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Role for Sit4p-dependent mitochondrial dysfunction in mediating the shortened chronological lifespan and oxidative stress sensitivity of Isc1p deficient cells

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Summary

Saccharomyces cerevisiae cells lacking Isc1p, an orthologue of mammalian neutral sphingomyelinase 2, display a shortened lifespan and an increased sensitivity to oxidative stress. A lipidomic analysis revealed specific changes in sphingolipids that accompanied the premature aging of Isc1p deficient cells under severe calorie restriction conditions, including a decrease of dihydrosphingosine levels and an increase of dihydro-C₂₆-ceramide and phyto-C₂₆-ceramide levels, the latter raising the possibility of activation of ceramide-dependent protein phosphatases. Consequently, deletion of the *SIT4* gene, which encodes for the catalytic subunit of type 2A ceramide-activated protein phosphatase in yeast, abolished the premature aging and hydrogen peroxide sensitivity of *isc1Δ* cells. *SIT4* deletion also abolished the respiratory defects and catalase A deficiency exhibited by *isc1Δ* mutants. These results are consistent with catabolic derepression associated with the loss of Sit4p. The overall results show that Isc1p is an upstream regulator of Sit4p and implicate Sit4p activation in mitochondrial dysfunction leading to the shortened chronological lifespan and oxidative stress sensitivity of *isc1Δ* mutants.

Keywords

Isc1p; Sit4p; ceramide-activated protein phosphatase; oxidative stress; chronological aging

Introduction

Sphingolipids are ubiquitous structural components of cell membranes, and their metabolites such as sphingosine, sphingosine-1-phosphate and ceramide play important roles in the regulation of several cellular processes. Sphingosine, a hydrophobic long chain base that provides the backbone of sphingolipids, can be phosphorylated into sphingosine-1-

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phosphate by sphingosine kinases or acylated to ceramide by ceramide synthase. The fatty acids added to sphingosine can possess different chain lengths. Therefore, ceramides constitute a large and complex group of molecules that share a common structure. Ceramides with different fatty chain lengths and localizations may play distinct biological functions. Ceramide may also be generated through hydrolysis of complex sphingolipids; for example, in mammalian cells, sphingomyelin is hydrolyzed by sphingomyelinases. Sphingosine and ceramide modulate cellular processes such as apoptosis, cell senescence and cell cycle arrest whereas sphingosine-1-phosphate plays a key role in proliferation, mitogenesis, cell migration, and protection from apoptosis. Changes in the relative amounts of sphingosine-1-phosphate and sphingosine/ceramide are, therefore, major factors that determine cell fate (Hannun and Obeid, 2008).

In yeast, as described for mammalian cells (Won and Singh, 2006), changes in sphingolipid metabolism affect oxidative stress resistance and life span (Jiang *et al.*, 2004; Aerts *et al.*, 2006; Almeida *et al.*, 2008; Aerts *et al.*, 2008). Furthermore, 5 genes (*LAG1*, *YPC1*, *YSR3*, *IPT1*, and *LCB5*) involved in sphingolipid metabolism are differentially expressed in both senescent and apoptotic yeast cells (Laun *et al.*, 2005). We have recently shown that Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase-2 (nSMase2) which acts on yeast inositol phosphosphingolipids, plays a key role in oxidative stress resistance and chronological lifespan, modulating redox homeostasis, iron levels and cell death by caspase-dependent apoptosis (Almeida *et al.*, 2008). Mitochondrial deficits leading to an increased production of reactive oxygen species and accumulation of oxidative damages have been implicated in the aging process (Murphy, 2009). Consistent with the shortened lifespan of *isc1Δ* mutants, several lines of evidence suggest that Isc1p is important for mitochondrial function. Isc1p is post-translationally regulated by translocation from the endoplasmic reticulum into mitochondria when aerobic respiration is induced (e.g. when glucose-grown cells reach the post-diauxic phase; Vaena de Avalos *et al.*, 2004). Yeast mitochondria are enriched in α -hydroxylated phytoceramides generated by Isc1p and these lipids contribute to the normal function of mitochondria (Kitagaki *et al.*, 2007). Cells lacking Isc1p preserve normal intrinsic mitochondrial functions, including intact mitochondrial DNA and oxygen consumption, but display a defective aerobic respiration in the post-diauxic phase associated with the incapacity to upregulate genes required for non-fermentable carbon source metabolism (Vaena de Avalos *et al.*, 2005; Kitagaki *et al.*, 2009).

Changes in the level of sphingolipid metabolites during cell aging and the modulation of its downstream targets and signal transduction pathways remain poorly characterized. Several protein targets, including ceramide-activated protein kinases and phosphatases, protein kinase C, cathepsin D and JNK (Hannun and Obeid, 2008) have been suggested to mediate the effects of bioactive sphingolipids. In yeast, Sit4p is the catalytic subunit of the ceramide-activated protein phosphatase, a heterotrimeric complex that also includes Tpd3p and Cdc55p as regulatory subunits (Nickels and Broach, 1996). Sit4p is a serine-threonine protein phosphatase related to type 2A family of protein phosphatases (PP2A), and it has a high homology to other protein phosphatases, including the fission yeast PP2A and human protein phosphatase 6 that are involved in cell cycle regulation (Bastians and Ponstingl, 1996; Shimanuki *et al.*, 1993). Sit4p has a role in the control of the Swi4p factor that regulates the transcription of G1 cyclin genes (Fernandez-Sarabia *et al.*, 1992; Di Como *et al.*, 1995) and is required for down-regulation of cell functions that depend on Pkc1p, such as cell wall integrity pathway, cytoskeleton organization, and ribosomal gene expression (Angeles de la Torre-Ruiz *et al.*, 2002). Sit4p has also been functionally linked to the regulation of the ubiquitin-proteasome system (Singer *et al.*, 2003), silencing in the subtelomeric region (Hayashi *et al.*, 2005), monovalent ion and pH homeostasis (Masuda *et al.*, 2000), nutrient signaling (Tate *et al.*, 2006; Di Como and Arndt, 1996) and to reinitiate endocytosis under stress in cells deficient in amphiphysin function (McCourt *et al.*, 2009).

Interestingly, respiration is derepressed in *sit4Δ* cells grown in glucose medium, and these mutants are unable to grow under anaerobic conditions, indicating that energy produced by mitochondrial respiration is essential for their viability (Jablonka *et al.*, 2006).

Here we show that the shortened chronological lifespan of *isc1Δ* cells submitted to a severe calorie restriction regime is accompanied by altered levels of bioactive sphingolipids, including an increase in dh-C₂₆-Cer and phyto-C₂₆-Cer. Consistent with ceramide activating protein phosphatases, loss of the ceramide-activated protein phosphatase Sit4p suppresses the shortened chronological lifespan, oxidative stress sensitivity and mitochondrial dysfunctions of *isc1Δ* cells.

Results

Levels of sphingolipids during chronological aging

Sphingolipids have been shown to play essential roles in both cell death and survival, and the balance between sphingosine-1-phosphate and sphingosine/ceramide is a key determinant of cell fate. To identify sphingolipid species that may be involved in the shortened chronological lifespan (CLS) of *isc1Δ* mutant cells, we analyzed changes in the levels of 27 sphingolipid species, including long chain sphingoid bases (DHS, PHS and its 1-phosphate forms), and ceramides [dihydroceramides (dh-Cer), phytoceramides (phyto-Cer), and α -hydroxylated phytoceramides (α OH-phyto-Cer)] in *S. cerevisiae* BY4741 and *isc1Δ* cells during chronological aging. The main ceramide species found were α OH-C₂₆-phyto-Cer and C₂₆-phyto-Cer (C₂₆ indicates an acyl chain of 26 carbon atoms). A map of sphingolipid metabolism in yeast is shown in Fig. 1.

These studies were performed using cells cultured to post-diauxic phase, washed and resuspended in water. These experimental conditions model a severe calorie restriction (CR) regime that induces a low metabolism stationary phase, resulting in the longest survival for wild type yeast strains. Yeast optimized for chronological survival allows establishing a better correlation between aging and cellular responses, including the modulation of bioactive molecules that regulate signaling pathways, stress resistance and mitochondria function (Fabrizio and Longo, 2003). The parental strain showed increasing levels of DHS (9.7-fold), DHS-1-P (18-fold) and PHS (1.8-fold) in cells aged during 10 days, but PHS-1-P decreased 5-fold (Fig. 2). Total dihydroceramide levels of parental cells increased 1.5-fold by day 10 (Fig. 3A), with C₂₄- (4.5-fold) and C₂₆-dh-Cer (2.8-fold) increasing the most (Table S1; Fig. 3B). Regarding total phytoceramides (Fig. 3C), no significant changes were observed in parental cells by day 10, but individual changes in specific species were observed; thus, phyto-C₁₆-Cer levels increased 2-fold, and phyto-C₂₆-Cer levels decreased 2.2-fold (Table S2; Fig. 3D). The level of total α -hydroxylated phytoceramides increased 5.9-fold during aging of parental cells (Fig. 3E). The greatest increases were seen in α OH-phyto-C₁₈-Cer (4.4-fold), α OH-phyto-C₂₄-Cer (4.4-fold) and α OH-phyto-C₂₆-Cer (9.6-fold) (Table S3). These results demonstrate significant changes in several distinct species of yeast sphingolipids during chronologic aging.

In contrast with parental cells, DHS and DHS-1-P levels did not change from day 0 to day 10 in *isc1Δ* cells. The final DHS levels were 5-fold lower in *isc1Δ* mutants compared to parental cells (Fig. 2). The constitutive levels of PHS were elevated in *isc1Δ* cells, but then did not change over time, a dynamic similar to that observed in the parental strain. As observed in parental cells, PHS-1-P levels decreased from day 0 to day 10 in *isc1Δ* cells (Fig. 2).

Isc1p deficiency also affected changes in ceramide levels associated with chronological aging (Fig. 3). Compared to aged parental cells, total dihydroceramides and phytoceramides

were 1.5-fold and 2.8-fold higher in day 10 *isc1Δ* mutants. The analysis of the different ceramide species showed a 2- to 9-fold increase in the levels of dh-C₁₆-Cer, dh-C₂₀-Cer, dh-C₂₆-Cer, phyto-C₂₆-Cer, and phyto-C_{26:1}-Cer in aged *isc1Δ* cells, compared to parental cells, whereas the levels of dh-C₂₄-Cer and phyto-C₁₆-Cer were 3-fold lower (see Tables S1-S2; Fig. 3A-D). Despite a 3.8-fold increase from day 0, the levels of total α -hydroxylated phytoceramides in day 10 *isc1Δ* mutants were 2-fold lower compared to parental cells (Fig. 3E). The most significantly decreased species were α OH-phyto-C₁₆-Cer (2-fold) and α OH-phyto-C₂₆-Cer (4-fold) (see Table S3). Consistent with published data, the constitutive levels of several α -hydroxylated phytoceramides were significantly reduced in cells lacking Isc1p (Kitagaki *et al.*, 2007). Thus taken together, there were several changes noted in senescent cells whose levels were differentially regulated in the *isc1Δ* strain.

One of these prominent lipids that showed differential changes was DHS-1-P. The low levels of DHS-1-P observed in *isc1Δ* cells suggest that it might be associated with the premature aging of this mutant strain. To test this hypothesis, chronological lifespan and a lipidomic analysis were performed using cells deficient in Lcb4p, the major long chain base kinase that phosphorylates DHS into DHS-1-P. The results showed that chronological lifespan was not affected in *lcb4Δ* cells (Fig. S1). As expected, in the *lcb4Δ* strain, the baseline levels of DHS-1-P (3.8-fold) and PHS-1-P (5-fold) were significantly reduced compared to the parental, whereas DHS (2.8-fold) and PHS (6.1-fold) were increased. Furthermore, in 10 days old cells, DHS levels in the *lcb4Δ* mutants were similar to those of parental cells but DHS-1-P were 18-fold lower (Fig. 2). These results implicate Lcb4p in DHS phosphorylation during cell aging but suggest that the shortened lifespan of *isc1Δ* cells is not associated with a decrease in DHS-1-P. The lipidomic analysis also showed higher levels of phytoceramides in *lcb4Δ* cells, compared to the levels of parental cells, both at day 0 and in aged cells (Fig. 3). All phytoceramide species contributed to this increase with the exception of phyto-C₂₆-Cer and phyto-C_{26:1}-Cer (Table S2; Fig. 3D).

The overall results indicate that there were significant changes specifically associated with the premature aging of *isc1Δ* cells: (i) decreases in the levels of DHS and most α OH-phytoceramides; (ii) increases in the levels of dh-C₂₆-Cer and phyto-C₂₆-Cer (Fig. 3B and D).

SIT4 disruption suppresses the shortened CLS and oxidative stress sensitivity of *isc1Δ* cells

Ceramide-activated protein phosphatase (CAPP) has been implicated in cell signaling elicited by ceramide. In yeast, Sit4p is the primary catalytic subunit of CAPP, being activated by a dose-dependent increase in ceramide levels (Nickels and Broach, 1996). Our results show that several ceramide species were increased in aged *isc1Δ* cells. These changes are probably implicated as a cause, rather than a consequence, of death/senescence, since cells remain metabolically active as suggested by the progressive increase of DNA fragmentation and caspase-like activity in *isc1Δ* cells from day 0 to day 14 of aging (Almeida *et al.*, 2008). This led us to wonder if Sit4p was involved downstream of the changes in ceramide. To begin to probe this, we first assessed whether loss of Sit4p could suppress the phenotypes of these mutant cells. Chronological lifespan and hydrogen peroxide resistance were measured in parental (BY4741), *isc1Δ*, *sit4Δ* and *isc1Δ sit4Δ* cells. As previously shown (Almeida *et al.*, 2008), *isc1Δ* cells displayed a shortened chronological lifespan associated with increased levels of protein carbonyls, a biomarker of oxidative damage (Fig. 4A-B). *SIT4* disruption abolished the reduced lifespan of *isc1Δ* cells: at day 14, cell viability was 62% in parental cells, 1% in *isc1Δ* cells, 55% in *sit4Δ* cells, and 46% in *sit4Δ isc1Δ* cells. This protective effect was accompanied by a suppression of protein carbonylation in cells aged for 14 days (Fig. 4A-B, Fig. S2). Notably, loss of Sit4p extended the chronological lifespan of yeast cells: at day 35, cell viability in parental and *sit4Δ* mutants was 3 and 32%, respectively.

The lifespan assay used in this study (cell grown to post-diauxic phase and then transferred into water) models a severe CR condition known to increase chronological survival (Fabrizio and Longo, 2003). To test if *SIT4* deletion also increased lifespan under non-CR conditions, similar studies were performed using cells grown to post-diauxic phase on SC-2% glucose and survival of cells kept in the medium was followed over time. As expected, under these conditions, parental cells displayed a shorter lifespan (Fig. S3), compared to the observed under CR (Fig. 4A). The lifespan of *isc1Δ* cells also decreased under non-CR. Loss of Sit4p significantly increased chronological survival of both parental and *isc1Δ* cells, as observed for CR cultures. These results indicate that CR is not required for the effect of *SIT4* deletion on lifespan extension.

The *sit4Δ* mutants were also hyper-resistant to H₂O₂, and *SIT4* disruption suppressed the H₂O₂ sensitivity of *isc1Δ* cells (Fig. 5A): cellular viability was 20% in parental cells, 5% in *isc1Δ* cells, 80% in *sit4Δ* cells and 82% in *isc1Δ sit4Δ* double mutants. The increased resistance to oxidative stress observed in *sit4Δ* and *isc1Δ sit4Δ* cells was accompanied by a decrease in oxidative stress markers, namely intracellular oxidation (assessed using a molecular probe sensitive to reactive oxygen species, H₂DCFDA) and protein carbonylation (Fig. 5B–C). The increase in H₂O₂-induced intracellular oxidation and protein carbonylation was higher in *isc1Δ* cells (5.4- and 1.7-fold, respectively), compared to the observed in parental cells (4.5- and 1.5-fold, respectively). Remarkably, the protein carbonyl content in *sit4Δ* cells was always lower to that of parental cells. Moreover, *SIT4* deletion significantly decreased oxidative stress markers in Isc1p deficient cells. These results suggest that Sit4p is a key downstream mediator of the effects of Isc1p on lifespan and oxidative stress.

Based on the above, it became important to investigate if Sit4p is activated in response to deletion of *ISC1*. Sit4p is negatively controlled by the target of rapamycin complex 1 (TORC1), which is inhibited by rapamycin, and Sit4p in turn regulates the dephosphorylation of the Gln3p transcription factor and its translocation into the nucleus (Tate *et al.*, 2006). To assess if Sit4p/CAPP activity was increased in *isc1Δ* cells, we measured the Gln3p-dependent *MEP2* expression in parental (BY4741), *isc1Δ*, *sit4Δ* and *isc1Δsit4Δ* cells transformed with a *MEP2-lacZ* reporter. Consistent with an induction of the Gln3p-reporter in *isc1Δ* cells, β-galactosidase activity increased 55% in this mutant strain, an effect that was suppressed by *SIT4* disruption (Fig. S4). In addition, the analysis of *isc1Δ* microarrays data (Almeida *et al.*, 2008) revealed that 2.8%, 33.3% and 53.2% of the genes differentially expressed in *isc1Δ* mutants are regulated by Gln3p based on direct, indirect, or potential evidence, respectively (Table S4). These results are consistent with an increase of Sit4p/CAPP activity in Isc1p deficient cells.

SIT4 disruption relieves mitochondrial dysfunction and catalase A deficiency of *isc1Δ* cells

Cells lacking Isc1p display a mitochondrial dysfunction (Vaena de Avalos *et al.*, 2005; Kitagaki *et al.*, 2007; Kitagaki *et al.*, 2009), and respiration is derepressed in a *sit4Δ* mutant grown in glucose (Jablonka *et al.*, 2006). Therefore, we raised the hypothesis that the effect of *SIT4* deletion on the phenotypes of *isc1Δ* cells could be due to the modulation of mitochondrial function. To test this hypothesis, we measured oxygen consumption, cytochrome c oxidase (COX) activity and capacity to grow on glycerol as non-fermentable carbon source (Fig. 6). In exponential phase cells, loss of Isc1p had no significant effect on oxygen consumption and COX activity, probably due to the fact that metabolism in parental and *isc1Δ* cells relies on fermentation for growth. In agreement with the catabolite derepression described for *sit4Δ* cells, oxygen consumption increased 60% and COX activity increased 9.2-fold in this strain. Moreover, COX activity increased 5.3-fold in *sit4Δ isc1Δ* double mutants. In the post-diauxic phase, as expected, oxygen consumption and COX activity increased in parental cells. However, both oxygen consumption and COX

activity were almost undetected in *isc1Δ* cells. The incapacity of *isc1Δ* cells to upregulate genes required for non-fermentable carbon source metabolism (Vaena de Avalos *et al.*, 2005; Kitagaki *et al.*, 2009) likely contributes to the decrease of oxygen consumption and the suppression of COX activation observed in *isc1Δ* mutants grown from exponential to post-diauxic phase. Notably, *SIT4* disruption abolished the respiratory defect of *isc1Δ* cells: oxygen consumption in *sit4Δ isc1Δ* double mutant was 57% of that in parental cells and COX activity was similar in both strains. In *sit4Δ* single mutants, oxygen consumption was not affected but COX activity increased 50% compared to that in post-diauxic phase parental cells. Consistent with the increase in mitochondrial function, *sit4Δ isc1Δ* cells, in contrast with *isc1Δ* cells, were able to grow on a non-fermentable carbon source (Fig. 6C). It should be noted that catabolite derepression per se does not seem to account for the increased H₂O₂ resistance of *sit4Δ* cells since oxidative stress resistance was not affected in *mig1Δ* and *hxx2Δ* mutants that are also known to be derepressed (Fig. S5).

Antioxidant defenses play a key role in cellular defense against oxidative stress and aging by preventing oxidative damages (Moradas-Ferreira and Costa, 2000). We have previously shown that glutathione levels and the activity of catalase T (Ctt1p; cytosolic form) and superoxide dismutases (Sod) is not affected in *isc1Δ* cells (Almeida *et al.*, 2008). However, Kitagaki *et al.* (2009) showed that *isc1Δ* cells fail to induce *CTA1* gene expression in the post-diauxic phase. In agreement, we observed a decrease of 66% in total catalase activity in *isc1Δ* cells that was associated with a decrease in the activity of Cta1p, the catalase A form present in mitochondria and peroxisomes (Petrova *et al.*, 2004). In addition, *SIT4* disruption in *Isc1p* deficient cells restored catalase activity by increasing Cta1p levels (Figs. 7A and B) and, thus, the capacity to detoxify H₂O₂. The overexpression of *CTA1* gene in *isc1Δ* cells partially suppressed the shortened lifespan of this mutant (Figs. 7C and D). This result indicates that catalase A deficiency contributes to the premature aging of cells lacking *Isc1p*, although it is not the only factor leading to this phenotype. A recent study showed that *CTA1* overexpression shortens chronological lifespan whereas inactivation of catalases promotes longevity by increasing hydrogen peroxide levels (Mesquita *et al.*, 2010). Under the severe CR conditions used in the current study, the viability of parental cells overexpressing *CTA1* or transformed with the empty vector was 100% up to 14 days of aging (Figs. 7C and D). The viability of these strains was not evaluated at longer time points in this study.

Discussion

Isc1p, the yeast orthologue of mammalian neutral sphingomyelinase-2, is important for oxidative stress resistance and chronological lifespan (Almeida *et al.*, 2008). The hydrolysis of inositolphosphosphingolipids by *Isc1p* generates ceramide, a signaling molecule conserved during evolution. However, the mechanisms and downstream mediators of these actions of *Isc1p* are not known. Elucidation of these mechanisms is further complicated by the interconnectivity of bioactive sphingolipids. As such, enzymes of sphingolipid metabolism function as a network that regulates the levels of individual bioactive lipids and their metabolic interconversion (Hannun and Obeid, 2008). The interaction between *de novo* sphingolipid biosynthesis versus *Isc1p*-mediated sphingolipid production is shown by the finding that *ISC1* deletion is synthetically lethal with the *lcb1-100* mutation (Coward *et al.*, 2006). Moreover, the *LAC1* gene that encodes for a ceramide synthase component is upregulated in *isc1Δ* cells (Almeida *et al.*, 2008). Given this metabolic interconnectivity, we performed a lipidomic analysis to identify specific changes in sphingolipids that might mediate the shortened lifespan of *isc1Δ* cells. To extend the monitoring of these changes, we used a severe CR regime (incubation in water) that results in the longest survival for wild type yeast strains (Fabrizio and Longo, 2003). This treatment seems to activate an anti-aging response similar to the observed under CR conditions induced by growing the cells in

medium containing low glucose concentrations such as 0.05% or 0.5%, instead of the standard 2% (Smith *et al.*, 2007).

In day 10 cells of the parental strain, the sphingoid bases had increases in DHS, DHS-1-P and PHS along with a decrease in PHS-1-P. Ceramides increased in total dh-Cer and α OH-phyto-Cer. Under these conditions, cell viability remained high; therefore, these changes may reflect an adaptive response to maintain stationary phase viability. In *isc1 Δ* mutants, lower levels of DHS and higher levels of dh-C₂₆-Cer and phyto-C₂₆-Cer are the changes that best correlate with loss of viability during aging. The low levels of DHS observed in aged *isc1 Δ* cells are probably a limiting factor for DHS-1-P formation, but the decrease in the levels of this long chain base does not seem to be associated with cell aging. Indeed, the formation of DHS-1-P during aging was also suppressed in Lcb4p deficient cells, which displayed a lifespan similar to that of parental cells. Other changes associated with aging of *isc1 Δ* mutants included an increased accumulation of dh-C₁₆-Cer and dh-C₂₀-Cer, and lower levels of phyto-C₁₆-Cer, α OH-phyto-C₁₆-Cer and α OH-phyto-C₂₆-Cer. The decrease in α OH-phyto-Cer is in agreement with recent data showing that yeast mitochondria are enriched in these lipids, which are generated by Isc1p and contribute to mitochondrial function (Kitagaki *et al.*, 2007). Mitochondrial dysfunction has been implicated in the aging process (Murphy, 2009). Thus, alterations in the levels of α -hydroxylated phytoceramides may also function in the modulation of lifespan.

Ceramides containing different length fatty acids seem to have distinct effects in cell physiology, by either affecting the biophysical properties of the membrane lipid bilayer or by interacting with specific downstream components in signaling pathways (Hannun and Obeid, 2008). Here we present evidence that Sit4p, a serine-threonine phosphatase that is also a component of ceramide-activated PP2A, functions downstream of Isc1p. *SIT4* disruption suppressed the shortened lifespan and H₂O₂ sensitivity of *isc1 Δ* cells and decreased oxidative stress markers in these mutants. *SIT4* disruption also increased chronological lifespan and H₂O₂ resistance of parental cells. Sit4p seems to be specifically associated with oxidative stress since its mutation or deficiency confers resistance to thiol-specific oxidants (Lopez-Mirabal *et al.*, 2008), t-butyl-hydroperoxide and menadione (a generator of superoxide radicals), but not to heat shock or cadmium (Fig. S6).

Sit4p functions downstream of Tor1p, a conserved nutrient responsive protein kinase associated with lifespan regulation in organisms ranging from yeast to mice (Di Como and Arndt, 1996). Inhibition of TOR contributes to the beneficial effect of CR on cell longevity (Wei *et al.*, 2008). In agreement with published data, our results show that chronological survival increased in cells submitted to CR. However, *SIT4* deletion further delayed aging of calorie restricted cells, indicating that Sit4p functions, at least in part, through a mechanism that is not modulated by CR.

Several lines of evidence were consistent with CAPP activation in *isc1 Δ* cells. The Gln3p transcription factor is controlled by Sit4p and the expression of a Gln3p-regulated LacZ reporter increased in *ISC1*-deleted cells. Moreover, an in silico analysis suggested that many of the genes differentially expressed in *isc1 Δ* cells (Almeida *et al.*, 2008) are probably regulated by Gln3p. Since *TOR1* or *GLN3* deletion significantly increases chronological lifespan (Powers *et al.*, 2006; Wei *et al.*, 2009), it is likely that the activation of Gln3p associated with Isc1p deficiency contributes to the premature aging phenotype of this mutant strain. Interestingly, TORC2 complex controls ceramide synthase activity as well as the turnover of complex sphingolipids through the activation of Slm1p and Slm2p that inhibit Isc1p (Tabuchi *et al.*, 2006; Dickson, 2008). The characterization of changes in TOR signaling in *isc1 Δ* cells will contribute to our understanding of the role of sphingolipids in the regulation of oxidative stress responses and chronological lifespan.

The identification of a key role of Sit4p activation downstream of Isc1p suggests that the most relevant changes in sphingolipids are the elevations in the levels of ceramides which would be capable of activating of Sit4p. However, significant more studies are needed to conclusively establish the specific downstream effects mediated by specific sphingolipids. Notably, the reciprocal changes in two subgroups of ceramides (increased phytoceramides and decreased α OH-phytoceramides) illustrate the complexity and interconnection of sphingolipid metabolism as one would have expected primarily a decrease in ceramides in the *ISC1* deletion.

During exponential growth on glucose, yeast cells are under catabolite repression: oxygen consumption is low and respiratory metabolism is repressed (Santangelo, 2006). Sit4p is involved in catabolite repression and, therefore, respiration is derepressed in *sit4* Δ cells (Jablonka *et al.*, 2006; Jin *et al.*, 2007; Fig. 6A–B). Notably, *SIT4* disruption alleviated mitochondrial dysfunction and catalase A (Cta1p) deficiency of *isc1* Δ cells. The expression of *CTA1* gene is repressed by glucose but *isc1* Δ cells are unable to derepress the *CTA1* gene after the diauxic shift when glucose decreases to low levels (Kitagaki *et al.*, 2009). The induction of Cta1p activity probably promotes oxidative stress resistance and lifespan in *isc1* Δ *sit4* Δ double mutants by decreasing oxidative damages. Indeed, *CTA1* overexpression partially suppressed the shortened lifespan of *isc1* Δ cells.

The identification of proteins related to mitochondria function that are Sit4p targets will contribute to our understanding of how Sit4p controls chronological lifespan. Published data show that Sit4p interacts with two mitochondrial proteins, namely the Atp3p subunit of the ATP synthase and Nde1p, the external NADH dehydrogenase. Moreover, a mutation in Atp3p (*ATP3-1*) or the deletion of Nde1p are both capable of increasing chronological lifespan of yeast cells (Li *et al.*, 2006; Francis *et al.*, 2007). Thus, Sit4p may affect mitochondria function through modulation of mitochondrial proteins. Mitochondria have been also linked to replicative lifespan, which is measured by counting the number of daughter cells generated by a mother cell before cell division stops. Mitochondria function is required for the maintenance of an age asymmetry between the mother and daughter yeast that prevents the inheritance of damaged molecules by the daughter cell. A retrograde response signals mitochondria dysfunction and induces cellular adaptations that compensate for age changes. This response links metabolism with genome stability during aging (Jazwinski, 2005). It should be noticed that Sit4p also regulates replicative lifespan. Specifically, Sit4p dephosphorylates Sir3p, which plays a key role in maintaining a repressed chromatin structure near telomeres, and loss of Sit4p decreases replicative lifespan (Hayashi *et al.*, 2005). These results point to distinct mechanisms by which Sit4p controls replicative and chronological lifespan of yeast cells.

In summary, our data suggest that Isc1p functions upstream of Sit4p through the modulation of ceramide levels and implicate Sit4p in mitochondrial dysfunction, premature aging and oxidative stress sensitivity. *SIT4* deletion restores mitochondrial function, increasing the longevity of *isc1* Δ cells (Fig. 8). These results offer new insights on the regulation of redox homeostasis by sphingolipid signaling, a link that has been suggested in several studies (Won and Singh, 2006).

Experimental procedures

Yeast strains, plasmids and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Yeast cells were grown aerobically at 26°C in a gyratory shaker (at 140 rpm), with a ratio of flask volume / medium volume of 5:1, to early exponential phase ($OD_{600}=0.6$) or to post-diauxic phase ($OD_{600}=7-8$). The growth media used were YPD (1% (w/v) yeast extract, 2% (w/v)

bactopeptone, 2% (wt/vol) glucose), YPGlycerol (1% (wt/vol) yeast extract, 2% (wt/vol) bactopeptone, 4% (vol/vol) glycerol) or synthetic complete (SC) drop-out medium containing 2% (wt/vol) glucose 0.67% yeast nitrogen base without amino acids and supplemented with appropriate amino acids (80 mg histidine l⁻¹, 400 mg leucine l⁻¹, 80 mg tryptophan l⁻¹). For *ISC1* disruption in the *sit4Δ* strain, a deletion fragment containing *URA3* and the flanking regions of *ISC1* was amplified by PCR using pYES2 (Invitrogen) and the following primers: F (ATTTGCGCTTTCCGCGTAAAAAGGGAAAAAAGCAGATATCTAGCTTTTCAATTCAATTC) and R (TCAGTAATTTTTTTTACATATGCTAAAGAAAATCGATAATATTAGTTTTGCTGGCCGCATC). Cells were transformed by electroporation, and *isc1Δsit4Δ* double mutants were selected in minimal medium-GLC (0.67% (wt/vol) yeast nitrogen base without amino acids, 2% (wt/vol) glucose supplemented with appropriate amino acids (40 mg histidine l⁻¹, 80 mg leucine l⁻¹, 40 mg methionine l⁻¹). For *CTA1* gene overexpression, BY4741 and *isc1Δ* cells were transformed by electroporation with pPGK-M28-I (empty vector) or p*CTA1-GFP* (Petrova *et al.*, 2004) and selected in minimal medium lacking uracil. For Gln3p activity, yeast cells were transformed by electroporation with YCp*MEP2-lacZ* plasmid (Marini *et al.*, 1997), and selected in minimal medium lacking uracil.

Sphingolipid profiling

ESI/MS/MS analysis of endogenous phyto- and dihydrosphingosine bases, their phosphates, α -hydroxylated phytoceramide, and non-hydroxylated phyto- and dihydroceramide species were performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring positive ionization mode, as described (Kitagaki *et al.*, 2007). The mass of each species was normalized to total lipid phosphate.

Stress resistance and chronological lifespan

Stress resistance was determined in cells treated with H₂O₂, menadione, tert-butyl hydroperoxide, cadmium or submitted to heat shock (45°C), as indicated in figure legends, during 30 min. Chronological lifespan was assayed as described previously (Almeida *et al.*, 2008). Briefly, overnight cultures were diluted to OD₆₀₀=0.5 and grown for 24h (to post-diauxic phase). Cells were then centrifuged at 4000 rpm for 5 min, washed twice with water, resuspended in water, and incubated at 26°C for the indicated times. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % agar. Colonies were counted after growth at 26°C for 3 days. Viability was expressed as the percentage of the colony-forming units.

Enzymatic activities and oxygen consumption

All the procedures were carried out at 0–4°C. Yeast cells were harvested by centrifugation. For enzyme activities, yeast extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannheim), by vigorous shaking of the cell suspension in the presence of glass beads for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Cell debris was removed by centrifugation at 13,000 rpm for 15 min and protein content was determined by the method of Lowry, using bovine serum albumin as a standard. Catalase activity was analyzed *in situ*, in the presence of 3,3'-diaminobenzidine tetrahydrochloride, using the H₂O₂/peroxidase system (Conyers and Kidwell, 1991) or spectrophotometrically as described previously (Aebi, 1984). Cytochrome c oxidase activity was determined as previously described (Poyton *et al.*, 1995), by measuring cytochrome c oxidation. Oxygen consumption rate was measured for 7.5 × 10⁸ cells in the culture media using an oxygen electrode (Oxygraph, Hansatech). Data was analyzed using the Oxyg32 V2.25 software. For the β -galactosidase assay, yeast cells containing YCp*MEP2-lacZ* were

grown in SC-glucose medium lacking uracil to exponential phase, and the activity was measured as previously described (Ausubel *et al.*, 1998), with the following modifications: a cellular extract was prepared, as described above, in 100 mM Tris-HCl, 1 mM DTT, 10% (vol/vol) glycerol, and 40 µg of total protein was used in the assay.

Oxidative stress markers

Protein oxidation was determined by immunodetection of protein carbonyls, as previous described (Costa *et al.*, 2002). Protein content of cellular extracts was estimated as described above. Protein carbonylation assays were performed by slot blot analysis or SDS-PAGE using 12.5 % gels followed by protein electrotransfer onto nitrocellulose membranes using rabbit IgG anti-DNP (Dako, Glostrup, Denmark) at a 1:5,000 dilution, as the primary antibody, and goat anti-rabbit IgG-peroxidase (Sigma, St. Louis, MO, USA) at a 1:5,000 dilution, as the secondary antibody. Immunodetection was performed by chemiluminescence, using a kit from GE Healthcare (RPN 2109). Quantification of carbonyls was performed by densitometry (GS-800, BioRad). Intracellular oxidation was measured by using the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (H₂DCF-DA), as described before (Almeida *et al.*, 2008), and values were expressed as fluorescence units mg⁻¹ protein.

Statistical analysis

Data are expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's *t*-test. The 0.05 probability level was chosen as the point of statistical significance throughout.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CAPP	ceramide-activated protein phosphatase
CLS	chronological lifespan
CR	calorie restriction
H₂DCFDA	2',7'-dichlorodihydrofluorescein diacetate
PP2A	type 2A protein phosphatase
ROS	reactive oxygen species
DHS	dihydrosphingosine
PHS	phytosphingosine
dh-Cer	dihydroceramides, phyto-Cer, phytoceramides
αOH-phyto-Cer	α-hydroxylated phytoceramides

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Fig. 1.

The *de novo* synthesis and catabolism of sphingolipids in yeast. LCFA, long chain fatty acid (C_{16-18}); MLCFA, medium long chain fatty acid (C_{20-22}); VLCFA, very long chain fatty acid (C_{24-26}), FFA, free fatty acid.

Fig. 2.

Levels of long chain sphingoid bases during chronological aging. *S. cerevisiae* BY4741, *isc1*Δ and *lcb4*Δ cells were grown in YPD medium to the post-diauxic phase, washed twice with H₂O and kept in H₂O at 26°C. (A) DHS; (B) PHS; (C) DHS-1-P; (D) PHS-1-P. Data are expressed as pmol of lipid/nmol lipid Pi and are means ± SD of three independent experiments.

Fig. 3.

Levels of ceramides during chronological aging. *S. cerevisiae* BY4741, *isc1*Δ and *lcb4*Δ cells were grown in YPD medium to the post-diauxic phase, washed twice with H₂O and kept in H₂O at 26°C. (A) total dihydroceramide (dh-Cer); (B) dihydro-C₂₆-ceramide (dh-C₂₆-Cer); (C) total phytoceramide (phyto-Cer); (D) phyto-C₂₆-ceramide (phyto-C₂₆-Cer); (E) total α-hydroxylated-phytoceramide (αHO-phyto-Cer). Data are expressed as pmol of lipid/nmol lipid Pi and are means ± SD of three independent experiments. See Tables S1–S3 for levels of individual ceramide species.

Fig. 4.

SIT4 disruption suppresses the shortened chronological lifespan of *isc1*Δ cells. *S. cerevisiae* BY4741, *isc*Δ, *sit4*Δ and *sit4*Δ*isc1*Δ mutant cells were grown in YPD medium to post-diauxic phase. (A) Cells were washed twice with H₂O, and kept in H₂O at 26°C. The viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units at time 0h. (B) Protein carbonylation. Protein extracts were derivatized with DNPH and slot-blotted into a PVDF membrane. Immunodetection was performed using an anti-DNP antibody, as described in Materials and Methods. Quantitative analysis of total protein carbonyl content was performed by densitometry using data taken from the same membrane. Data are expressed as the carbonyl content ratio between cells of day 14 and cells of day 0. Values are means ± SD of three independent experiments.

*p<0.05.

Fig. 5.

SIT4 deletion suppresses hydrogen peroxide sensitivity of *isc1Δ* cells. *S. cerevisiae* BY4741, *isc1Δ*, *sit4Δ* and *sit4Δisc1Δ* mutant cells were grown in YPD medium to the exponential phase ($OD_{600}=0.6$) (white bars) and exposed to 1.5 mM H_2O_2 for 30 min (black bars). (A) Cell viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units of non-stressed cells. (B) Intracellular oxidation. Cells were labeled with the molecular probe H_2DCFDA and lysed as described in Materials and Methods. Data was normalized for protein content. (C) Protein carbonylation. Proteins were derivatized with DNPH and slot-blotted into a PVDF membrane. Immunodetection was performed using an anti-DNP antibody, as described in Materials and Methods. Quantitative analysis of total protein carbonyl content was performed by densitometry using data taken from the same membrane. Values are means $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Fig. 6. *SIT4* disruption abolishes mitochondrial dysfunctions seen in *isc1*Δ cells. *S. cerevisiae* BY4741, *isc1*Δ, *sit4*Δ and *sit4*Δ*isc1*Δ mutant cells were grown in YPD medium to exponential (log) or post-diauxic phase (PDS). (A) Oxygen consumption rates were measured as described in Materials and Methods. (B) Cytochrome C Oxidase (COX) specific activity. Cells were lysed, and enzyme activity was measured as described in Materials and Methods. (C) Cells were plated in 5-fold dilution series on glucose or glycerol as carbon source. Values are means ± SD of three independent experiments. **p<0.01.

(D)

BY4741 pCTA1-GFP
*isc1*Δ pCTA1-GFP

Fig. 7.

SIT4 disruption suppresses catalase A deficiency seen in *isc1Δ* cells and *CTA1* overexpression partially suppresses the shortened chronological lifespan of *isc1Δ* cells. *S. cerevisiae* BY4741, *isc1Δ*, *sit4Δ* and *sit4Δisc1Δ* mutant cells were grown in YPD medium to post-diauxic phase (PDS). Catalase activity was determined (A) spectrophotometrically or (B) detected *in situ* after non-denaturing polyacrylamide gel electrophoresis, using the H₂O₂/peroxidase system. (C) *S. cerevisiae* BY4741 and *isc1Δ* mutant cells transformed with pPGK-M28-I (empty vector) or p*CTA1-GFP* were grown in SC medium lacking uracil to post-diauxic phase. Cells were washed twice with H₂O, and kept in H₂O at 26°C. The viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units at time 0h. (D) *CTA1* overexpression increases Cta1p activity in both *S. cerevisiae* BY4741 and *isc1Δ* cells. Enzyme activity was detected as in (b). Values are means ± SD of three independent experiments. **p<0.01.

Fig. 8.

Proposed model for Sit4p-dependent oxidative stress sensitivity and shortened chronological lifespan of *isc1*Δ cells. Cells lacking *Isc1p* display an increase in dh-C₂₆-Cer and phyto-C₂₆-Cer species, probably due to *de novo* biosynthesis, that activate Sit4p. Dephosphorylation of Sit4p target proteins causes mitochondrial dysfunction, leading to oxidative stress sensitivity and shortened chronological lifespan in *isc1*Δ cells. *SIT4* deletion restores mitochondrial function of *isc1*Δ cells, increasing oxidative stress resistance and chronological lifespan.

Table 1*Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/source
BY4741	Mata <i>his3</i> Δ1, <i>leu2</i> Δ0, <i>met15</i> Δ0, <i>ura3</i> Δ0	EUROSCARF
<i>isc1</i> Δ	BY4741 <i>isc1</i> Δ:: <i>KanMX4</i>	EUROSCARF
<i>sit4</i> Δ	BY4741 <i>sit4</i> Δ:: <i>KanMX4</i>	EUROSCARF
<i>isc</i> Δ <i>sit4</i> Δ	BY4741 <i>sit4</i> Δ:: <i>KanMX4</i> <i>isc</i> Δ:: <i>URA3</i>	This study
<i>lcb4</i> Δ	BY4741 <i>lcb4</i> Δ:: <i>KanMX4</i>	EUROSCARF
BY4741 YCpMEP2-lacZ	BY4741 carrying YCpMEP2-lacZ	This study
<i>isc1</i> Δ YCpMEP2-lacZ	<i>isc1</i> Δ carrying YCpMEP2-lacZ	This study
<i>sit4</i> Δ YCpMEP2-lacZ	<i>sit4</i> Δ carrying YCpMEP2-lacZ	This study
<i>isc1</i> Δ <i>sit4</i> Δ YCpMEP2-lacZ	<i>isc1</i> Δ <i>sit4</i> Δ carrying YCpMEP2-lacZ	This study
<i>hxx2</i> Δ	BY4741 <i>hxx2</i> Δ:: <i>KanMX4</i>	EUROSCARF
<i>mig1</i> Δ	BY4741 <i>mig1</i> Δ:: <i>KanMX4</i>	EUROSCARF
BY4741 pPGK-M28-I	BY4741 carrying pPGK-M28-I	This study
BY4741 pCTA1-GFP	BY4741 carrying pPGK-M28-I <i>CTA1-GFP</i>	This study
<i>isc1</i> Δ pPGK-M28-I	<i>isc1</i> Δ carrying pPGK-M28-I	This study
<i>isc1</i> Δ pCTA1-GFP	<i>isc1</i> Δ carrying pPGK-M28-I <i>CTA1-GFP</i>	This study