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REPORT

The ceramide-activated protein phosphatase Sit4p controls lifespan, mitochondrial function and cell cycle progression by regulating hexokinase 2 phosphorylation

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ABSTRACT

Sit4p is the catalytic subunit of a ceramide-activated PP2A-like phosphatase that regulates cell cycle, mitochondrial function, oxidative stress resistance and chronological lifespan in yeast. In this study, we show that hexokinase 2 (Hxk2p) is hyperphosphorylated in *sit4*Δ mutants grown in glucose medium by a Snf1p-independent mechanism and Hxk2p-S15A mutation suppresses phenotypes associated with *SIT4* deletion, namely growth arrest at G1 phase, derepression of mitochondrial respiration, H_2O_2 resistance and lifespan extension. Consistently, the activation of Sit4p in *isc1*Δ mutants, which has been associated with premature aging, leads to Hxk2p hypophosphorylation, and the expression of Hxk2p-S15E increases the lifespan of *isc1*Δ cells. The overall results suggest that Hxk2p functions downstream of Sit4p in the control of cell cycle, mitochondrial function, oxidative stress resistance and chronological lifespan.

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Introduction

Bioactive sphingolipid metabolites, such as ceramide, sphingosine and sphingosine-1-phosphate, participate actively in the regulation of signal transduction pathways and modulate a variety of cellular processes, including growth, stress responses, apoptosis and aging. Protein targets, such as ceramide-activated protein kinases and phosphatases, protein kinase C, cathepsin D and JNK, have been suggested to mediate the effects of bioactive sphingolipids.[1]

The yeast Sit4p is the catalytic subunit of the ceramide-activated protein phosphatase (CAPP), a heterotrimeric complex that also includes Tpd3p and Cdc55p as regulatory subunits.[2] 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.[3,4] As expect for a protein phosphatase, Sit4p regulates a wide range of biological processes, including cell functions that are controlled by Pkc1p, such as cell integrity pathway, cytoskeleton organization and ribosomal gene expression, the Swi4p factor that controls the transcription of G1 cyclin genes,[5-7] the ubiquitin-proteasome system,[8] silencing in the subtelomeric region,[9] monovalent ion and pH homeostasis,[10] nutrient signaling,[11,12] endocytosis [13] and traffic from the endoplasmic reticulum to the Golgi complex.[14]

Sit4p also plays a central role in the regulation of mitochondrial function. Sit4p deficiency prevents yeast cells to grow on respiratory substrates due to a shift of carbohydrate metabolism flux into gluconeogenesis and glycogen storage at the expense of intermediates of the Krebs cycle.[15,16] Wild type cells grown in glucose medium rely on fermentation for energy production and repress the transcription of genes associated with mitochondrial function and the utilization of alternative carbon sources.[17] Interestingly, fermentation is reduced in *sit4*Δ cells due to a decrease of pyruvate decarboxylase activity.[18] Moreover, respiration is derepressed in *sit4*Δ cells grown in glucose medium and mitochondrial respiration is essential for their viability since these mutants are unable to grow under anaerobic conditions.[16] In wild type cells grown under repressing conditions, both Mig1p and Hxk2p are dephosphorylated by the Reg1p-Glc7p protein phosphatase complex, imported into the nucleus and form a Mig1p-Hxk2p complex that represses the expression of genes associated with growth on non-fermentable carbon sources.[19,20] The interaction between Hxk2p and Mig1p under repressing conditions inhibits Mig1p phosphorylation at Ser311 by the Snf1p kinase and its export from the nucleus into the cytosol by Msn5p.[19] Under these conditions, Snf1p is dephosphorylated and inhibited by Reg1p-Glc7p and Sit4p.[21] The catabolite derepression in *sit4*Δ cells is correlated with the degradation of the Mig1p transcription factor.[22]

Mitochondria are the main source of reactive oxygen species in cells and play a central role in oxidative stress resistance and chronological lifespan.[23] A recent study showed that deletion of Sit4p protects cells from defects

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associated with mitochondrial DNA damage, including reduced proliferation and decreased mitochondrial protein import and electrochemical potential, by mechanisms that require Snf1p.[24] Consistent with Sit4p being a negative regulator of mitochondrial function, *sit4*Δ cells show an increased chronological lifespan.[25] This protective effect is observed even in cells submitted to severe calorie restriction,[25] a regime that results in the longest survival for wild type yeast strains.[26] Thus, Sit4p seems to partially function through a mechanism that is not modulated by calorie restriction. Sit4p is a downstream target of Tor1p, a conserved nutrient protein kinase associated with lifespan regulation in several organisms. Sit4p deficiency also increases cellular resistance to thiol-specific oxidants and H_2O_2.[12,27] Consistent with a role for Sit4p/CAPP in the regulation of lifespan and oxidative stress resistance, the increase of dihydro-C26-ceramide and phyto-C26-ceramide levels is associated with the activation of Sit4p in *isc1*Δ cells, leading to mitochondrial dysfunction, H_2O_2 sensitivity and a shortened chronological lifespan.[25,28] Isc1p is an ortholog of mammalian neutral sphingomyelinase 2[29] and plays an important role in mitochondrial function. When aerobic respiration is induced, Isc1p is translocated from the endoplasmic reticulum into mitochondria, where it generates α-hydroxylated phytoceramides that contribute to its normal function.[30,31] The defective aerobic respiration in *isc1*Δ cells is associated with its incapacity to upregulate genes required for non-fermentable carbon source metabolism.[32,33]

Here we provide evidence that *SIT4* deletion leads to Hxk2p hyperphosphorylation and that Hxk2p-Ser15 phosphorylation is required for cellular effects such as growth arrest at G1 phase, derepression of mitochondrial respiration, H_2O_2 resistance and lifespan extension in *sit4*Δ cells. The role of Hxk2p phosphorylation on the regulation of aging is supported by data showing that Sit4p-dependent Hxk2p hypophosphorylation correlates with premature aging in *isc1*Δ cells and the expression of Hxk2p with a phosphomimetic glutamate residue at position 15 (Hxk2p-S15E) increases mean chronological lifespan of this mutant.

Material and methods

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% (wt/vol) yeast extract, 2% (wt/vol) bactopeptone, 2% (wt/vol) glucose), or synthetic complete (SC) drop-out medium containing 2% (wt/vol) glucose and 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco Laboratories, Detroit, USA). The deletion of *ISC1* in the *hxk2*Δ strain was performed using a deletion fragment containing *LEU2* and the flanking regions of *ISC1*. The deletion of *SIT4* in the *snf1*Δ and WAY.78–1 strains was performed using a deletion fragment containing *MX4HIS3 or KanMX4* and the flanking regions of *SIT4*, respectively. Cells were transformed by electroporation, and double mutants were selected in minimal medium lacking histidine or YPD containing 200 μg geneticin ml^{-1}. Gene deletion was confirmed by PCR. To generate pRS316-*HXK2*-S15 and pRS316-*HXK2*-S15E plasmids, YIp*HXK2*-S15 and YIp*HXK2*-S15E[34] were digested with *Bam*HI and *Eco*RI and the fragments containing *HXK2-S15* and *HXK2-S15E* were cloned into the pRS316 *Bam*HI and *Eco*RI sites. Cells transformed with pRS316-*HXK2*-S15, pRS316-*HXK2*-S15E or YEp352-*HXK2-GFP6*[35] were selected in minimal medium lacking uracil.

2D-gel electrophoresis and protein phosphorylation

Yeast cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannhein) and phosphatase inhibitors (50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate), and lysed by vigorous shaking of the cell suspension in the presence of glass beads for

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

Strain	Genotype	Reference/source
BY4741[a]	Mata *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	EUROSCARF
*sit4*Δ[a]	BY4741 *sit4*Δ::*KanMX4*	EUROSCARF
*cdc55*Δ[a]	BY4741 *cdc55*Δ::*KanMX4*	EUROSCARF
*isc1*Δ	BY4741 *isc1*Δ::*KanMX4*	EUROSCARF
*isc1*Δ*sit4*Δ	BY4741 *sit4*Δ::*KanMX4 isc1*Δ::*URA3*	[25]
*snf1*Δ[a]	BY4741 *snf1*Δ::*KanMX4*	EUROSCARF
*sit4*Δ*snf1*Δ[a]	BY4741 *sit4*Δ::*MXHIS3 snf1*Δ::*KanMX4*	This study
*hxk2*Δ[b, c]	BY4741 *hxk2*Δ::*KanMX4*	EUROSCARF
*isc1*Δ*hxk2*Δ[b, c]	BY4741 *hxk2*Δ::*KanMX4 isc1*Δ::*LEU2*	This study
WAY.78-1 YIpHXK2-S15	Matα *ura3-52 leu 2–3,112 trp1-289 hxk1*Δ::*HIS3 hxk2*Δ::*LEU2 glk1*Δ::*LEU2 MAL2-8[c] MAL3 SUC3* YIp*HXK2(S15)*	[34]
WAY.78-1 YIpHXK2-S15A	Matα *ura3-52 leu 2–3,112 trp1-289 hxk1*Δ::*HIS3 hxk2*Δ::*LEU2 glk1*Δ::*LEU2* MAL2-8c MAL3 SUC3 YIp*HXK2(S15A)*	[34]
WAY.78-1 YIpHXK2-S15 *sit4*Δ	WAY.78-1 YIp*HXK2-S15 sit4*Δ::*KanMX4*	This study
WAY.78-1 YIpHXK2-S15A *sit4*Δ	WAY.78-1 YIp*HXK2-S15A sit4*Δ::*KanMX4*	This study
BY4742	MATα *his3*Δ*1, leu2*Δ*0, lys2*Δ*0, ura3*Δ*0*	EUROSCARF
*sit4*Δ[a]	BY4742 *sit4*Δ::*KanMX4*	EUROSCARF
*pph21*Δ*pph22*Δ[a]	BY4742 *pph21*::*KanMX4*Δ*pph22*Δ::*KanMX4*	[64]

Note. Cells harboring YEp352-HXK2-GFP (a), pRS316-HXK2-S15 (b) or pRS316-HXK2-S15E (c) are indicated with "a", "b" and "c" superscript.

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. The proteins (200 μg) were solubilized in IEF solution (9 M Urea, 2% (wt/vol) CHAPS, 2% (vol/vol) ß-mercaptoethanol, 0.8% (vol/vol) Pharmalytes pH 3–10) and separated by 2D-gel electrophoresis, using 13 cm immobilized pH 3–10 nonlinear gradient (IPG) dry strips (GE Healthcare) in the first dimension, as previously described.[36] After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (Hybond-C, GE Healthcare, Little Chalfont, United Kingdom). A replica 2D-gel was silver stained and used for protein identification. For the analysis of phosphorylated proteins, the nitrocellulose membrane was incubated with the primary antibody, specific for the phosphorylated serine residues (rabbit anti-phosphoserine, Zymed Laboratories, Invitrogen, Waltham, USA), at a 1:1,000 dilution, and subsequently with the secondary antibody, goat anti-rabbit IgG-linked to horseradish peroxidase (Sigma-Aldrich, St. Louis, USA), at a 1:5,000 dilution. Immunodetection was performed by chemiluminescence, using a kit from GE Healthcare (RPN 2109). The film and the gel were scanned using a densitometer (GS-800, Bio-Rad). Images were converted to tagged image file format (TIFF) and the PDQuest v7.3 (Bio-Rad) software was used for quantification of spot intensities. Two normalization steps were performed to determine the fold changes in (phospho)protein levels. First, sampled spot intensities were divided by the intensities of all spots. Second, for each protein, normalized phosphorylation level was divided by normalized protein intensity. The relative phosphoprotein level was expressed as the ratio between the *sit4*Δ and BY4741 strains. Proteins that changed at least 2-fold were considered for further analysis. All values are means of the expression profiles of 3 experiments with similar results, using independent cultures grown under the same conditions.

Protein identification

Silver-stained protein spots were excised and *in gel* digested with trypsin (Promega, USA). Peptide extraction was performed by a 60% acetonitrile/0.1% trifluoroacetic acid solution. Protein digests were desalted and concentrated using ZipTips (Millipore, USA) and crystallized onto a MALDI plate using α-Cyano-4-hydroxycinnamic acid as a matrix. Samples were analyzed using the 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, USA), as previously described.[37]

Western blotting

To evaluate Snf1p phosphorylation, cells were grown to exponential phase and proteins were extracted as previously described.[38] For Western blotting 40 μg of proteins were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were incubated with anti-Snf1 (Santa Cruz Biotechnology, Inc., Dallas, USA) at a 1:500 dilution, or anti-AMPK (phospho T172; Cell Signaling Technology, Beverly, MA, USA) at a 1:1,000 dilution followed by incubation with appropriate secondary antibody. For analysis of Hxk2p phosphorylation, Hxk2p was immunoprecipitated by incubating protein extracts from exponential phase cells with anti-GFP (Roche, Basel, Switzerland) for 2 h at 4°C. Protein A-Sepharose beads (GE Healthcare, Little Chalfont, United Kingdom) were then added and incubated for 2 h at 4°C. After extensive washes (150 mM NaCl, 50 mM Tris, pH 7.5, 1mM EDTA, 2% (vol/vol) Triton X-100, containing protease and phosphatase inhibitors as before), immunoprecipitated samples were boiled in 2X SDS-loading buffer and the supernatant was subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with anti-phosphoserine (Invitrogen, Waltham, USA) at a 1:10,000 dilution followed by incubation with appropriate secondary antibody. After stripping, membranes were reprobed with anti-GFP (Roche) at a 1:10,000 dilution followed by incubation with the secondary antibody. Where mentioned, stripped membranes were also reprobed with anti-phosphotyrosine (Zymed Laboratories, Invitrogen, Waltham, USA) at a 1:3,000 dilution. Immunodetection was performed by chemiluminescence using a kit from GE Healthcare. Western-blots shown are representative of at least 3 independent experiments with similar results.

Oxygen consumption

Oxygen consumption rate was measured for 7.5×10^8 cells in the culture media using an oxygen electrode (Oxygraph, Hansatech). Data was analyzed using the Oxyg32 V2.25 software (Oxygraph, Hansatech).

Oxidative stress resistance and chronological lifespan

Oxidative stress resistance was determined in yeast cells grown to exponential phase ($OD_{600nm} = 0.6$) and treated with 0.5 mM H_2O_2 (Merck, Darmstadt, Germany) for 30 min. Chronological lifespan was assayed as previously described.[39] Briefly, overnight cultures were diluted to $OD_{600nm} = 0.5$ and grown for 48h (stationary phase; considered t0 in the lifespan assay) and kept in culture media 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 d. Viability was expressed as the percentage of the colony-forming units.

Cell cycle analysis

10^7 cells in exponential phase were collected and fixed with ethanol 70 % overnight at 4°C. Cells were washed twice in 1 ml of 50 mM sodium citrate pH 7.0 buffer and resuspended in the same buffer. RNase A was added to a final concentration of 0.25 mg ml^{-1} and incubated at 50°C for 1 h. Cells were washed in 50 mM sodium citrate pH 7.0 and sonicated for 2 min at output 2, duty cycle 20% for 2 min, incubated with propidium iodide (16 μg ml^{-1}), incubated for 30 min at room temperature, and analyzed by flow cytometry. Fluorescence was measured on the FL-2 channel of a Becton-Dickinson FACSort flow cytometer (excitation and emission 488 and 585 nm, respectively). The data was analyzed using the FlowJo software (Tree Star).

Results

Phosphoproteomic analysis of sit4Δ cells

The reversible phosphorylation of proteins plays a major role in signal transduction, transcriptional regulation, and metabolic control by affecting protein function, activity, localization or interactions. Since Sit4p is the catalytic subunit of a type 2A ceramide-activated protein phosphatase that was previously implicated in the regulation of oxidative stress resistance and chronological lifespan,[25] we raised the hypothesis that changes in protein phosphorylation could mediate the phenotypes of *sit4*Δ cells. To identify alterations in the (phospho)proteome associated with loss of Sit4p, protein extracts prepared from parental and *sit4*Δ cells were separated by 2D-gel electrophoresis and silver stained or blotted into a nitrocellulose membrane. Proteins phosphorylated at serine residues were analyzed by immunodetection, using an anti-phosphoserine antibody.

The analysis of total protein patterns showed that the deficiency in Sit4p led to an increase of the levels of 6 proteins (9 spots) whereas that of 27 proteins (30 spots) decreased (Fig. 1A and Supplemental Table 1). Proteins differentially expressed were sorted into functional categories according to MIPS (Munich information center for protein sequences; Table 2). The most significantly affected cell functions were related to carbohydrate metabolism and energy production. Notably, 5 out of the 6 induced proteins and 30% of repressed proteins were associated with carbohydrate metabolism. Some of these changes, such as the increase in the levels of Eno1p (expression repressed by glucose) and the decrease in the levels of Eno2p (expression induced by glucose), are consistent with the catabolite derepression in *sit4*Δ mutants.[16] Remarkably, we also

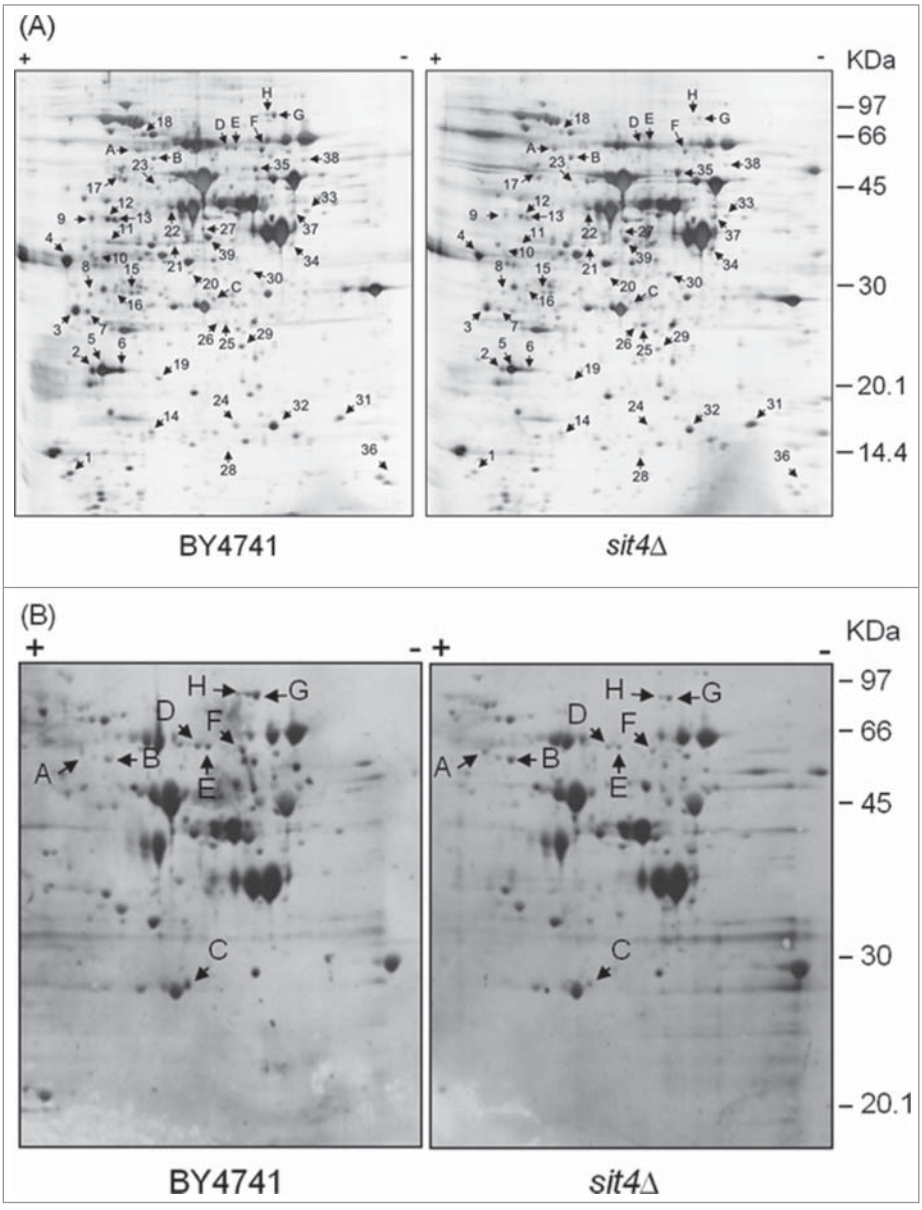

Figure 1. Analysis of changes in the proteome of *sit4*Δ cells. Yeast extracts were prepared from *S. cerevisiae* BY4741 and *sit4*Δ cells grown in YPD medium to exponential phase. Proteins were separated by 2-dimensional gel electrophoresis and visualized by silver staining (A) or blotted into a nitrocellulose membrane. Immunodetection of proteins phosphorylated in serine residues was performed using an anti-phosphoserine antibody (B), as described in Materials and Methods. The experiment was reproduced 3 times, using independent samples. A representative gel/blot is shown. Arrows indicate proteins differentially expressed in *sit4*Δ cells compared to BY4741 cells.

Table 2. Functional categories of proteins differentially expressed in *sit4*Δ cells. Proteins differentially expressed (*sit4*Δ vs parental cells) were sorted into functional categories according to MIPS (Munich information center for protein sequences). Proteins belonging to each category are indicated, together with their fold-change (in parentheses). Upregulated proteins are shown in bold.

Functional category	P-VALUE	Proteins
Purine metabolism	3.29E-04	Ade1p (0.27) Guk1p (0.38) Ado1p (0.39) Shm2p (0.54)
C-compound and carbohydrate metabolism	1.27E-06	**Tdh3p (3.2–4.2) Tdh2p (2.8–6.9) Pgk1p (3.6) Eno1p (2.5) Adh1p (13.0)** YDL124wp (0.28) Sec53p (0.39) Dys1p (0.41) Eno2p (0.46) Shm2p (0.54) Rki1p (0.40) Idh2p (0.47) Ald6p (0.56)
Energy	188E-05	**Tdh3p (3.2–4.2) Tdh2p (2.8–6.9) Pgk1p (3.6) Eno1p (2.5) Adh1p (13.0)** Pdb1p (0.35) Eno2p (0.46) Rki1p (0.40) Idh2p (0.47) Ald6p (0.56)
Glycolysis and gluconeogenesis	6.69E-07	**Tdh3p (3.2–4.2) Tdh2p (2.8–6.9) Pgk1p (3.6) Eno1p (2.5)** Pdb1p (0.35) Eno2p (0.46)
Fermentation	2.76E-02	**Adh1p (13.0)** Ald6p (0.56)
Protein synthesis	1.45E-02	**Rps10bp (> 10)** Efb1p (0.53) Tma19p (0.36) Mrp8p (0.28) Rps0bp (0.54) Hcr1p (0.30) Ssb2p (0.10)
Stress response	9.99E-02	Cpr1p (0.41–0.47) Hmf1p (0.53) Sod1p (0.41) Ahp1p (0.2–0.45) Ssb2p (0.10)
Oxidative stress response	3.69E-02	Sod1p (0.41) Ahp1p (0.2–0.45)
Homeostasis of cations	5.97E-02	Sod1p (0.41) Ahp1p (0.2–0.45) Vma4p (0.39)

observed a decrease in the levels of 5 proteins associated with stress responses, including the antioxidant defenses Sod1p and Ahp1p that catalyze the disproportionation of superoxide radicals [40] and the reduction of alkyl hydroperoxides,[41] respectively.

The proteomic data also showed a decrease in the levels of several proteins associated with nucleotide metabolism and protein translation that may contribute to the low cellular growth rate observed in the *sit4*Δ strain.[42] One of these proteins, Tma19p, is the yeast ortholog of the translationally controlled tumor protein (TCTP) and it is translocated from cytoplasm to the outer surface of the mitochondria after induction of apoptosis by oxidative stress or replicative aging. Notably, *TMA19* deletion increases the resistance to hydrogen peroxide and mother cell-specific lifespan.[43] The decrease in Tma19p may therefore contribute to oxidative stress resistance and increased lifespan of *sit4*Δ cells.

In the analysis of the phosphoproteome, 57 protein spots were detected in parental cells, 8 of them being differentially phosphorylated in *sit4*Δ cells (Fig. 1B). These spots were identified by mass spectrometry and correspond to 6 proteins (Table 3). The levels of phospho-Hxk2p and phospho-Dug1p increased, as expected in a phosphatase deficient strain. The decrease in the phosphorylation status of the other proteins may result from indirect effects. Changes in the phosphorylation of Dug1p and Cys4p, 2 proteins associated with glutathione metabolism,[44,45] may explain the increased levels of this tripeptide in *sit4*Δ cells ([27]; our results, data not shown). However, glutathione deficiency does not affect oxidative stress resistance of a *sit4-110* mutant.[27]

A Western blot analysis of Hxk2p immunoprecipitated from protein extracts of cells expressing Hxk2p-GFP confirmed that serine phosphorylation of Hxk2p increases in cells lacking Sit4p (Fig. 2). This effect seems to be specific for serine since no changes in phospho-Hxk2p levels were observed using an anti-phosphotyrosine antibody. Notably, the deletion of *CDC55* gene, which encodes a regulatory subunit B of the CAPP complex,[2] also promoted serine phosphorylation of Hxk2p. Moreover, the levels of Hxk2p phosphorylation in *cdc55*Δ mutants (2.9-fold relative increase) were higher to those observed in *sit4*Δ cells (1.9-fold relative increase) (Fig. 2). This is probably due to the fact that Cdc55p is also the regulatory subunit B of type 2A protein phosphatase (PP2A), a heterotrimeric complex that contains Pph21p or Pph22p as catalytic subunit and Tpd3p as regulatory subunit A.[46-48] Indeed, downregulation of Pph21p and Pph22p also increased serine phosphorylation of Hxk2p (1.9-fold relative increase) to levels similar to those of *sit4*Δ cells (Fig. 2), suggesting that both CAPP and PP2A contribute to the regulation of Hxk2p phosphorylation.

*Hxk2p-S15 phosphorylation is essential for oxidative stress resistance, lifespan extension and G1 arrest in sit4*Δ *cells*

Together with Mig1p and Snf1p, Hxk2p is part of an interconnected network that regulates both replicative and chronological lifespan in yeast.[49-51] The phosphorylation of Hxk2p in serine-15 was previously implicated in glucose signaling.[52,53] To assess the importance of Hxk2p-S15 phosphorylation in the increase of oxidative stress resistance and chronological lifespan in *sit4*Δ cells, *SIT4* gene was deleted in *S. cerevisiae* WAY.78-1 cells expressing wild type hexokinase 2 (Hxk2p-S15) or a mutant protein with an alanine instead of serine at position 15 (Hxk2p-S15A; phosphoresistant mutation). This

Table 3. Identification of proteins differentially phosphorylated in *sit4*Δ mutants.

Gel spot	Accession no.	Protein name	Protein Function	MW (kDa)	pI	Fold Change	t-test
A	gi|6321184	Hxk2p	Hexokinase isoenzyme 2	54.1	5.16	2.3	$p < 0.01$
B	gi|14318569	Dug1p	Probable di- and tri-peptidase	53.0	5.43	2.6	$p < 0.01$
C	gi|6324759	Gsp2p	GTP binding protein	25.1	6.22	0.37	$p < 0.01$
D	gi|460064	Gua1p	GMP synthase	58.7	6.05	0.39	$p < 0.01$
E	gi|460064	Gua1p	GMP synthase	58.7	6.05	0.45	$p < 0.01$
F	gi|6321594	Cys4p	Cystathionine β-synthase	56.0	6.25	0.31	$p < 0.01$
G	gi|6325331	Tkl1p	Transketolase	73.8	6.51	0.25	$p < 0.01$
H	gi|6325331	Tkl1p	Transketolase	73.8	6.51	0.18	$p < 0.01$

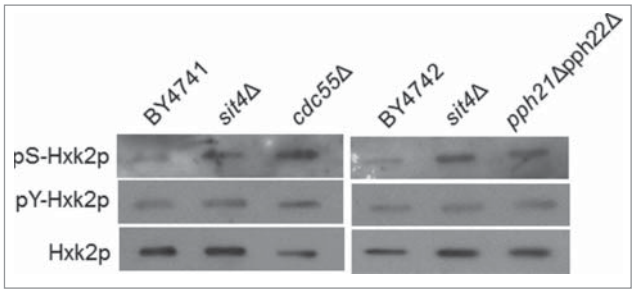

Figure 2. Hxk2p is hyperphosphorylated in cells with compromised CAPP or PP2A activity. Hxk2p-GFP was immunoprecipitated from the indicated mutants and immunodetected using anti-phosphoserine, anti-phosphotyrosine or anti-GFP antibodies, as described in Material and Methods. A representative experiment is shown.

strain is isogenic to the ENY.WA parental strain (K.-D. Entian, Frankfurt, Germany). We have used a different genetic background to confirm that the phenotypes of *sit4*Δ cells were not specific of the BY4741 strain. Moreover, WAY.78-1 cells do not express the other 2 yeast hexokinases and one of them, Hxk1p, was also previously described as a phosphoprotein.[53] Therefore, the use of this strain allowed us to specifically test the role of Hxk2p phosphorylation on the phenotypes of *sit4*Δ cells. As observed in the BY4741 background,[25] *SIT4* deletion in WAY.78-1 cells expressing wild type hexokinase (Hxk2p-S15) increased oxygen consumption, H_2O_2 resistance and chronological lifespan (Fig. 3A-C). These effects were significantly decreased when *SIT4* was deleted in cells expressing Hxk2p-S15A. Interestingly, the Hxk2p-S15A mutation decreased

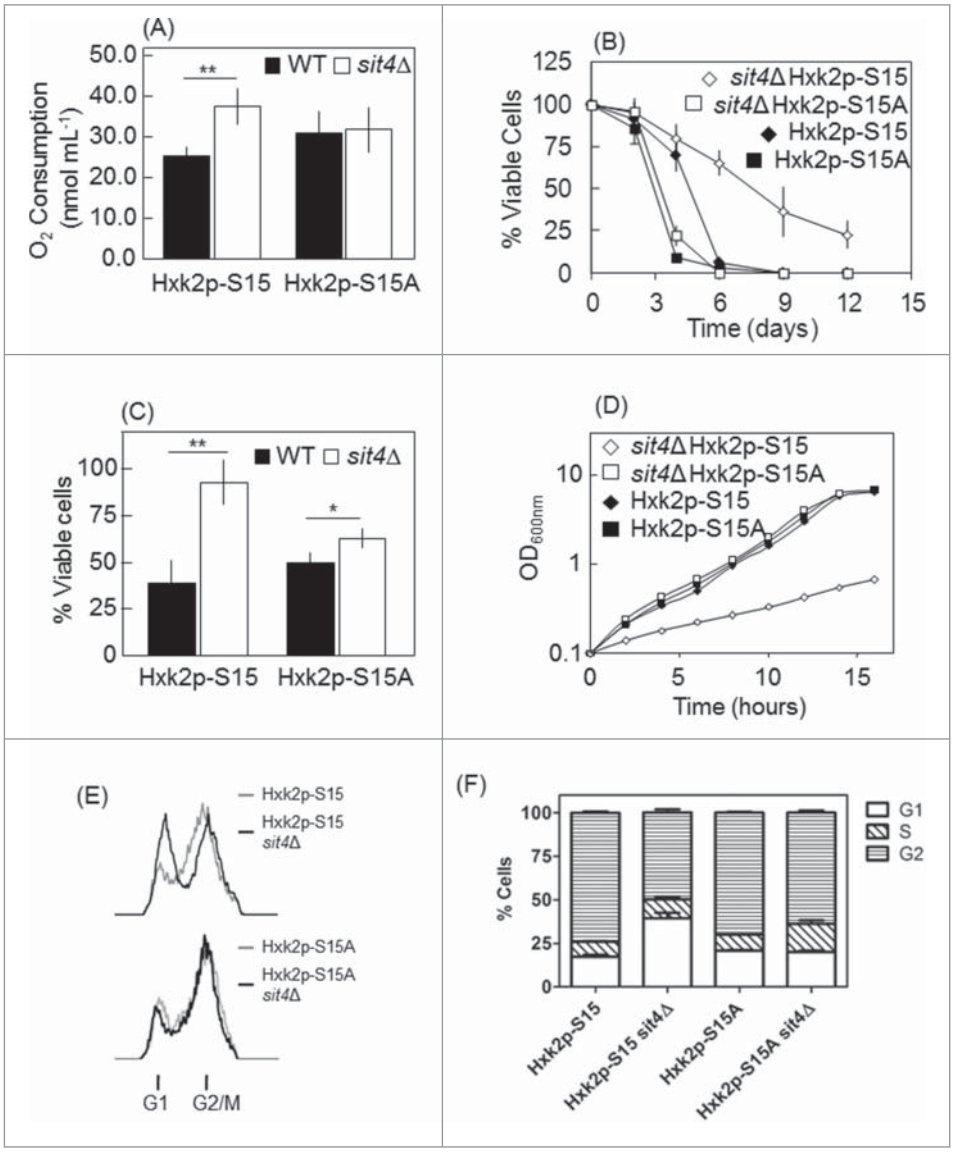

Figure 3. Hxk2p-S15 to Hxk2p-A15 mutation suppresses the phenotypes of *sit4*Δ cells. *SIT4* gene was deleted in *S. cerevisiae* WAY.78-1 cells expressing wild type (Hxk2p-S15) or mutant (Hxk2p-S15A) hexokinase 2. (A) Oxygen consumption rates were measured as described in Materials and Methods using exponential phase cells grown in YPD medium. (B) Chronological lifespan was assessed by following the survival of cells maintained in the growth medium overtime. Cellular viability was measured at 2 to 3 d intervals and was expressed as % colony forming units (aged vs day 0). Values are mean ± SD of at least 3 independent experiments. (C) Oxidative stress resistance was assessed in cells were treated with 0.5 mM H_2O_2 for 30 min. Cell viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units of non-stressed cells. Values are means ± SD of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$; Student's *t*-test. (D) Growth curves. (E) Cell cycle. Yeast cells were labeled with propidium iodide and analyzed by flow cytometry. The experiments were reproduced 3 times, using independent samples. A representative experiment is shown. (F) Quantification of the percentage of cells in the different phases of the cell cycle [from (E)].

chronological lifespan even in the presence of Sit4p (Fig. 3B), further supporting the importance of Hxk2p-S15 phosphorylation for cell survival during aging.

It was previously shown that cells lacking Sit4p exhibit slow growth due to cell cycle arrest in G_1 phase.[42] To investigate if this phenotype is associated with changes in Hxk2p phosphorylation, we measured the effect of *SIT4* deletion on the growth rate of cells expressing Hxk2p-S15 or Hxk2p-S15A. As expected, *sit4*Δ Hxk2p-S15 cells presented a slow growth phenotype (generation time = 405min) but cell growth was not affected in *sit4*Δ Hxk2p-S15A cells, compared to the correspondent parental cells (Hxk2p-S15A; g = 150 min) (Fig. 3D). To correlate these results with G_1 arrest, we analyzed the cell cycle of these cells. The *sit4*Δ Hxk2p-S15A strain exhibited an increased number of cells in the G2/M phase, compared with that in *sit4*Δ Hxk2p-S15 cells (Fig. 3E, F). These results show that Hxk2p functions downstream of Sit4p in the control of oxidative stress resistance, chronological lifespan and cell cycle.

*Hxk2p hypophosphorylation contributes to premature aging in isc1*Δ *cells*

As Sit4p is activated in cells lacking Isc1p,[25] we postulated that the phosphorylation of Hxk2p and Dug1p decrease in those mutant cells by a Sit4p-dependent mechanism. Indeed, a phosphoproteomic analysis revealed that *isc1*Δ cells display lower levels of phospho-Hxk2p and phospho-Dug1p that were suppressed in *isc1*Δ*sit4*Δ cells (Fig. 4A). These results further support a role of Sit4p in the regulation of Hxk2p and Dug1p.

To examine whether Hxk2p hypophosphorylation contributes to the shortened lifespan of *isc1*Δ cells, we expressed wild type hexokinase (Hxk2p-S15) or this protein with a phosphomimetic serine to glutamate mutation at position Ser-15 (Hxk2p-S15E) in *isc1*Δ*hxk2*Δ cells. As shown in Figure 4B, the lifespan of *isc1*Δ*hxk2*Δ cells increased upon expression of Hxk2p-S15E vs Hxk2p-S15. However, the phosphomimetic Hxk2p-S15E mutation did not significantly affect the lifespan of *hxk2*Δ cells (Fig. 4B). Hxk2p phosphorylation increases in wild type cells upon glucose depletion,[53] which also occurs before yeast cells reach stationary phase when chronological lifespan is measured. This may explain why the expression of the phosphomimetic Hxk2p-S15E did not further enhance the lifespan of parental cells. Moreover, *hxk2*Δ Hxk2p-S15E cells did not show the slow growth phenotype exhibited by *sit4*Δ mutants (data not shown). The overall results suggest that Hxk2p functions downstream of Sit4p in the control of oxidative stress resistance, chronological lifespan and cell cycle, but other unidentified protein(s) may be involved.

*Hxk2p is not a direct target of Sit4p or phosphorylated by Snf1p in sit4*Δ *cells*

Our results led us to postulate that Sit4p may directly dephosphorylate Hxk2p. However, co-immunoprecipitation assays using Sit4p-TAP and Hxk2p-GFP constructs did not reveal a

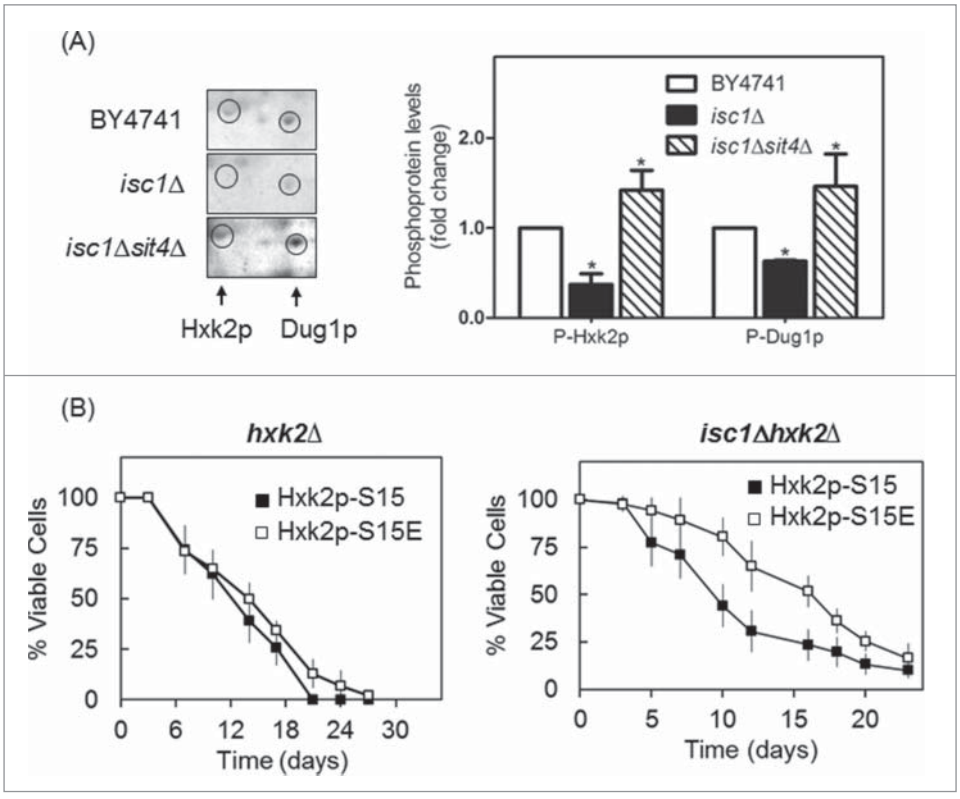

Figure 4. Hxk2p is hypophosphorylated in *isc1*Δ cells by a Sit4p-dependent mechanism. (A) Yeast extracts were prepared from *S. cerevisiae* BY4741, *isc1*Δ and *isc1*Δ*sit4*Δ cells and a phosphoproteome analysis was performed, as described in legend to Figure 1. The region of the blots showing Hxk2p and Dug1p was selected. Spot intensities were quantified by densitometry. Values are means ± SD of 3 independent experiments. $^{*}p < 0.05$. (B) Chronological lifespan of *S. cerevisiae hxk2*Δ and *isc1*Δ*hxk2*Δ cells expressing Hxk2p-S15 or Hxk2p-S15E. Cellular viability was determined as described in legend to Figure 2B. Values are means ± SD of 4 independent experiments.

direct interaction between these proteins. *In vitro* assays using a Sit4p-GST construct also suggest that Hxk2p is not a direct target of Sit4p (data not shown). Sit4p is known to dephosphorylate a Thr-210 on the activation loop of Snf1p, inhibiting this kinase.[21] Thus, Snf1p activation in *sit4*Δ cells could mediate Hxk2p phosphorylation. In fact, it was previously reported that Hxk2p is a substrate of Snf1p,[54] a protein kinase that senses and regulates energy homeostasis.[17] However, other studies indicate that Snf1p is not essential for Hxk2p phosphorylation [34] although it seems to positively modulate Hxk2p phosphorylation.[55] Consistently, our results showed an increase of Snf1p phosphorylation in *sit4*Δ cells (Fig. 5A). However, Hxk2p phosphorylation did not decrease (it even increased) in *sit4*Δ*snf1*Δ when comparing with *sit4*Δ cells (Fig. 5B), suggesting that Snf1p activation does not contribute to Hxk2p phosphorylation in *sit4*Δ cells.

Discussion

The modulation and coordination of cell signaling pathways plays critical roles in oxidative stress resistance and cell longevity. Signal transduction often involves the reversible phosphorylation of proteins. Ceramide is an evolutionary conserved molecule that affects cell functions through the regulation of protein kinases or phosphatases.[1] We have shown that loss of Sit4p, the catalytic subunit of a ceramide-activated type 2A protein phosphatase, increases oxidative stress resistance and extends chronological lifespan.[25] In this study, we adopted a phosphoproteomic approach in order to define potential candidate substrates for Sit4p that could mechanistically mediate the actions of this phosphatase. Importantly, we found that hexokinase 2 (Hxk2p) and Dug1p are hyperphosphorylated in *sit4*Δ cells. The increase in phospho-Dug1p suggests that Sit4p has a function, direct or indirect, in the dephosphorylation of Dug1p, a protein associated with a glutathione degradation pathway.[44] Interestingly, the phosphorylation of Cys4p (cystathionine β-syntethase), a protein involved in the biosynthesis of cysteine that is required for glutathione production,[45] decreased in *sit4*Δ cells. Although none of these proteins have been described as phosphoproteins, changes in its phosphorylation status are probably related to the increase of glutathione levels in *sit4*Δ cells. However, the oxidative stress resistance of *sit4*Δ cells is glutathione-independent.[27]

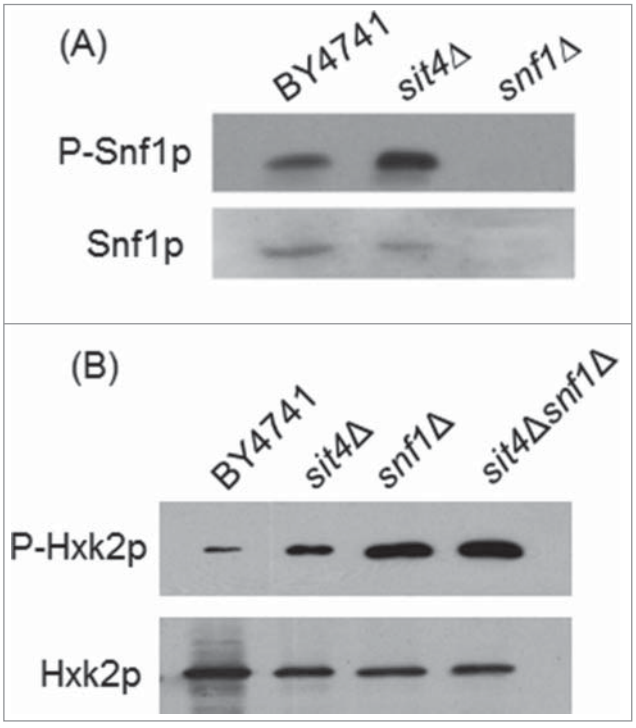

Figure 5. Snf1p activation does not mediate Hxk2p phosphorylation in *sit4*Δ cells. (A) Protein extracts from *S. cerevisiae* BY4741, *sit4*Δ and *snf1*Δ cells were analyzed by immunoblotting using anti-Snf1 or anti-AMPK (phospho T172) as described in Materials and Methods. (B) Hxk2p-GFP was immunoprecipitated from protein extracts of *S. cerevisiae* BY4741, *sit4*Δ, *snf1*Δ and *sit4*Δ*snf1*Δ cells and analyzed by immunoblotting using anti-GFP or anti-phosphoserine as described in Materials and Methods. Representative blots are shown (out of 3 independent experiments).

Hxk2p is a glycolytic enzyme involved in glucose signaling and aging.[49-51,56] Hxk2p phosphorylation has a regulatory function in the Mig1p-dependent glucose signaling pathway. Under derepressing conditions, Hxk2p is phosphorylated.[34] Under repressing conditions, Hxk2p is dephosphorylated by the Reg1p-Glc7p protein phosphatase complex and forms an active dimer that is imported into the nucleus where it interacts with the Mig1p transcription factor and represses genes associated with growth on non-fermentable carbon sources.[20] The phosphorylation and inhibition of Mig1p mediated by the Snf1p kinase is prevented by Snf1p dephosphorylation mediated by Reg1p-Glc7p or Sit4p.[21]

Our results suggest that Sit4p also has an indirect function in Hxk2p dephosphorylation and Hxk2p-S15 phosphorylation is critical for mitochondrial derepression and for cell survival upon H_2O_2 stress and during chronological aging. Mechanistically, the mutation of S15 to A15 in Hxk2p abolished the protective effect of *SIT4* deletion, thus demonstrating the critical role for this phosphorylation site in mediating the response to loss of Sit4p. In mammalian cells, the Akt-dependent phosphorylation of hexokinase 2 promotes its association with the mitochondrial outer membrane, suppressing apoptotic cell death.[57,58] A similar mechanism may exist in yeast, but it remains to be demonstrated.

Ceramide can be produced via *de novo* biosynthesis or through the hydrolysis of complex sphingolipids. We have shown that cells lacking Isc1p, an ortholog of mammalian neutral sphingomyelinase 2, present high levels of dihydro-C26-ceramide and phyto-C26-ceramide, and that the activation of Sit4p mediates mitochondrial dysfunction, oxidative stress sensitivity and premature aging of *isc1*Δ cells.[25] Notably, Hxk2p was hypophosphorylated in *isc1*Δ cells by a Sit4p-dependent mechanism. This result supports a role for Hxk2p phosphorylation in mitochondria function, H_2O_2 resistance and chronological lifespan. The regulation of Hxk2p by CAPP is further supported by our data showing that Hxk2p phosphorylation increases in cells lacking Cdc55p. Our results show that PP2A is also involved in the modulation of Hxk2p phosphorylation, probably in response to metabolic cues unrelated to sphingolipid signaling.

Our proteomic data also showed a decrease in the level of several proteins associated with purine metabolism and protein synthesis that may contribute to the slow growth phenotype of *sit4*Δ cells. Notably, our results show that the

cell cycle arrest in G_1 phase, characteristic of *sit4*Δ mutant cells, is also mediated by Hxk2p phosphorylation. The cell cycle arrest in *sit4*Δ cells may promote cell survival by providing more time to repair molecular damages and prevent mutagenesis, as previous suggested.[59] This probably contributes to the cellular protection observed in *isc1*Δ*sit4*Δ cells since oxidized proteins and lipids accumulate to higher levels in *isc1*Δ mutants when stressed or aged.[28] DNA repair also seems to be critical for survival of *isc1*Δ cells.[60] In addition, Isc1p protects yeast from genotoxic stress by regulating Swe1p, a protein kinase that regulates the G2/M transition,[61] through Cdc55p, allowing cell cycle progression.[62,63]

In summary, our data suggests that Hxk2p phosphorylation is required for phenotypes associated with *SIT4* deletion, namely mitochondrial derepression, growth arrest in G1 phase of the cell cycle, oxidative stress resistance and lifespan extension. We propose that the activation of Sit4p in *isc1*Δ cells indirectly promotes Hxk2p dephosphorylation, leading to mitochondrial dysfunction, premature aging and oxidative stress sensitivity (Fig. 6). These results offer new insights on the regulation of mitochondrial function and aging by hexokinase 2 and sphingolipid signaling.

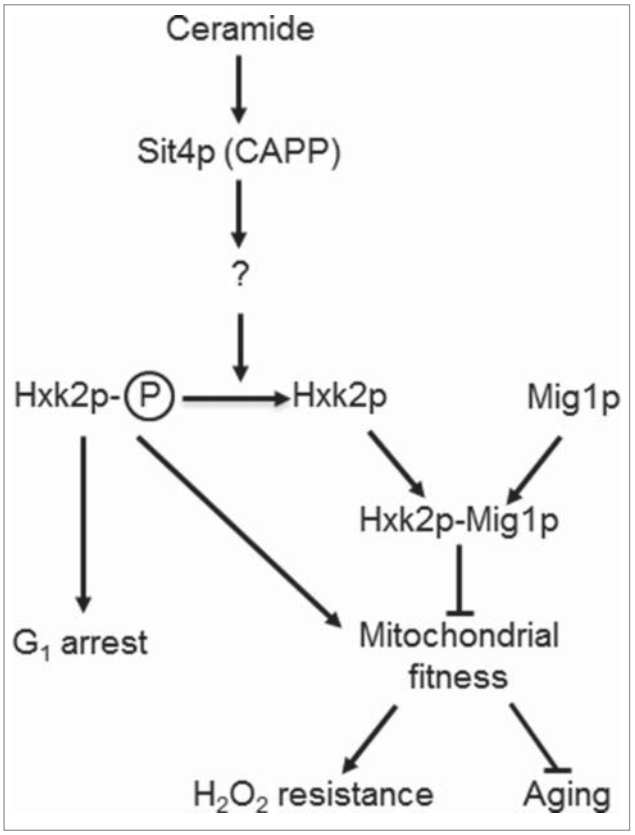

Figure 6. A model for the role of Sit4p in the regulation mitochondrial function. In parental cells, dihydroceramides (dh-Cer) and phytoceramide (phyto-Cer) levels are kept low, preventing the dephosphorylation of hexokinase 2 (Hxk2p) in serine-15, indirectly regulated by the ceramide-activated protein phosphatase Sit4p. In *sit4*Δ cells, the increase of Hxk2p phosphorylation contributes to mitochondrial fitness, increasing oxidative stress resistance and chronological lifespan. In *isc1*Δ cells, the higher levels of dh-Cer- and phyto-Cer activate Sit4p leading to Hxk2p dephosphorylation. This results in mitochondrial dysfunction, leading to a disturbed redox homeostasis, premature aging and oxidative stress sensitivity. Other proteins regulated by Sit4p may also contribute to these phenotypes.

Abbreviations

CAPP ceramide-activated protein phosphatase
PP2A type 2A protein phosphatase

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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