Down-Regulating Sphingolipid Synthesis Increases Yeast Lifespan

Xinhe Huang
University of Kentucky, xinhe.huang@uky.edu

Jun Liu
University of Kentucky, jun.liu-scu@uky.edu

Robert C. Dickson
University of Kentucky, bobd@uky.edu

Follow this and additional works at: https://uknowledge.uky.edu/biochem_facpub

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Repository Citation
Huang, Xinhe; Liu, Jun; and Dickson, Robert C., "Down-Regulating Sphingolipid Synthesis Increases Yeast Lifespan" (2012). Molecular and Cellular Biochemistry Faculty Publications. 43.
https://uknowledge.uky.edu/biochem_facpub/43

This Article is brought to you for free and open access by the Molecular and Cellular Biochemistry at UKnowledge. It has been accepted for inclusion in Molecular and Cellular Biochemistry Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
Down-Regulating Sphingolipid Synthesis Increases Yeast Lifespan

Digital Object Identifier (DOI)
http://dx.doi.org/10.1371/journal.pgen.1002493

Notes/Citation Information
Published in PLOS Genetics, v. 8, issue. 2, e1002493.

© 2012 Huang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Introduction

Most of us dream of a long life and wonder if we can increase our time on earth. Except for caloric restriction (CR), there has been little prospect for increasing lifespan. But recent studies offer hope for developing drugs that increase lifespan by reducing the incidence of age-related diseases that kill most humans. The immunosuppressant drug rapamycin has been shown to reduce age-related diseases and increase lifespan in yeasts, flies and mice [1–3] and these results suggest that pharmacological down-regulation of one or more sphingolipids may provide a means to reduce age-related diseases and increase lifespan in other eukaryotes.

Abstract

Knowledge of the mechanisms for regulating lifespan is advancing rapidly, but lifespan is a complex phenotype and new features are likely to be identified. Here we reveal a novel approach for regulating lifespan. Using a genetic or a pharmacological strategy to lower the rate of sphingolipid synthesis, we show that *Saccharomyces cerevisiae* cells live longer. The longer lifespan is due in part to a reduction in Sch9 protein kinase activity and a consequent reduction in catabolic processes, such as autophagy, become more active in order to promote biosynthetic processes such as protein synthesis [10,23]. Longevity also arises in ways that are independent of Sch9 or caloric restriction, and we speculate on ways that sphingolipids might mediate these aspects of increased lifespan. Sch9 and its mammalian homolog S6 kinase work downstream of the target of rapamycin, TOR1, protein kinase, and play evolutionarily conserved roles in regulating lifespan. Our data establish Sch9 as a focal point for regulating lifespan by integrating nutrient signals from TOR1 with growth and stress signals from sphingolipids. Sphingolipids are found in all eukaryotes and our results suggest that pharmacological down-regulation of one or more sphingolipids may provide a means to reduce age-related diseases and increase lifespan in other eukaryotes.
Sphingolipids Control Lifespan

Author Summary

Studies with rats in the 1930s showed a surprising increase in lifespan when the diet contained 30%-40% fewer calories than normal. This experiment has been repeated on many organisms and is the gold standard for extending lifespan. While we are beginning to understand how calorie restriction regulates lifespan, the mechanisms are complex and much remains to be learned. In the work presented here, we demonstrate a novel way to increase lifespan in the budding yeast Saccharomyces cerevisiae. Our strategy is to lower the rate of sphingolipid synthesis either by reducing the synthesis of the first enzyme in the biosynthesis pathway or by using a drug to reduce enzyme activity. This strategy works in part by lowering the activity of a protein kinase, termed Sch9 in yeast and S6K in mammals, both of which are involved in the processes affected by calorie restriction and that control lifespan. In addition, we find that reducing sphingolipid synthesis increases lifespan in ways that are independent of Sch9 and calorie restriction. Since sphingolipids are found in all eukaryotes, our results suggest that pharmacological down-regulation of one or more sphingolipids may provide a means to reduce age-related diseases and increase lifespan in other eukaryotes.

Results

Genetic strategy for down-regulating sphingolipid synthesis

In one strategy to determine if sphingolipids regulate CLS we used a tetracycline-repressible promoter cassette (tetO7) to regulate gene transcription [28]. The utility of this strategy for studying CLS was verified by using cells having transcription of the SCH9 gene (tetO7-SCH9) or the RIM15 gene (tetO7-RIM15) controlled by the tetO7 promoter. Based on the roles of Sch9 and Rim15 in lifespan [7], we expected CLS to increase in doxycycline (Dox)-treated tetO7-SCH9 cells and to decrease in Dox-treated tetO7-RIM15 cells. We find that a low concentration of Dox (100 ng/ml) significantly increases the CLS of tetO7-SCH9 cells (Figure 1B) while a higher dose of Dox (1000 ng/ml) is needed to decrease the CLS of tetO7-RIM15 cells (Figure 1C). In control experiments we find that Dox treatment has no effect on the CLS of the parental strain R1158 (Figure 1B and 1C, WT). Thus, this experimental strategy is able to recapitulate the CLS phenotype of sch9Δ and rim15Δ mutant cells. It also recapitulates the increased oxidative stress resistance of sch9Δ cells and the reduced resistance of rim15Δ cells [7] (Figure 1D).

Down-regulating tetO7-LCB1 and tetO7-LCB2 increases CLS

The essential LCB1 and LCB2 genes encode subunits of serine palmitoyltransferase (SPT), the first and rate-limiting enzyme in sphingolipid biosynthesis (Figure S1 in Text S1) [15] and we expected that by lowering expression of LCB1 or LCB2 it should be possible to lower the level of sphingolipids and extend lifespan via reduction of the sphingolipid-controlled Pkh1/2-Sch9 pathway. We identified concentrations of Dox that reduce the initial rate of growth but not the final cell density of strains with tetO7-LCB1 or tetO7-LCB2 alleles, consistent with a lowered rate of sphingolipid synthesis but not low enough to affect the final cell density or mass yield (Figure 2A and 2B). Under these conditions Dox-treated and untreated cells should be in a similar metabolic state upon entering stationary phase and any effect of Dox on lifespan should be due to down-regulation of LCB1 or LCB2 rather than to off target effects.

We find that the CLS of tetO7-LCB1 or tetO7-LCB2 cells treated with 100 or 300 ng/ml of Dox increases significantly (Figure 2C and 2D). Control experiments demonstrate that this increase in CLS is due to down-regulation of LCB1 or LCB2 and not to off target effects of Dox. First, Dox treatment has no effect on the CLS of wild-type R1158 cells, the parent of tetO7-LCB1 and tetO7-LCB2 cells (Figure 1B and 1C). Next, we reasoned that introduction of a plasmid-borne wild-type LCB1 gene into tetO7-LCB1 cells should preclude Dox from increasing CLS, since the wild-type LCB1 allele will not respond to Dox and SPT activity should be normal. As expected, the CLS of Dox-treated tetO7-LCB1 cells transformed with a single-copy vector carrying the LCB1 gene is reduced compared to cells transformed with just the vector and is similar to cells not treated with Dox (Figure 2E). Analogous experiments with tetO7-LCB2 cells give similar results (Figure 2F). We conclude from these data that Dox treatment of cells carrying a tetO7-LCB1 or tetO7-LCB2 allele increases CLS because expression of the LCB1 or LCB2 gene is reduced.

Finally, most experimental manipulations enhance lifespan in part by increasing stress resistance. Thus we anticipated that down-regulating the tetO7-LCB1 or tetO7-LCB2 genes by Dox treatment would increase stress resistance and, indeed, resistance to heat and hydrogen peroxide stress did increase (Figure 2G).

Myriocin treatment increases CLS

A second strategy for determining if sphingolipids regulate CLS uses myriocin, an inhibitor of SPT, to down-regulate enzyme activity. We examined the effect of low doses of myriocin on three strains: DBY746, used extensively to study CLS [7], BY4741, the parent of one of the non-essential yeast gene deletion sets which is often used to study lifespan [8], and a derivative of BY4741, R1158, the parent strain for making tetO7-regulated genes [29]. The initial rate of R1158 cell growth is the least impaired by myriocin treatment while growth of DBY746 and BY4741 cells is more impaired (Figure 3A, 3C and 3E). The highest concentration of myriocin used for each strain causes a delay in initial cell growth and increases the time it takes for DBY746 and BY4741 cells to enter stationary phase. However, the final cell density is either the same as or within 20% of the cell density found in the absence of drug, which argues that differences in final cell density or failure to enter stationary phase are not likely to explain the effect of myriocin on CLS. Using these same drug concentrations, there is a robust, dose-dependent and statistically significant increase in the CLS of each strain (Figure 3B, 3D and 3F) with DBY746 cells showing the largest increase (Figure 3B). DBY746 cells treated with 400 ng/ml of myriocin grow slowly and, unlike the other cells, do not stop growing at 72 hrs (CLS day 1), but keep growing up to about the 120 hr time point. This causes an increase in viable cells on CLS days 3 and 6 (Figure 3B, solid red line) and overstates the increase in CLS. To compensate for this extended growth phase, we set the 120 hr time point as CLS day 1.
(compared to the 72 hr time point for the other cultures). This corrected survival curve still shows an increase in lifespan (Figure 3B, dashed red line), but not as large an increase as the uncorrected curve (Figure 3B, solid red line).

Myriocin treatment, similar to down-regulation of LCB1 or LCB2 expression, increases resistance of DBY746 cells to both heat and hydrogen peroxide stress (Figure 3G). In summary, the data presented in Figure 3 provide additional and independent support for the hypothesis that sphingolipids regulate stress resistance and CLS.

Wild-type yeast cells normally grow to a specific size, but mutation of SCH9 has the unusual effect of reducing cell size [29]. If a reduction in sphingolipid synthesis increases CLS by reducing Sch9 activity as we predict (Figure 1A), then cell size ought to be reduced. We find that the volume of myriocin-treated DBY746 cells on CLS day 1 is 28% lower than that of untreated cells (Figure S2 in Text S1, 59 versus 82 μm³). This reduction is less than the 40% reduction reported for sch9A cells compared to wild-type DBY746 cells [30], but probably reflects just a partial reduction of Sch9 activity in myriocin-treated cells (see below) compared to no activity in sch9A cells.

Acetic acid accumulation in the diauxic shift has been proposed as a major cause of cell death, but it can be alleviated by buffering the medium [31]. To eliminate the possibility that myriocin treatment extends CLS by preventing acetic acid killing, we compared the effect of myriocin treatment in buffered and unbuffered medium. Medium was buffered with succinate (200 mM), pH 4.5, because this compound is not metabolized by yeast cells. Myriocin treatment extended CLS in either buffered or unbuffered media (Figure 3H). Thus, myriocin treatment does not extend lifespan simply by increasing resistance to acetic acid and additional support for this conclusion is provided below. Also, we found that the rate of cell death increased because the concentration of iron (1.23 μM) in SDC medium was limiting and we added 3-fold more iron to the medium (indicated in figure legends) to eliminate this variable. The succinate-buffered medium

Figure 1. The tetO7 promoter can be used to regulate CLS. (A) Model showing how sphingolipids are proposed to regulate lifespan. In this model LCBs and perhaps other sphingolipids activate the Pkh1 and Pkh2 protein kinases which phosphorylate residue T570 in the activation loop of the Sch9 protein kinase. Activation of Sch9 also requires phosphorylation of C-terminal residues by the TORC1 kinase. Ceramide-mediated activation of the Sit4 protein phosphatase may also play roles in regulating lifespan as outlined in the Discussion. (B) CLS of tetO7-SCH9 (RCD952) and WT (R1158) cells +/- Dox treatment. Data show the mean ± SEM of surviving cells (** p<0.01, No Dox vs Dox treatment). Triplicate cultures were used in these and all other CLS experiments. (C) CLS of tetO7-RIM15 (RCD994) and WT (R1158) cells +/- Dox treatment. Data show the mean ± SEM of surviving cells (p<0.05, ** p<0.01, No Dox vs Dox treatment). (D) Oxidative stress (H₂O₂) resistance of cells from CLS day 1. Strains are: WT, R1158; tetO7-SCH9, RCD952; tetO7-RIM15, RCD994.

doi:10.1371/journal.pgen.1002493.g001
Figure 2. Down-regulating sphingolipid synthesis increases CLS. (A) Growth rate of tetO7-LCB1 cells (RCD956) treated with Dox to down-regulate expression of the tetO7promoter and reduce the rate of sphingolipid synthesis. (B) Growth rate of tetO7-LCB2 cells (RCD957) +/− Dox treatment. (C) CLS of tetO7-LCB1 cells +/− Dox treatment. Data show the mean ± SEM of surviving cells (* p<0.05, ** p<0.01, No Dox vs 100 ng/ml Dox). (D) CLS of tetO7-LCB2 cells +/− Dox treatment. Assays are similar to those presented in (C). (E) Verification that Dox-treatment extends CLS by reducing expression of the tetO7-LCB1 target gene. CLS of tetO7-LCB1 cells transformed with an empty vector (Vec1 = pRS315ADE2) or a vector carrying LCB1 (pLCB1 = pRS315ADE2-LCB1-S) is shown. Data represent the mean ± SEM of surviving cells (* p<0.05, ** p<0.01, for Vec1, 100 ng/ml Dox).
Dox vs pLCB1, 100 ng/ml Dox). (F) Control experiment showing that Dox-treatment extends CLS by reducing expression of the tetO7-LCB2 gene. CLS of tetO7-LCB2 cells transformed with a vector carrying LCBl (pLCB1 = pRS315-LCBl-B7) or with an empty vector (Vec2 = pRS315) is shown. Data represent the mean ± SEM of surviving cells (*p < 0.05, **p < 0.01, for Vec2, 100 ng/ml Dox vs pLCB2, 100 ng/ml Dox. (G) Resistance of Dox-treated WT (R1158), tetO7-LCB1 (RCD956) and tetO7-LCB2 (RCD957) cells to heat (35°C) or hydrogen peroxide (H2O2) stress. Photographs show a ten-fold dilution series of cells (from left to right).

doi:10.1371/journal.pgen.1002493.g002

may have a small affect on lifespan due to higher osmolarity as noted before [32], since we found that reducing the concentration from 200 to 50 mM caused a slight reduction in CLS, but no change in pH during the assay. The low pH of this medium is advantageous because the solubility of iron increases greatly at lower pHs [33].

Down-regulating LCB1 or SPT lowers sphingolipid levels

If sphingolipids mediate increased CLS, then we predict that down-regulating tetO7-LCB1 expression by Dox treatment or reducing SPT activity by myriocin treatment should lower sphingolipid levels. We measured LCBs, made primarily as intermediates in the de novo sphingolipid synthesis pathway, as well as the terminal complex sphingolipids found primarily in the plasma membrane. We also measured LCBPs made by phosphorylation of LCBs and thought to primarily represent intermediates in the degradation of LCBs (Figure S1 in Text S1).

We find that Dox treatment of tetO7-LCB1 cells, grown the same way as in a CLS assay, reduces total LCBs (C16-, C18- and C20-dihydrosphingosine and C18- and C20-phytosphingosine) by 84% with individual species of LCBs responding uniquely to drug treatment (Figure 4A). The much less abundant LCBPs are also reduced in toto by about 70% (Figure 4A). Dox treatment of wild-type R1158 cells does not produce a statistically significant change in the level of LCBs and LCBPs (data not shown).

Similarly, myriocin treatment of DBY746 cells, grown in the same way as in a CLS assay, reduces total LCBs by 64% and total LCBPs 62% (Figure 4B).

Next, we examined the effect of down-regulating sphingolipid synthesis on complex sphingolipids including inositol-phosphoceramide (IPC), mannosyl-inositol-phosphoceramide (MIPC) and mannosyl-inositol-P2-ceramide (M(IP)2C) (Figure S1 in Text S1). There is a statistically significant reduction in IPCs in both Dox treated tetO7-LCB1 cells and myriocin-treated DBY746 cells (Figure 4C, 4D and 4E). Because MIPCs are not well separated from IPCs (Figure 4C), both types of sphingolipids, not just IPCs, may be reduced in drug-treated cells. There is a tendency for M(IP)2C8s to decrease in Dox-treated tetO7-LCB1 cells but to increase in myriocin-treated DBY746 cells. However, neither change is statistically significant, suggesting that M(IP)2C8s are not playing roles in CLS. Our data show that down-regulating the rate of sphingolipid synthesis by impairing activity of the first enzyme in the biosynthesis pathway lowers the concentration of LCBs, LCBPs, IPCs and perhaps MIPCs and some or all of these changes could influence CLS.

Phk2 regulates CLS

As further support for our hypothesis that sphingolipids regulate CLS by controlling the Pkh1/2-Sch9 signaling pathway, we sought to show that PKH1 or PKH2 or both genes regulate CLS. Recently, deletion of PKH2 in the diploid BY4743 strain background was shown to produce a small but significant increase in CLS [34]. To verify these data and extend them to a haploid strain background and culture conditions with a different pH and buffering ions, we measured the CLS of a pkb2A mutant made in the R1158 strain background. We find a small but statistically significant increase in the CLS of pkb2A cells (Figure 5A). Our data are very similar to results obtained with BY4743 cells where the mean survival interval (area under a survival curve) was 3.10 for wild-type cells compared to 4.05 (34% increase) for pkb2A cells whereas our values are 4.14 verses 5.23 (29% increase).

Together, the published data and our data establish a role for Pkh2 in regulating CLS.

Phosphorylation of residue T570 in Sch9 is critical for protein kinase activity and is mediated in vivo and in vitro by Pkh1/2 [20,25] and is stimulated by LCBs [21]. If Pkh1/2 regulate Sch9 activity by phosphorylating residue T570, we anticipate less phosphorylation in pkb2A cells, since this would reflect reduced Sch9 activity and would explain the increase in CLS of pkb2A cells. We find a statistically significant 20% reduction in T570 phosphorylation in pkb2A cells compared to wild-type PKH2 cells which supports the hypothesis that Pkh2 phosphorylates this key Sch9 residue (Figure 5B and 5C).

Another prediction of the proposed signaling pathway going from sphingolipids to Pkh1/2 to Sch9 and other downstream kinases (Figure 1A) is that growth of cells with a low level of Pkh kinase activity ought to be more sensitive to myriocin (reduced SPT activity) compared to cells with a higher level of kinase activity if sphingolipids regulate Pkh activity. Comparison of the myriocin sensitivity of cells with varying combinations of mutant and wild-type PKH1 and PKH2 alleles shows that cells with a temperature-sensitive PKH1 allele or a deletion allele are very sensitive to myriocin at a permissive temperature compared to cells with PKH1 or PKH2 or both wild-type genes (Figure 5D). Likewise, deletion of either gene in a tetO7-LCB1 strain reduces Pkh activity and increases sensitivity to myriocin (Figure S4A in Text S1), moreover, Dox treatment further reduces Pkh activity by lowering sphingolipid levels and impairs growth compared to the parent tetO7-LCB1 (PKH1 PKH2) strain (Figure S4B in Text S1). These data all support the proposed role of sphingolipids as upstream activators of the Pkh1/2 kinases.

Sphingolipids control phosphorylation of Sch9-T570 and Sch9 substrates

To explore further the hypothesized role of sphingolipids in regulating CLS via the Pkh1/2-Sch9 signaling pathway, we examined phosphorylation of Sch9 residue T570. If sphingolipids control T570 phosphorylation, then reducing SPT activity by myriocin treatment or by Dox treatment of tetO7-LCB1 cells should decrease phosphorylation. Cells were grown as in a CLS assay, but because drug-treated cells grow slower than untreated cells, comparisons were made with cells grown to similar OD600 nm values (~) rather than for similar times.

We find a statistically significant 35% reduction in T570 phosphorylation in log phase DBY746 cells treated for 7–8 cell doublings with myriocin compared to untreated cells (Figure 5E and 5F, n = 4 cultures). Also, in parental R1138 (LCBl) and tetO7-LCB1 cells myriocin treatment reduces T570 phosphorylation by 40% (Figure 5G, compare lane 1 with 2 and lane 3 with 4). Lastly, there is a 40% reduction in T570 phosphorylation in Dox-treated tetO7-LCB1 cells (Figure 5H, compare lanes 3 and 4) but no effect in parental R1138 cells, as expected since LCBl is not regulated by the tetO7 promoter (Figure S5H, compare lanes 1 and 2). These data
support the hypothesis that down-regulating sphingolipid levels by genetically or pharmacologically lowering SPT activity reduces T370 phosphorylation and consequently Sch9 activity thereby promoting increased CLS.

In all cases examined (Figure 5E, 5G and 5H), the total level of Sch9 protein is not changed by drug treatment. However, drug treatment does cause a shift of the Sch9 band to a slightly faster migrating form, similar to what is seen when phosphorylation of Sch9 is reduced [20]. In the case where Pkh activity was reduced by deleting PHK2 rather than by down-regulating sphingolipid synthesis, there was no shift in the mobility of Sch9 to a faster migrating form even though phosphorylation of T370 decreased (Figure 5B). Thus, the band shift we observe suggests that a reduction in sphingolipids triggers dephosphorylation of other Sch9 amino acid residues in addition to T370 and further work will be required to identify the residues involved.

The effect of myriocin treatment on the phosphorylation level of the Sch9 substrate Dot6 was also examined. Sch9 regulates ribosome biogenesis in part by phosphorylating transcriptional repressor proteins including Dot6, Tod6 and Sth3 [35,36]. Dot6 controls transcription of ribosome biogenesis [37] genes that are essential for ribosome formation. We find that Dot6-5HA in myriocin-treated cells migrates faster on a denaturing gel than it does in untreated cells (Figure 5I), indicating reduced phosphorylation by Sch9 [36]. To directly demonstrate reduced Sch9 activity in these myriocin-treated cells, the blot shown in Figure 5I was reprobed for T570 phosphorylation. As found in other settings, the band shift we observe suggests that a reduction in sphingolipids triggers dephosphorylation of other Sch9 amino acid residues in addition to T370 and further work will be required to identify the residues involved.

Sch9 also affects aging and lifespan by controlling genome stability [40,41]. For example, cells display premature age-dependent genome instability that is prevented by deletion of SCH9 [42]. Sgs1 is a member of the RecQ helicase family of DNA unwinding proteins which maintain genome stability. Sgs1 is related to human RecQ helicases WRN, defective in the premature aging disorder Werner’s syndrome, and BLM, defective in the cancer-prone disorder Bloom’s syndrome [42]. If sphingolipids control Sch9 activity, then age-dependent genome instability in a sgs1Δ strain should be prevented by treating cells with myriocin.

A genome instability was quantified by measuring the frequency of Can’ mutations. As in previous studies [42], we find Can’ mutations accumulating at a faster rate in sgs1Δ cells during chronological aging and by day 12 they are 4-fold more abundant than in wild-type cells (Figure 6D). Deletion of SCH9 reverses the effect of the sgs1Δ mutation since cultures of sgs1Δ sch9Δ cells have fewer Can’ mutants than the wild-type cultures. Notably, treating sgs1Δ cells with myriocin reduces the frequency of Can’ mutants to the wild-type level at all times during a CLS assay (Figure 6D).

In a second assay of genome instability the frequency of gross chromosomal rearrangements (GCRs) was measured [42]. By day 12 there are five times more GCRs in sgs1Δ cells than in wild-type or sgs1Δ sch9Δ cells (Figure 6E). Again, myriocin treatment reduces the frequency of GCRs about 75% at day 12 in sgs1Δ cells, very similar to the reduction obtained by deleting SCH9 (sgs1Δ sch9Δ cells, Figure 6E). In summary, the data presented in Figure 6 support the hypothesis that sphingolipids regulate CLS by Sch9-dependent mechanisms.

Finally, if myriocin enhances CLS entirely by mechanisms requiring Sch9, then myriocin should not enhance CLS in sch9Δ cells. We find that a dose of 25 or 100 ng/ml myriocin has no effect on the CLS of sch9Δ cells, but a higher dose of 300 ng/ml produces a statistically significant increase in CLS starting around day 22 (Figure 2A). Thus, myriocin most likely extends CLS in the early and middle time-frame of a CLS assay by Sch9-dependent mechanisms, but at later times the enhancement in lifespan is independent of Sch9. Possible mechanisms for the Sch9-independent effects on CLS are presented in the Discussion.

Down-regulating sphingolipid synthesis extends CLS in a caloric-restricted cells

Since calorie restriction increases the CLS of sch9Δ cells [30], we determined if myriocin produces an effect similar to CR. CR

Figure 3. Myriocin treatment extends CLS. (A) Growth rate of DBY746 cells +/- myriocin (Myr) treatment. Myriocin (300 ng/ml) treatment reduces cell size and there are fewer cells/A600 nm in the treated than in the untreated cultures (1.6 x 10^7 cells/A600 nm in untreated cultures. (B) CLS of DBY746 cells treated as described in (A). Data represent the mean +/- SEM of surviving cells (* p < 0.05, ** p < 0.01, No Myr vs 200 and 400 ng/ml Myr). To compensate for the slow growth and extended growth phase of cells treated with 400 ng/ml of myriocin (solid red line), the viability at the 120 hr time point, CLS day 3, was set as CLS day 1 (dashed red line). (C) Growth rate of R1158 cells +/- myriocin (Myr) treatment. Cells/A600 nm unit are similar to those described in (A). (D) CLS of R1158 cells treated as described in (C). Data represent the mean +/- SEM of surviving cells (* p < 0.05, ** p < 0.01, No Myr vs 300 ng/ml Myr). (E) Resistance of myriocin-treated DBY746 cells to heat (55 °C). (F) Resistance of myriocin-treated DBY746 and R1158 cells to heat (55 °C) or hydrogen peroxide (H2O2) stress. Photographs show a ten-fold dilution series of cells (left to right). (H) CLS of DBY746 cells grown in SDC medium buffered to pH 6 or 4.5 +/- myriocin (Myr) treatment. Data represent the mean +/- SEM of surviving cells (* p < 0.05, ** p < 0.01, No Myr vs 300 ng/ml Myr).
Figure 4. Myriocin or Dox treatment reduces sphingolipid levels. (A) LCBs and LCBPs were quantified in cells grown from an A600 nm of 0.005 to 1 in the presence of absence of Dox (300 ng/ml). Average values for triplicate cultures of tetO7-LCB1 (RCD956) cells are shown (* p < 0.05, ** p < 0.01, *** p < 0.001, No Dox vs Dox-treated). (B) LCBs and LCBPs were quantified in DBY746 cells treated or not treated with Myr (400 ng/ml) grown the same way as in (A). Average values for triplicate cultures are shown (* p < 0.05, ** p < 0.01, *** p < 0.001, No Myr vs Myr-treated). (C) Complex sphingolipids (IPCs, MIPCs and M(IP)2Cs) in untreated and Dox-treated RCD956 cells were grown as in (A) in the presence of [3H]-myoinositol and cpm were quantified by scanning the thin-layer chromatogram on a Bioscan apparatus. Glycerol –P-inositol represents the deacylation product of phosphatidylinositol and serves as an internal control. (D) Average cpm +/- SD for [3H]-labeled lipids from three cultures analyzed as in (C). (E) Average cpm +/- SD for [3H]-labeled lipids from three cultures of DBY746 cells treated or not treated with Myr (400 ng/ml). Cells were grown and analyzed as in (C).

doi:10.1371/journal.pgen.1002493.g004
was implemented by switching cells on day 3 from culture medium to water. This extreme form of CR was used because it prevents adaptive regrowth and eliminates regrowth as an explanation for myriocin-induced CSL extension [40]. We find that myriocin produces a dose-dependent increase in the CSL of cells subjected to extreme CR (Figure 7B). Thus, myriocin extends CSL in sch9A cells similar to CR treatment, extends CSL even under conditions where adaptive regrowth does not occur, and programs cells for an extended lifespan prior to the end of the diauxic shift (prior to shifting cells to water). In addition, these data further support the conclusion that myriocin does not simply extend CSL by protecting cells from acetic acid killing, since replacing the culture medium with water and changing the water every third day eliminates acetic acid accumulation. Finally, we find that myriocin also enhances CSL when CR is applied by reducing the concentration of glucose from 2 to 0.5% (Figure S3 in Text S1).

Discussion

The desire for a long and healthy life is universal, but most of us will succumb sooner than hoped for due to age-related disorders, particularly cancer, cardiovascular disease and diabetes. Fortunately, strategies that enhance lifespan in model organisms work to a large degree by delaying the onset of these diseases. A striking example of this relationship is seen in monkeys during CR where the incidence of age-related diseases decreases while lifespan increases [43,44]. CR is not a practical strategy to extend lifespan in humans, but pharmacological agents are, and the rapamycin-type compounds seem quite promising [4–6]. As promising as rapamycin and related drugs are for improving human health and extending lifespan, they may have unforeseen drawbacks and be suitable only for individuals with specific genotypes. Hence, other strategies are needed.

Sphingolipids regulate yeast lifespan

Our data show that down-regulating sphingolipid synthesis produces an increase in the CSL of S. cerevisiae cells (Figure 2, Figure 3, and Figure 7) and that part of this increase depends on the Sch9 protein kinase (Figure S2 in Text S1, Figure 5, Figure 6, and Figure 7A), an established regulator of lifespan. We show that CSL can be increased by lowering sphingolipid synthesis via manipulation of SPT, the initial enzyme in sphingolipid biosynthesis (Figure 2, Figure 3, and Figure 7). We propose that lowering SPT activity increases CSL, at least in part, by reducing the concentration of LCBs thereby lowering the activity of Phk1/2 and Sch9 which programs cells for an extended lifespan (Figure 1A). This scenario is supported by data showing that myriocin treatment or down-regulation of tetO7-LCB1 gene expression diminishes Phk1/2-dependent phosphorylation of Sch9 residue T570, essential for activity (Figure 5) and reduces phosphorylation of the Sch9 substrate Dot6 (Figure 5E). In addition, as a surrogate for Sch9 activity in vivo, we measured processes that play key roles in lifespan and are regulated by Sch9. All processes including resistance to heat, oxidative and acetic acid stress (Figure 2, Figure 3, Figure 6A and 6B), cell size (Figure S2 in Text S1), mitochondrial oxygen consumption (Figure 6C), and genome stability (Figure 6D and 6E) responded to down-regulation of sphingolipid synthesis in a manner indicative of reduced Sch9 activity.

Deletion of PKH2 was recently shown to extend CSL [34]. We verified this finding (Figure 5A) and show that phosphorylation of Sch9 T570 is reduced in phk2A cells (Figure 5B). Finally, we show that cells with low Phk kinase activity are much more sensitive to growth inhibition by myriocin or Dox than are cells with higher Phk activity (Figure 5D and Figure S4 in Text S1). Together these data add further support for a signaling pathway comprised of sphingolipids->Phk1/2->Sch9->CSL.

While most of our data suggest that down-regulating sphingolipid synthesis increases lifespan by Sch9-dependent mechanisms, the finding that myriocin causes a mobility shift in Sch9, indicative of reduced phosphorylation, and increases the CSL of sch9A cells argues for some aspects of CSL extension operating by Sch9-independent mechanisms. Down-regulation of the protein kinase A [45] pathway is the most likely way for sphingolipids to affect lifespan independently of Sch9, since these two pathways have been known to interact ever since the initial discovery of Sch9 [46]. Also, the pathways are known to interact in a way that extends CSL, since naï2A sch9A double mutant cells have a longer lifespan than the single mutants [30]. This interaction phenotype is similar to what we find with myriocin treatment of sch9A cells (Figure 7A). Recent publications have detected biochemical links between the Phk1/2/Sch9 and PKA pathways [24,25,47] and these may explain how myriocin increases the lifespan of sch9A cells, but further effort is needed to determine if this is actually the case.

In addition to LCBs, complex sphingolipids (IPCs and perhaps MIPCs) may be regulating CSL, since their concentration is lowered along with LCBs when SPT activity is down-regulated (Figure 4C, 4D, and 4E). A multi-membrane spanning protein Nce102 has been proposed to sense complex sphingolipids and negatively regulate Phk1/2 [26]. According to this proposal, a reduction in complex sphingolipids should increase Phk1/2 activity by releasing Nce102-mediated down-regulation. As a consequence, Sch9 activity should increase; however, this would not enhance CSL and is contrary to our finding that reducing sphingolipids decreases Sch9 activity. Thus, Nce102 is unlikely to be mediating the increase in CSL that we observe.
Another group has also questioned the role of LCBs in activating Pkh1/2 kinase activity [27]. These authors found that short-term, acute dose myriocin treatment of cells failed to reduce Pkh1/2-mediated phosphorylation of residue T504 in the Ypk1 kinase. And recently, short-term, acute dose treatment of cells with myriocin did not decrease Pkh1/2-mediated phosphorylation of T570 in Sch9 [25]. In contrast to these results, we show that lowering the rate of sphingolipid synthesis by long-term, low dose myriocin treatment or down-regulation of LCB1 expression reduces Pkh1/2-mediated phosphorylation of T570 (Figure 5) and, in addition, myriocin treatment reduces phosphorylation of the Sch9 substrate Dot6 (Figure 5I). These results clearly support the idea that sphingolipids regulate lifespan by reducing Pkh1/2-mediated phosphorylation of T570 thereby reducing Sch9 activity.

Further work is required to determine which sphingolipids control Sch9 activity and CLS (Figure 1A).

Studies of the ISC1 gene also implicate sphingolipids in controlling lifespan. Isc1 hydrolyzes the polar head group from complex sphingolipids to yield ceramides. The CLS of isc1Δ cells is reduced partly because of mitochondrial dysfunctions [48], but CLS can be extended in these cells even beyond the wild-type value by deleting the SIT4 gene. Sit4 is the catalytic subunit of a PP2A-type protein phosphatase whose activity is down-regulated by TORC1 but up-regulated by ceramides (Figure 1A). In our experiments, enhancement of CLS by down-regulating sphingolipid synthesis in both wild-type and sch9Δ cells may involve Sit4, because reducing the abundance of IPCs, and perhaps MIPCs (Figure 4), may lower the amount of ceramide generated by Isc1.
Sphingolipids Control Lifespan

This should in turn lower Sit4 activity and increase CLS just like deletion of \textit{STT4} (Figure 1A). Finally, other studies implicate complex sphingolipids in CLS, since 	extit{ipt1A} and \textit{ska1A} cells, which lack the most complex sphingolipid M(IP)$_2$C, have a slight increase in CLS and deletion of both genes yields an additive increase in CLS [49]. It remains to be determined how a reduction in M(IP)$_2$C increases CLS, but this effect is probably real as \textit{ska1A} cells ranked high in a survey of lifespan in the yeast single-deletion strain set [1].

Sphingolipids have been implicated also in RLS. The first \textit{S. cerevisiae} gene found to regulate RLS, \textit{LAG1} (Longevity-Assurance Gene 1) [50], was later shown to encode a component of ceramide synthase which catalyzes ceramide synthesis from LCBs and fatty acids (Figure S1 in Text S1) [51,52]. Deleting \textit{LAG1} increases RLS but has no effect on CLS [1]. Since the mechanism for increased RLS without increased CLS is unknown, it is difficult to relate these data to our CLS data.

In cases where a decrease in LCBs has perturbed a cellular process in yeast it has been possible to restore the process by adding LCBs to the culture medium [53–55]. This strategy suggests that exogenous PHS should prevent the increase in CLS produced by Dox treatment of \textit{tetO7-LCB1} cells. In actuality, low levels of PHS (10–500 nM) did not thwart the increase in CLS and higher doses (10 \textmu M) actually enhanced CLS. Exogenous PHS is likely to enhance CLS by inducing the general stress response via activation of genes with stress response elements (STREs) in their promoter, as previously reported [53,56]. In addition, exogenous PHS is known to down-regulate amino acid and other nutrient transporters in yeast [57,58] and this is likely to increase lifespan since amino acid or glucose deprivation enhance yeast lifespan [1,59].

Lifespan regulation by myriocin and CR

Because CR is the standard for judging interventions that extend lifespan, we determined if myriocin enhances lifespan in CR-treated cells. We found that myriocin treatment does enhance CLS in cells grown under moderate (0.5% glucose, Figure S3 in Text S1) or extreme CR (water) conditions (Figure 7B). These results are similar to the recent finding that rapamycin treatment increases the lifespan of caloric restricted Drosophila [3] and they have implications for human health. For example, it may be possible to reduce disease incidence and increase human lifespan by combining a drug with low level CR or with a CR mimetic such as rapamycin. Furthermore, a combination regime may reduce disease incidence in genotypic backgrounds which are unresponsive to single treatment regimes.

Sphingolipids and lifespan in multicellular eukaryotes

Increasing lifespan by using a pharmacological agent to down-regulate sphingolipid synthesis may be applicable to other organisms in addition to yeasts such as rapamycin treatment has been used to down-regulate the TOR pathway and increase lifespan in yeast, worms, flies and mice [1–3,10,18]. The sphingolipids that regulate lifespan in yeast may not be present in mammals and, thus, lifespan extension may require modulating another type of mammalian sphingolipid [11]. Ceramides and sphingosine-1-phosphate are the most widely appreciated sphingolipid signaling molecules in mammals [11,12]. Ceramides are thought to promote age-related diseases since their concentration increases with age in many mammalian cells and tissues where they regulate processes that may control lifespan [60–64]. Recently long-chain hexosylceramides and lactosylceramides have been implicated in aging and lifespan in mice and cultured human cells [65]. The challenge will be to determine if mammalian lifespan can be extended by preventing the increase in ceramides or whether up or down-modulating the concentration of other sphingolipids will extend lifespan. There is, however, precedent for extending lifespan in multicellular eukaryotes through modulation of sphingolipids: inactivation of the Dacer gene, which encodes an
alkaline ceramidase, has been shown to increase lifespan in *Drosophila* [66].

As mutations and chromosomal rearrangements promote mammalian tumor cell formation, our demonstration that myriocin treatment reduces the rate of mutation and gross chromosomal rearrangements in yeast (Figure 3D and 3E) could lead to novel preventive measures and treatments for human cancers and thereby enhance lifespan.

**Materials and Methods**

**Yeast strains, media, and plasmids**

Yeast strains used in our studies are listed in Table S1 in Text S1. Viability assays were done by using YPD agar plates (2% yeast extract, 1% peptone, 2% dextrose and 2% agar). For CLS and stress resistance assays, cells were grown in SDC medium supplemented with a 4-fold excess of the tryptophan, leucine, uracil and histidine to avoid possible artifacts due to auxotrophic deficiencies of the strain [30]. For initial experiments, SDC medium was lightly buffered to pH 6.0 (1.6 gm NaH2PO4 • H2O/liter), while in later experiments it was more strongly buffered with succinic acid (200 mM, final pH 4.5). Initially the concentration of iron in SDC medium was 1.23 μM (1 x yeast nitrogen base) but in later experiments the concentration was raised 3-fold to avoid iron-starvation and reduced viability. In summary, SDC medium, pH 6.0, 1 x iron, was used for the experiments shown in Figure 1, Figure 2, and Figure 3A-3F, while the same medium, but with 3x iron, was used for the experiments shown in Figure 3H, Figure 4, and Figure 6A. For the experiments shown in Figure 3G, 3H, Figure 5, Figure 6C–6E, Figure 7, and Figures S2, S3, S4 in Text S1 the medium was SDC, pH 4.5, 3 x iron.

The pRS315 and pRS316 vectors [67] were used to make pRS315ADE2 by ligating a 2.3 Kb *PstI*-*SpeI* fragment carrying *ADE2* into the corresponding sites of pRS315. Similarly, a genomic *EcoRI*-*XbaI* fragment was ligated to pRS316 cut with the same enzymes to make pRS316-PKH2. pLCB1-5 was made by ligating a 4 Kb *NruI*-SacI chromosomal fragment containing *LCB1* and its promoter [68] to pRS315ADE2 digested with *SacI* and *Nael* (made blunt ended). pRS315-LCB2-B7 was made by ligating a 7 Kb *BamHI* chromosomal fragment containing *LCB2* and its promoter [69] to pRS315 digested with *BamHI*. The plasmid encoding HA-tagged Dot6 has been described [36].

**Lifespan assays**

CLS was measured as previously described [30]. Drug treatments were performed in medium containing a final concentration of 0.3% ethanol (ethanol plus drug). This was done be first adding a calculated volume of 95% ethanol to the medium followed by diluting a stock solution of myriocin (200 μg/ml in 95% ethanol, Sigma, # M1177) or doxycycline (100 μg/ml in 50% ethanol, Sigma, # D9891) to yield the desired concentration of drug (indicated in figure legends). Drugs were stored at −20°C and warmed to room temperature before dilution into medium, followed by thorough mixing. Cells grown overnight at 30°C in SDC medium were diluted into 25 ml of medium (125 ml flask) to give an initial A600 nm of 0.002 or as indicated in figure legends. Cultures were incubated at 30°C in an air bath shaker (220 rpm) for three days and cell viability was measured (CLS day 1) by diluting and spreading cells on YPD plates. After 2–3 days of incubation at 30°C colonies were counted and expressed a fraction of the day 1 value. Statistical significance was determined by using the two-tailed Student’s *t*-test.

**Stress resistance and oxygen consumption assays**

Cells grown in SDC medium for the time indicated in figure legends were diluted to an A600 nm of 1.0 in K-phosphate buffer (pH 6.0), treated with hydrogen peroxide for 60 min at room temperature with mixing, serially diluted (10-fold), spotted onto YPD plates, and incubated at 30°C for 2–3 days. For thermal stress resistance, cells were serially diluted (10-fold dilution starting with an A600 nm = 1), spotted onto YPD plates, and incubated at either 35°C (heat-shocked) or 30°C (control) for 45–100 min. Plates were incubated for 2–3 days at 30°C. For acidic stress resistance, cells from day 1 (72 hrs of incubation) of a CLS assay were treated with acetic acid as described in the legend to Figure 6 for 2 hours at 30°C, followed by dilution and plating on YPD plates to measure colony forming units (CFUs). CFUs of the treated samples were expressed as a percentage of the untreated samples.

Oxygen consumption was measured on cells grown from an A600 nm of 0.005 to 1 at which time the cell concentration was quantified by counting cells in a Petroff-Hauser counting chamber, cell size was measured as described in Supplemental Information and oxygen consumption was measured by using an Oxyntherm System [38]. Data are expressed as nmol of oxygen consumed per min per total volume of 10⁶ DBY746 cells.

**Age-dependent DNA damage measurements**

The frequency of spontaneous canavanine-resistant mutants (Can™) mutants was measured by using a published assay [42]. Cells were grown as described for CLS experiments and assayed for CFUs except that cultures were started at a density of 0.05 A600 nm units/ml and the SDC medium was buffered with succinate (200 mM, pH 4.5) and contained 3.69 μM iron (3x normal). These changes were necessary to prevent parental cells from dying so rapidly that they behaved like gsp1 mutant cells. Mutant gsp1 cells were grown in medium containing 300 mg/ml of myriocin. The frequency of Can™ mutants was measured on the days indicated in Figure 6D. To identify Can™ mutants, a sample of the culture was centrifuged and the cell pellet was washed once with sterile water and plated on selection medium (SDC-ARG supplemented with 60 μg/ml L-canavanine sulfate). After 3–4 days of incubation at 30°C, the colonies were counted and expressed as the ratio to total viable cells. GCRs were assayed as described previously [42]. Strains carried a *URA3* cassette at the *HXT13* locus which is located 7.5 kb from the *CAN1* gene towards the telomeric of chromosome V. The experimental protocol was similarly to the one for measuring Can™ mutations but the frequency of simultaneous loss of *CAN1* and *URA3* was measured by plating cells on SDC plates containing 1 mg/ml 5-fluoroorotic acid (5FOA) and 60 μg/ml L-canavanine sulfate [70].

**Analysis of LCBs, LCBPs, and complex sphingolipids**

To extract LCBs and LCBPs, cells were grown as described for CLS assays and at the indicated time points 10 A600 nm units of cells were treated by slowly adding cold TCA to a final concentration of 6%. After incubating on ice for 5 min, cells were collected by centrifugation, washed twice with cold water, and suspended in 500 μl of fresh 80 mM triethylamine (made in HPLC grade absolute ethanol). Samples were sonicated in a water bath for 5 min at 37°C, heated for 30 min at 65°C, centrifuged and the supernatant fluid (150 μl) was transferred to an HPLC sample vial insert. AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) reagent (30 μl) was added and samples were treated at
room temperature for 40 min followed by addition of 20 μl of 1.5 N KOH (made in MeOH) and incubation at 37 °C for 30 min. The pH was adjusted by adding 20 μl of 1.74 M acetic acid (made in MeOH) and 40 μl of the sample were assayed by HPLC [71].

Complex sphingolipids were analyzed as described previously [72] except for three changes. First, cells were grown in SDC medium [pH 6.5, 3.69 mM Fe] from 0.005 to 1 A600 mm units to produce uniform radiolabeling. Second, the concentration of [2-3H]myoinositol (American Radiochemicals, Inc., catalog #ART116) in the growth medium was 40 μCi/ml. Finally, each lane on the thin-layer plate contained 20 μl of non-radioactive yeast sphingolipids (0.5 mg/ml), which after charring, were used to verify the location of radioactive sphingolipids.

Protein extraction and Western blotting analysis

Cells were grown as described for a CLS assay and cell-free yeast extracts were prepared by using a modified published procedure [73]. Ten A600 mm units of cells were treated with cold TCA as described above, suspended in 400 μl of water following by the addition of 400 μl of 0.2 M NaOH. Thoroughly mixed samples were incubated 5 min at room temperature, concentrated by centrifugation and suspended in 200 μl of lysis buffer (0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 1% β-mercaptoethanol). After heating at 95°C for 5 min, samples were centrifuged and the protein concentration of the supernatant fluid was determined by using the DC protein assay kit (Bio-Rad Laboratories). Samples (50 μg of protein) electrophoresed on a 9% SDS-PAGE gel were transferred for 1.5 hours onto a PVDF membrane (Millipore Immobilon) by using a Bio-Rad semi-dry transfer system. For the blots shown in Figure 5I and 5J the acrylamide-bisacrylamide ratio was changed from the normal 37.5:1 ratio to 172:1. Membrane blocking and antibody binding were done in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% nonfat dry milk. Membranes were incubated for 2 hrs at room temperature with custom made polyclonal rabbit anti-Sch9 antibodies (Batch 2872, 1:1000, OpenBiosystems), phospho-specific anti-Sch9T570 antibodies (1:10,000 dilution) [20], anti-HA antibodies (clone 3F10, 1:5000, Roche) or commercial monoclonal mouse anti-Vma2 (1:3000 dilution) or anti-Vph1 (1:1000 dilution) antibodies (Molecular Probes). Other antibodies included alkaline phosphatase-linked anti-rabbit or anti-mouse IgG (1:5000, Sigma-Aldrich). Fluorescent signals from membranes transferred for 1.5 hours onto a PVDF membrane (Millipore) by using a Bio-Rad semi-dry transfer system. For the blots shown in Figure 5I and 5J the acrylamide-bisacrylamide ratio was changed from the normal 37.5:1 ratio to 172:1. Membrane blocking and antibody binding were done in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% nonfat dry milk. Membranes were incubated for 2 hrs at room temperature with custom made polyclonal rabbit anti-Sch9 antibodies (Batch 2872, 1:1000, OpenBiosystems), phospho-specific anti-Sch9T570 antibodies (1:10,000 dilution) [20], anti-HA antibodies (clone 3F10, 1:5000, Roche) or commercial monoclonal mouse anti-Vma2 (1:3000 dilution) or anti-Vph1 (1:1000 dilution) antibodies (Molecular Probes). Other antibodies included alkaline phosphatase-linked anti-rabbit or anti-mouse IgG (1:5000, Sigma-Aldrich). Fluorescent signals from membranes exposed to an ECF substrate (Amersham Biosciences) were analyzed by using a PhosphorImager (Amersham Biosciences) and quantified by using ImageQuant NT Software.

References

1. Powers RW, 3rd, Kaederlein M, Caldwell SD, Kennedy BK, Fields S (2006) Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev 20: 174–104
2. Harrison DE, Strong R, Sharp ZD, Nelson JF, Asle CM, et al. (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 460: 392–395
3. Biedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, et al. (2010) Mechanisms of lifespan extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab 11: 35–46
4. Blagosklonny MV (2009) Validation of anti-aging drugs by treating age-related diseases. Aging (Albany NY) 1: 201–208
5. Kaederlein M (2010) Resveratrol and rapamycin: are they anti-aging drugs? Bioessays 32: 96–99
6. Goldberg AA, Richard VR, Kyrakov P, Bourque SD, Beach A, et al. (2010) Chemical genetic screen identifies histococholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes. Aging (Albany NY) 2: 393–414
7. Fabrizio P, Pozza F, Fletcher SD, Gendron CM, Longo VD (2001) Regulation of longevity and stress resistance by Sch9 in yeast. Science 292: 288–290
8. Kaederlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, et al. (2005) Regulation of Yeast Replicative Life Span by TOR and Sch9 in Response to Nutrients. Science 310: 1193–1196
9. Selman G, Tufet JM, Wieser D, Irvine E, Lingard SJ, et al. (2009) Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science 326: 140–144
10. Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, et al. (2010) With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab 11: 453–465
11. Hannun YA, Obeid LM (2000) Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol 9: 139–150
12. Maceyka M, Milstien S, Spiegel S (2009) Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling. J Lipid Res 50 Suppl: S272–276
13. Stanciuc A, Boleviciute R (2010) Ceramide-rich platforms in transmembrane signaling. FEBS Lett 584: 1728–1740
14. Merrill AH, Jr., Wang MD, Park M, Sullards MC (2007) Glyco(sphingolipid)og-ogy: an amazing challenge and opportunity for systems biology. Trends Biochem Sci 32: 457–468
15. Dickson RC (2008) Thematic review series: sphingolipids. New insights into sphingolipid metabolism and function in budding yeast. J Lipid Res 49: 909–921
16. Cowart LA, Obeid LM (2007) Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. Biochim Biophys Acta 1771: 421–431.

17. Breslow BK, Weisman JS (2010) Membranes in balance: mechanisms of extracellular lipid transport. Mol Cell 40: 129–134.

18. Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Oroz L, et al. (2003) Genetics influence of TOR kinase on lifespan in C. elegans. Nature 426: 620.

19. Jia K, Chen D, Riddle DL (2004) The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. Development 131: 3897–3906.

20. Urban J, Soulard A, Huber A, Lippman S, Mukhopadhyay D, et al. (2007) Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell 26: 665–674.

21. Liu K, Zhang X, Lester RL, Dickson RC (2007) The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in Saccharomyces cerevisiae including Ypk1, Ypk2, and Sch9. J Biol Chem 282: 22679–22687.

22. Roelants FM, Torrance PD, Thorner J (2004) Differential roles of PDK1 and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Ypk2 and Sch9. Microbiology 150: 3289–3304.

23. Jancito E, Lorberg A (2008) TOR regulation of AGC kinases in yeast and mammals. Biochem J 410: 19–37.

24. Soulard A, Cremonesi A, Moes S, Schutz F, Jeno P, et al. (2010) The rapamycin-sensitive phosphoproteome reveals that TOR controls protein kinase A toward some but not all substrates. Mol Biol Cell 21: 3475–3486.

25. Voordeckers K, Kinne M, Hasendonckx S, Lounet W, Versielle M, et al. (2011) Ypk3, a sphingolipid-dependent protein kinase-1 (PDK1)-ortholog phosphorylates differentially regulated phosphorylation of protein kinase A (PKA) and the protein kinase B (PKB/NUK6 ortholog Sch9). J Biol Chem 286: 22017–22027.

26. Frohlich F, Moreira K, Aguilar PS, Hubner NC, Mann M, et al. (2009) A genome-wide screen for genes affecting eisosomes reveals Nce102 function in sphingolipid signaling. J Cell Biol 185: 1227–1242.

27. Roelants FM, Baltz AG, Trout AE, Fereres S, Thorner J (2010) A protein kinase C ortholog interacts with Ypk1 and regulates gene expression in Saccharomyces cerevisiae. Mol Microbiol 79: 1263–1276.

28. Mnaimneh S, Davierwala AP, Haynes J, Moffat J, Peng WT, et al. (2004) G protein-coupled receptor-mediated transcriptional regulation. Mol Cell 14: 569–582.

29. Roelants FM, Baltz AG, Trout AE, Fereres S, Thorner J (2010) A protein kinase C ortholog interacts with Ypk1 and regulates gene expression in Saccharomyces cerevisiae. Mol Microbiol 79: 1263–1276.

30. Liu K, Zhang X, Lester RL, Dickson RC (2007) The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in Saccharomyces cerevisiae including Ypk1, Ypk2, and Sch9. J Biol Chem 282: 22679–22687.

31. Roelants FM, Torrance PD, Thorner J (2004) Differential roles of PDK1 and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Ypk2 and Sch9. Microbiology 150: 3289–3304.

32. Weinberger M, Feng L, Paul A, Smith DL, Jr., Hontz RD, et al. (2007) DNA damage and transcriptional regulation of the yeast ATM ortholog Mre11. J Biol Chem 282: 37507–37517.

33. Kaplan CD, Kaplan J (2009) Iron acquisition and transcriptional regulation. J Biol Chem 284: 13729–13736.

34. Burtner CR, Murakami CJ, Olsen B, Kennedy BK, Kaeberlein M (2011) A molecular mechanism for the silent mating type locus in yeast. J Cell Biol 193: 141–155.

35. Burtner CR, Murakami CJ, Olsen B, Kennedy BK, Kaeberlein M (2009) A molecular mechanism for the silent mating type locus in yeast. J Cell Biol 193: 141–155.

36. Weinberger M, Feng L, Paul A, Smith DL, Jr., Hontz RD, et al. (2007) DNA damage and transcriptional regulation of the yeast ATM ortholog Mre11. J Biol Chem 282: 37507–37517.

37. Soulard A, Cremonesi A, Moes S, Schutz F, Jeno P, et al. (2010) The rapamycin-sensitive phosphoproteome reveals that TOR controls protein kinase A toward some but not all substrates. Mol Biol Cell 21: 3475–3486.

38. Lavoie H, Whiteway M (2008) Increased respiration in the sch9Delta mutant is related to, cAMP, dependent protein kinase catalytic subunits. Genes and Development 22: 517–527.

39. Zhang A, Shen Y, Gao W, Dong J (2011) Role of Sch9 in regulating Ras-cAMP signal pathway in Saccharomyces cerevisiae. FEBS Lett 585: 3026–3032.

40. Barbosa AD, Osorio H, Sims KJ, Almeida T, Alves M, et al. (2011) Role for Sir2p-dependent mitochondrial dysfunction in mediating the shortened chronological lifespan and oxidative stress sensitivity of Isi-lp-deficient cells. Mol Microbiol 81: 513–527.

41. Aerts AM, Francois IE, Bammens L, Cammue BP, Smetts B, et al. (2006) Level of MIP2/C2 sphingolipid affects plant defence sensitivity, oxidative stress response, and Piaseczny and Lipinski (2006) Cloning and characterization of LAG1, a longevity-assurance gene in yeast. J Biol Chem 281: 14519–14529.

42. Madia F, Gattazzo C, Wei M, Fabrizio P, Burhans WC, et al. (2008) Longevity under heat stress involves AKT1, a stress-activated protein kinase in Caenorhabditis elegans. Aging (Albany NY) 1: 131–145.

43. Guillas I, Kirchman PA, Chuard R, Pfefferli M, Jiang JC, et al. (2001) C26-CoA-dependent ceramide synthesis requires the yeast AGC kinase Ypk1. J Biol Chem 276: 8374–8381.

44. Anderson RM, Weindruch R (2010) Metabolic reprogramming, caloric restriction, and lifespan extension. J Cell Physiol 224: 1–15.
73. Kushnirov VV (2000) Rapid and reliable protein extraction from yeast. Yeast 16: 857–860.
74. Dickson RC, Lester RL (2002) Sphingolipid functions in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1583: 13–25.
75. Funato K, Vallee B, Riezman H (2002) Biosynthesis and trafficking of sphingolipids in the yeast *Saccharomyces cerevisiae*. Biochemistry 41: 15105–15114.