Spectra were averaged over 100 scans. MS data were analyzed using Xcalibur software from Finnigan (Aerts et al., 2006).

Trichloroacetic acid protein extraction and Western blot
Samples for protein extraction were washed with 20% trichloroacetic acid (TCA) and stored at −80°C for extraction. Cells were resuspended in 20% TCA and broken with glass beads. Extracts were spun down at 4°C for 15 min at 13,000 rpm, and the pellet was washed twice with ice-cold acetone. Equal amounts of protein (Bradford quantification) were mixed with sample buffer and loaded onto an SDS–PAGE gel. Detection of the 9Myc-tagged proteins was performed with the anti-Myc antibody (BIOKE, Leiden, the Netherlands), and detection of internal Adh2 levels with the anti-Adh2 antibody (Millipore, Overijse, Belgium). Signals were quantified using UVP VisionWorks LS software (VWR, Leuven, Belgium).

β-Galactosidase assay
Promoters of the LAG1, LAC1, YDC1, and YPC1 genes were amplified using primers listed in Supplemental Table S3 and cloned into the BamHI and SalI sites of the multicopy lacZ reporter YEp357 (Serrano et al., 2002), yielding plasmids FBp701–704. Subcloning of SCH9 constructs was performed by cutting the SCH9 coding regions from plasmids pJU675, pJU822, and pJU841 (Urban et al., 2007) with Xhol and HindIII and ligating them into the pRS415 vectors, generating plasmids FBp705, FBp706, and FBp707. Precultures were grown overnight in SD-URA or SD-URA-LEU, inoculated into YPD medium, and grown for 6–7 h, after which part of the culture was treated with 200 nM rapamycin (Sigma-Aldrich). After 1 h, samples of treated and untreated cells were pelleted at 4°C and stored at −80°C. Cell pellets were resuspended in breaking buffer (16.1 g of Na_{2}HPO_{4}·7H_{2}O, 5.5 g of NaH_{2}PO_{4}·H_{2}O, 0.75 g of KCl, 246 mg of MgSO_{4}·7H_{2}O per liter) containing proteinase inhibitor cocktail tablets (Complete Mini, EDTA free; Roche Diagnostics) and broken using glass beads. The clarified extract was diluted in breaking buffer to a total volume of 100 μl and incubated at 30°C for 5 min before the reaction was initiated by adding 20 μl of 4 mg/ml o-nitrophenylgalactoside (Sigma-Aldrich). At a precise time, the reaction was terminated by addition of 50 μl of 1 M Na_{2}CO_{3}. OD_{405nm} was measured, and protein concentration was determined according to the Bradford method. We calculated specific activity from 1.7OD_{405nm}/(0.0045 × protein concentration × extract volume × time), expressed as nmol/min mg protein. Data are the mean of three independent transformants. Error bars represent SD.

Fluorescence microscopy
To study intracellular localization of Isc1, we received a construct expressing Isc1-GFP under the galactose-inducible promoter of the pYES2 vector (FBp431; Vaena de Avalos et al., 2004). To study Isc1-GFP localization under conditions using glucose as a carbon source, we cloned ISC1-GFP behind its native promoter into plasmid pRS426 (2 μ, URA3). For this, we used plasmid FBp431 as a template for PCR of the ISC1-GFP fusion construct using primers ISC1-fus5 and ISC1-fus4. Yeast genomic DNA was used for PCR of the ISC1 promoter using primers ISC1-fus1 and ISC1-RVC. Both PCR products contain overlapping ends with the pRS426 vector and each other, and a cotransformation of both PCR products with EcoRI-linearized pRS426 into yeast resulted in in vivo recombination to yield plasmid FBp708 (pISC1pr-ISC1-GFPuv construct in pRS426). Correct cloning was confirmed by sequencing. Intracellular localization of Isc1-GFP was scored by assessing colocalization with mCherry markers for mitochondria (plasmid FBp459; mCherry targeted to the mitochondria by the Cox4 presequence) and the ER (plasmid FBp709). The latter plasmid was created in analogy with the strategy used by Okamoto et al. (2006). In short, an ER-targeted version of mCherry was made by fusing the Kar2p signal-peptide sequence (first 135 nucleotides) to the coding region of mCherry containing a C-terminal HDEL retention sequence. This was achieved by cotransformation of PCR products using primers KAR2-F1 and KAR2-F2 (genomic DNA template) and primers mCherry-F1 and mCherry-F2 (FBp459 template), with a BamHI-linearized pYX242 plasmid. After recomposition in yeast, plasmid FBp709 was formed, expressing the KAR2_{1-135}-mCherry-HDEL fusion from the TPI promoter. Plasmids used are listed in Supplemental Table S2 and primers in Supplemental Table S3. Colocalization of Isc1-GFP (plasmid FBp431 or FBp708) with mCherry (plasmid FBp459 or FBp709) was carried out using a Leica DM 4000B fluorescence microscope (Leica Microsystems, Diegem, Belgium), and images were taken with a Leica DFC 420c camera using Leica Application software. Cultures were pregrown overnight on SG medium lacking uracil and leucine and transferred to YPGAL (FBp431) or YPD (FBp708) medium at an initial OD_{600} of 0.5. Green and red fluorescence was monitored after 6–8 h (exponential phase) and after 28 h (postdiauxic phase).

Chromological life span analysis and determination of ROS
For the analysis of chronological life span, cultures were pregrown in SG complete growth medium and diluted in flasks to an OD_{600} of 0.1 in SG complete medium containing 2% glucose for 28 h, which was set as time zero for longevity assessment. At the indicated time points, cells were plated out on YPD agar plates, and colony-forming units were counted after 2 d of incubation at 30°C. For determination of ROS accumulation, cells were stained with dihydroethidium, pictures were taken with the fluorescence microscope, and
quantification was performed by fluorescence measurements (excitation, 535 nm; emission, 595 nm), normalized for cell number by measuring OD_{595}, using a Beckman DTX880 plate reader (Molecular Devices, Wokingham, UK).

Reproducibility of results
All experiments were performed at least three times with similar results. Where applicable, quantification was performed, and results are shown as mean values ± SD (shown as error bars). For other experiments, representative results are shown.

ACKNOWLEDGMENTS
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Supplemental Materials
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### Table S1: Strains used in this study.

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<tr>
<td>sch9Δ isc1Δ</td>
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<td>This study (1)</td>
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<td>This study (1)</td>
</tr>
<tr>
<td>JW 04 601</td>
<td>BY4741 with C-terminal 9MYC tagged LAG1</td>
<td>This study (2)</td>
</tr>
<tr>
<td>JW 04 602</td>
<td>JW 03 038 with C-terminal 9MYC tagged LAG1</td>
<td>This study (2)</td>
</tr>
<tr>
<td>JW 04 603</td>
<td>BY4741 with C-terminal 9MYC tagged LAC1</td>
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JW 04 604  JW 03 038 with C-terminal 9MYC tagged LAC1  This study (2)
JW 04 606  BY4741 with C-terminal 9MYC tagged ISC1  This study (2)
JW 04 607  JW 03 038 with C-terminal 9MYC tagged ISC1  This study (2)
JW 04 610  BY4741 with C-terminal 9MYC tagged YDC1  This study (2)
JW 04 612  JW 03 038 with C-terminal 9MYC tagged YDC1  This study (2)
JW 04 613  BY4741 with C-terminal 9MYC tagged YPC1  This study (2)
JW 04 615  JW 03 038 with C-terminal 9MYC tagged YPC1  This study (2)

(1) Strains were created by crossing JW 01 306 (BY4741 with sch9::HIS3) with BY4742 strains containing the desired KANMX4 deletions, followed by sporulation and tetrad dissection. Triple deletion strains were made using appropriate single and double deletion strains for crossing and sporulation.

(2) For C-terminal 9Myc tagging, the Euroscarf plasmid pYM20 (9Myc tag, hphNT1 marker) was used as a template for PCR using primers containing 60bp overlap with the region directly before and after the stop codon of the target gene, as described in (Janke et al., 2004). Transformants were selected on hygromycin B containing medium and checked with colony PCR for correct integration of the 9Myc tag.

Table S2: Plasmids used in this study.

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<thead>
<tr>
<th>Name</th>
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<th>marker</th>
<th>Reference</th>
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<tr>
<td>FBp87</td>
<td>Yeplac195</td>
<td>pSCH9pr-SCH9</td>
<td>URA3</td>
<td>(Reinders et al., 1998)</td>
</tr>
<tr>
<td>pCUP-LAG1</td>
<td>pYEX 4T-1</td>
<td>pCUP1pr-GST-LAG1</td>
<td>URA3</td>
<td>(Martzen et al., 1999)</td>
</tr>
<tr>
<td>pCUP-LAC1</td>
<td>pYEX 4T-1</td>
<td>pCUP1pr-GST-LAG1</td>
<td>URA3</td>
<td>(Martzen et al., 1999)</td>
</tr>
<tr>
<td>FBp431</td>
<td>pYES2</td>
<td>ISCl-GFPuv</td>
<td>URA3</td>
<td>(Vaena de Avalos et al., 2004)</td>
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<td>FBp459</td>
<td>pH512</td>
<td>prADHpr-preCox4-mCherry</td>
<td>LEU2</td>
<td>Addgene 25444 (Benjamin Glick)</td>
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<td>FBp701</td>
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<td>FBp702</td>
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<td>FBp704</td>
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<td>KAR2(1&gt;135)-mCherry-HDEL</td>
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Table S3: Primers used in this study.

<table>
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<tbody>
<tr>
<td>LAG1-lacZFw</td>
<td>CCGGGATCCGATCTGTGGCCTTCAACAGTGCGATTCC (bp -780 → -754, BamHI)</td>
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<tr>
<td>LAG1-lacZRv</td>
<td>CCGGTGCACTGATAGATTGGTGGCATGATGTC (bp 30 → 3, Sall)</td>
</tr>
<tr>
<td>LAC1-lacZFw</td>
<td>CCGGGATCCCTTCTTCTGTGCCTTTATGATCCGATTGG (bp -750 → -725, BamHI)</td>
</tr>
<tr>
<td>LAC1-lacZRv</td>
<td>CCGGGATCCCTTTCTGTGCCTTTATGATCCGATTGG (bp 18 → -21, 6bp mutated (C→G) to remove endogenous Sall site)</td>
</tr>
<tr>
<td>YDC1-lacZFw</td>
<td>CCGGGATCCACGGCAAGAGTGTTC (bp -750 → -727, BamHI)</td>
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</table>
YDC1-lacZrv  CCGGTCGACGGCTTCTGGATAAAGCCAGCTGAACAGC  (bp 30 → 3, SalI)
YPC1-lacZFw  CCGGGATCCGGCTTCTGGATAGTTCAAACGAAATATTTCCC  (bp 30 → 3, SalI)
YPC1-lacZrv  CCGGTCGACCTCTGGATAGTTCAAACGAAATATTTCCC  (bp 30 → 3, SalI)
ISC1-fus1  cccgccccccccccccctcgaggtcgacggtatcgataagcttgatatcgGCGCGAGGTCGCCCTGGACGG (ISC1: bp -795 → -774)
ISC1-RVC  GAGTCTTTTTTTACGGTGTTTG (ISC1: bp 180 → 158)
ISC1-fus5  ATGTACAACAGAAAGACAGAGATG (ISC1: bp 1 → 25)
ISC1-fus4  gcggtgctggctgtctgataactagtagctgatccccccgggcatgcGCAAATTAAGCCTTGAGCG (CYC1ter: bp 248 → 229)
KAR2-F1  gatttcaacctttgcctcgacagctaacgatgcacacgggATGTGGTTTCAACAGACTAAGCGC (KAR2: bp 1 → 23)
KAR2-F2  gaactctgtatcagtcagctaacgatgcacacgggATGTGGTTTCAACAGACTAAGCGC (KAR2: bp 135 → 112)
mCherry-F1  aatttctttgtctcctctatagtttgatagatgctgCAGAGG (mCherry 4 → 22)
mCherry-F2  ggaaacagttacttcttaacagctagctagctgatctgCTTGATCACGCTCGTCCATGC (mCherry: bp 705 → 686)
Figure S1: Genetic interaction of SCH9 with enzymes involved in sphingolipid metabolism.

(A) Serial dilutions of exponential growing wild type, single, double and triple deletion strains on YPD medium with or without the addition of 0.5 μg/ml myriocin. The wild type and the single and double deletion mutants with a BY background are segregants from a diploid strain, created by crossing the sch9Δ (JW 01 306; background BY4741) strain with the respective single deletion strains with background BY4742. Triple deletions are segregants from a diploid strain, created by crossing a double deletion mutant with the single deletion mutant carrying the third deletion. (B) Additional deletion of SUR4 or ISC1 in the sch9Δ strain increases myriocin resistance. Double deletion strains were spotted directly below the sch9Δ strain, to better observe the increase in myriocin resistance. (C) Synthetic sick phenotype of a sch9Δ sur4Δ strain. Diploid cells, heterozygous for SCH9 and SUR4 were sporulated, and tetrads were dissected on YPD (in horizontal rows). Genotypes are indicated. (D) Overexpression of the ceramide synthases LAG1 or LAC1 suppresses the myriocin
resistance of the \textit{sch9Δ} strain. Spottest on SD-URA with or without addition of myriocin of the WT (BY4741) and the \textit{sch9Δ} strain (JW 03 038) transformed with plasmids with Cu$^{2+}$ inducible over-expression of \textit{LAC1} and \textit{LAG1} (Martzen \textit{et al.}, 1999) or an empty vector (ev).

**Figure S2: Deletion of \textit{SCH9} or rapamycin treatment of cells does not affect \textit{Isc1} protein levels.**

The gene encoding the inositol phosphosphingolipid phospholipase C (\textit{ISC1}) was genomically tagged with a 9Myc tag in both WT BY4741 and \textit{sch9Δ} (JW 03 038) strains. Overnight precultures were inoculated at an initial OD$_{600}$ of 0.3 and grown to OD$_{600}$ 2.0, after which a sample was withdrawn for protein extraction (noted as -). The remainder of the culture was treated with 200nM rapamycin and grown for an additional hour, after which a second sample for protein extraction was taken (noted as +). After TCA protein extraction, equal protein amounts were run on a SDS-PAGE. The tagged enzyme was detected using an anti-Myc antibody, the yeast Adh2 serves as an internal control. A representative result for the western blot is shown in (A). Signals for Myc detection were quantified and normalized for Adh2 levels. Average results of two independent cultures are shown in (B), with standard deviations noted as error bars.

**Figure S3: Mitochondrial translocation of \textit{Isc1} on galactose medium is impaired in the absence of Sch9.**

Wild type (BY4741) and \textit{sch9Δ} (JW 01 306) expressing \textit{Isc1-GFP} (from a galactose-inducible promoter, plasmid FBp431) and a red fluorescent mitochondrial marker protein (plasmid FBp459, containing fluorescent mCherry targeted to the mitochondria by the Cox4 presequence) were grown in YPGAL medium and localization was analyzed at the early exponential and post-diauxic growth phase using fluorescence microscopy. Bright field, single channel and the corresponding merge are shown for both growth phases. Representative pictures are shown.
Figure S4: Sch9 and Isc1 modulate apoptotic cell death.
Tests for cell death markers of apoptosis and necrosis (Annexin/PI co-staining) were performed as described by (Buttner et al., 2008). The wild type (BY4741), sch9Δ (JW 01 306), isc1Δ and sch9Δisc1Δ strains were inoculated in fresh synthetic complete medium at an OD600 0.1. After 12h (exponential phase) and 36h (post-diauxic phase) cells were stained using Annexin-V-FITC and propidium iodide (PI), to detect phosphatidyl serine externalization and loss of membrane integrity, respectively, as described by (Buttner et al., 2008). Quantitative flow cytometric analysis, using a BD influx flow cytometer, on the apoptotic/necrotic markers was performed and analysis was carried out using FlowJo software. Results depicted are mean values ± SD of three independent cultures.
SUPPLEMENTARY REFERENCES


