The ceramide activated protein phosphatase Sit4 impairs sphingolipid dynamics, mitochondrial function and lifespan in a yeast model of Niemann-Pick type C1

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Abstract
The Niemann-Pick type C is a rare neurodegenerative disease that results from loss-of-function point mutations in *NPC1* or *NPC2*, which affect the homeostasis of sphingolipids and sterols in human cells. We have previously shown that yeast lacking Ncr1, the orthologue of human NPC1 protein, display a premature ageing phenotype and higher sensitivity to oxidative stress associated with mitochondrial dysfunctions and accumulation of long chain bases. In this study, a lipidomic analysis revealed specific changes in the levels of ceramide species in *ncr1Δ* cells, including decreases in dihydroceramides and increases in phytoceramides. Moreover, the activation of Sit4, a ceramide-activated protein phosphatase, increased in *ncr1Δ* cells. Deletion of *SIT4* or *CDC55*, its regulatory subunit, increased the chronological lifespan and hydrogen peroxide resistance of *ncr1Δ* cells and suppressed its mitochondrial defects. Notably, Sch9 and Pkh1-mediated phosphorylation of Sch9 decreased significantly in *ncr1Δsit4Δ* cells. These results suggest that phytoceramide accumulation and Sit4-dependent signaling mediate the mitochondrial dysfunction and shortened lifespan in the yeast model of Niemann-Pick type C1, in part through modulation of the Pkh1-Sch9 pathway.

Abbreviations:

CAPP, ceramide-activated protein phosphatase; CLS, chronological lifespan; COX, cytochrome c oxidase; dhCer, dihydroceramide; DHS, dihydrosphingosine; LCB, long chain sphingoid base; MAPK, mitogen activated protein kinase; NPC, Niemann-Pick type C; PDS, post-diauxic shift; phytoCer, phytoceramide; PHS, phytosphingosine; ROS, reactive oxygen species.

Keywords: Niemann-Pick type C; ceramide; sphingolipid signaling; mitochondria; Sit4; Sch9
1. Introduction

Sphingolipids are important structural components of cell membranes, highly conserved among species, and their bioactive metabolites can act as signaling molecules regulating many biological processes. Sphingosine, a long chain sphingoid base (LCB), and ceramide are involved in the regulation of actin cytoskeleton organization, endocytosis, apoptosis, cell senescence and cell cycle arrest whereas sphingosine-1-phosphate plays a key role in proliferation, mitogenesis, cell migration, cell survival and inflammation (reviewed in [1, 2]). The homeostasis of sphingolipids is affected in several age-associated neurological diseases, such as Alzheimer’s [3] and Parkinson’s [4] diseases, in cancer [5], as well as in sphingolipidoses, a group of lysosomal storage diseases that comprise several distinct defects in lysosomal enzymes and lipid transfer proteins [6]. A characteristic feature of the sphingolipidoses is the accumulation of other secondary storage products because of the lipid nature of the primary storage compound, leading to a traffic jam [7]. Niemann-Pick type C (NPC) is a sphingolipidosis caused by loss-of-function point mutations in NPC1 or NPC2 gene [8, 9] and is clinically characterized by a severe neurodegeneration and accentuated failure of systemic organs such as liver and spleen. The NPC1 protein is a large transmembrane protein that is located in the transient late endosome/lysosome system, while NPC2 protein is a soluble glycoprotein with high affinity for cholesterol [10, 11]. Both proteins seem to be involved in intracellular transport of endocytosed cholesterol through the endolysosomal system [12]. In addition to cholesterol accumulation concomitant with sphingomyelin and gangliosides storage [13], there is an increase in the levels of sphingosine that precedes cholesterol entrapment in the lysosome of NPC1 cells [14].

Sphingolipid metabolism is highly conserved from yeast to mammalian and has been extensively studied using this simple model organism (Fig.1) [15]. We have recently reported that yeast cells lacking Ncr1, an orthologue of human NPC1 [16], exhibit high levels of sphingosine and hyperactivation of the Pkh1-Sch9 pathway that are associated with severe mitochondrial dysfunctions, oxidative stress sensitivity and premature ageing [17]. Several studies suggest that sphingolipid homeostasis plays an important role in oxidative stress resistance and longevity in
yeast, as also described in mammals [18, 19]. Lag1, a component of ceramide synthase, regulates replicative lifespan [20, 21]. Yeast cells lacking Ydc1, a dihydroceramidase that hydrolyzes dihydroceramide into the LCB dihydrosphingosine, exhibit an increased chronological lifespan (CLS) whereas cells overexpressing \textit{YDC1} show mitochondria and vacuolar fragmentation, increased apoptosis and a shortened CLS [22]. The levels of LCBs increase in stationary phase cells due to a decrease in the activity of both ceramide synthase (Lag1) and LCB kinase (Lcb4) [23], and the down-regulation of sphingolipid synthesis extends CLS in part due to a reduction in LCB-mediated activation of Sch9 [24], the yeast homologue of mammalian ribosomal S6K protein kinase also related to Akt/protein kinase B [25, 26]. Moreover, yeast lacking Isc1, an orthologue of mammalian neutral sphingomyelinase-2 (nSMase2) that generates ceramide through the hydrolysis of inositol phosphosphingolipids, display a shortened CLS, oxidative stress sensitivity, mitochondrial dysfunction, iron overload and caspase-dependent apoptosis [27].

Defects in NPC1 function result in the accumulation of ceramide in the liver of NPC1 patients [28]. In addition, the deficiency of a NPC1-related protein in the intracellular parasite \textit{Toxoplasma} increases the accumulation of cholesteryl esters, sphingomyelin, as well as of ceramide [29]. In mammals, changes in ceramide levels have been implicated in apoptosis through the modulation of signaling proteins [30, 31], including protein kinase C (PKC), cathepsin D, JNK, ceramide-activated protein kinases and ceramide-activated protein phosphatases (CAPPs) [32-35]. The CAPPs are found in all eukaryotes and are composed by one catalytic subunit and two regulatory subunits [36]. The yeast CAPP is constituted by the Tpd3 and Cdc55 regulatory subunits and by the Sit4 catalytic subunit [37, 38]. Sit4 is a serine-threonine protein phosphatase with high homology to human protein phosphatase 6 [39]. It has been implicated in the regulation of the cell cycle [40], mitochondrial function [41-43], carbohydrate metabolism [44, 45], homeostasis of monovalent ion and pH [46], the Pkc1-MAPK pathway [47] and traffic from the ER to the Golgi complex [48].

Moreover, Sit4 is down regulated by the Target of Rapamycin Complex 1 (TORC1) [49, 50]. Interestingly, a recent study implicated NPC1 in mTORC1 regulation in response to lysosomal cholesterol. Castellano \textit{et al} showed that lysosomal cholesterol drives mTORC1 recruitment to
lysosomes and activation. Moreover, NPC1 mediates mTORC1 inhibition upon cholesterol
depletion and mTORC1 is constitutively active in NPC1 deleted cells [51]. Our laboratory has shown that SIT4 deletion increases CLS and H2O2 resistance through
modulation of mitochondrial function [52]. Moreover, we reported that Hog1 MAPK is a downstream
effector of Sit4 that regulates mitochondrial function, being activated in response to ceramide by a
Sch9-dependent mechanism [53, 54]. Both Sch9 and Sit4 are also modulators of sphingolipid
metabolism [55, 56]. These results support a functional cross talk between Sit4 and Sch9 in
response to ceramide changes.

In this report, we show that the activation of Sit4/CAPP associated with an increase in the levels of
long chain phytoceramide species impairs oxidative stress resistance, mitochondrial function and
chronological lifespan in ncr1Δ cells. Notably, the suppression of ncr1Δ phenotypes by SIT4
deletion is associated with downregulation of the Pkh1-Sch9 pathway.

2. Materials and Methods

2.1. Yeast strains and growth conditions

The Saccharomyces cerevisiae strains used in this work are listed in table 1. The growth media
used were YPD [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose], YPG [1 %
(w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (v/v) glycerol], synthetic complete (SC) drop-out
medium containing 2 % (w/v) glucose 0.67 % yeast nitrogen base without amino acids or minimal
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supplemented with appropriate amino acids [0.008 % (w/v) histidine, 0.04 % (w/v) leucine, 0.008 %
(w/v) tryptophan] and 0.008 % (w/v) uracil. Yeast cells were grown aerobically at 26 °C in an orbital
shaker (at 140 rpm), with a ratio of flask volume:medium volume of 5:1, to early exponential phase
(OD600nm=0.6) or to post-diauxic shift phase (PDS; OD600nm=7-9).

Deletion strains were generated using PCR-derived deletion cassettes containing URA3, HIS3 or
KanMX4 with flanking regions of each gene. Yeast cells were transformed using the lithium acetate/
single-stranded DNA/ polyethylene glycol protocol [57].
To evaluate the levels of Lag1 and Ypc1 expression, ncr1ΔLAG1-9MYC and ncr1ΔYPC1-9MYC cells were generated by disruption of the NCR1 gene in BY4741 cells with LAG1 and YPC1 genomically tagged with 9Myc, respectively [55]. All the disruptions were confirmed by standard PCR procedures.

Plasmids used in this work are detailed in table 2. To study Sit4-Gln3-dependent MEP2 expression, yeast cells were transformed with the YCpMEP2-lacZ plasmid [58] and selected in minimal medium lacking uracil. To assess mitochondrial morphology, cells were transformed with pYX222-mtDsRed plasmid [59] and selected in minimal medium lacking histidine. To evaluate the expression of ceramide synthases and ceramidases, cells were transformed with plasmids expressing lacZ reporter fusions of the promoters of LAG1, LAC1, YDC1 and YPC1 genes [55] and selected in minimal medium lacking uracil.

2.2 Sphingolipid analysis by HPLC-MS/MS

Yeast cells were grown in SC-glucose medium and 1.9x10^9 cells were collected at exponential and PDS phase. Cell pellets were re-suspended in 1mL of lipid extraction solvent: 50 % (v/v) isopropanol, 10 % (v/v) diethyl ether, 2 % (v/v) pyridine, 25 % (v/v) ammonia. A 200 µL volume of glass beads were added into a 2 ml screw cup plastic tubes. Tubes were shaken in a bead beater 5 times, 3 min on, 1 min off at 4 ºC. The content of the tubes was poured into a 13x100 mm glass tubes. An additional 1 mL solvent was used to wash the plastic tubes and was added into the glass tubes. The tubes containing 2 mL solvent with cells and glass beads were dried in an analytical nitrogen evaporator (N-EVAP). The dried samples were sent to the Lipidomic Core at the Medical University of South Carolina for lipid analysis. Levels of dihydroceramides, phytoceramides, α-hydroxylated phytoceramides, long-chain sphingoid bases and their phosphorylated forms were measured by the high-performance liquid chromatography/mass spectrometry (LC-MS/MS) methodology as previously described [60]. Analytical results of lipids were expressed as pmol sphingolipid/total cell number.
2.3. Oxidative stress resistance and chronological lifespan

For analysis of oxidative stress resistance, yeast cells were grown to exponential phase and treated with 1.5 mM H$_2$O$_2$ for 1 h. CLS was assayed as previously described [61]. Briefly, overnight cultures were diluted to OD$_{600nm}$=0.6 and grown for 24 h (PDS phase) or 48 h (stationary phase; considered t0 in the lifespan assay) and kept in culture media at 26 ºC. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % (w/v) agar. Colonies were counted after growth for 3 days at 26 ºC. Viability was expressed as a percentage of colony-forming units in relation to time 0 or to untreated cells, as indicated.

2.4. Enzymatic activities

All the procedures were carried out at 4 ºC. Yeast cells were grown to respiratory phase and harvested by centrifugation. Cytochrome c oxidase (COX) activity was determined as previously described [62], by measuring cytochrome c oxidation. For the β-galactosidase assay, yeast cells were grown in selective SC-glucose medium to PDS phase, and the activity was measured as previously described [52], with some modifications: a cellular extract was prepared in 100 mM Tris-HCl, 1 mM DTT, 10 % (v/v) glycerol, and 150 µg of total protein was used in the assay.

2.5. Oxygen consumption and growth in glycerol

Oxygen consumption rate was measured for 3x10$^8$ cells at 26 ºC in phosphate buffer using an oxygen electrode (Oxygraph, Hansatech). Data were analyzed using the Oxyg32 v2.25 software. For analysis of respiratory capacity, yeast cells were grown to exponential phase, diluted to an OD$_{600nm}$=0.1 and five-fold serial dilutions were plated in SC media containing glucose or glycerol (non-fermentable carbon source) supplemented with 1.5 % (w/v) agar.

2.6. Mitochondrial morphology

Mitochondrial morphology was analyzed in cells transformed with a plasmid expressing mitochondrial DsRed (pYX222-mtDsRed) [59]. Cells were grown in SC-glucose medium lacking histidine to PDS phase. The mitochondrial network was observed in live cells mounted on agarose
coated slides by fluorescence microscopy (Axioimager Z1, Carl Zeiss). Data image stacks were deconvolved by QMLE algorithm of Huygens Professional v3.0.2p1 (Scientific Volume Imaging B.V.). Maximum intensity projection was used to output final images using ImageJ 1.51n software. Final scientific figures were designed using Figure J tool.

2.7. Protein extraction and Western blotting analysis

Yeast cells were grown in SC-glucose medium to exponential phase and protein extracts were prepared as described [24] with minor modifications. Briefly, $9 \times 10^8$ cells were collected, suspended in 200 µL of water and 200 µL of 0.2 M NaOH. Samples were vortexed and incubated at room temperature for 5 min, centrifuged and the pellet was suspended in sample buffer and boiled for 5 min at 95 °C. Equal amounts of protein (BCA™ Protein Assay Kit quantification) were loaded onto SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare). Detection of the proteins was performed with the primary antibodies: mouse anti-yeast phosphoglycerate kinase (Pgk1) antibody (1:30000, Molecular Probes), rabbit anti-Sit4 antibody (1:5000, kindly provided by Dr Yu Jiang), mouse anti-c-myc antibody (1:100, Roche), rabbit anti-Sch9 antibody (1:1000, kindly provided by Dr Robert Dickson) or rabbit anti-P-T570-Sch9 antibody (1:10000, kindly provided by Dr Robbie Loewith), and with the secondary antibody, anti-mouse IgG-peroxidase (1:5000, Molecular probes) or anti-rabbit IgG-peroxidase (1:5000, Sigma). Immunodetection was performed by chemiluminescence (Advansta).

2.8. Statistical analysis

The results were represented by mean and standard deviation values of at least three independent experiments. Statistical analyses were carried out using GraphPad Prism Software v6.02 (GraphPad Software).
3. Results

3.1. Ceramide levels in Ncr1-deficient cells

In order to characterize changes in the levels of ceramide species associated with NCR1 deletion, a lipidomic analysis was performed in cells grown to post diauxic shift (PDS) phase (respiratory phase). At this phase, ncr1Δ mutant cells present mitochondrial dysfunction associated with the fragmentation of the mitochondrial network [17]. The levels of long chain (LC; C_{12} to C_{22}) and very long chain (VLC; C_{24} to C_{26}) dihydroceramides, phytoceramides and α-hydroxylated phytoceramides were measured.

The total levels of dihydroceramides (dhCer) were 40% lower in ncr1Δ cells (Fig. 2A-B) due to a decrease in the levels of most (C_{18} to C_{26}) dhCer species (Fig. S1A-B). Concerning phytoceramides (phytoCer), the levels of C_{14} to C_{20}-species were 2-fold higher in ncr1Δ cells comparing with parental levels (Fig. S1C-D), but no major differences were observed for VLC phytoCer (Fig. 2C-D). Notably, the levels of C_{14} to C_{20} phytoCer also increased in ncr1Δ cells grown to exponential phase (data not shown). These results show significant changes in ceramide levels, including an increase of long chain phytoCer and a decrease of dhCer species in cells lacking Ncr1.

We postulated that the mitochondrial defects of ncr1Δ cells could result from a decrease in α-hydroxylated phytoceramides (αOH-phytoCer), which are particularly enriched in mitochondria [63]. However, the levels of LC αOH-phytoCer were slightly higher in Ncr1-deficient cells (Supplementary Fig. S1E-F). Thus, the mitochondrial dysfunction displayed by ncr1Δ cells does not seem to result from a decrease in αOH-phytoCer, but we cannot rule-out the hypothesis that a decrease of these species may occur specifically in mitochondria of ncr1Δ cells.

To assess changes in the expression of genes related to ceramide synthesis and turnover, namely ceramidases (YPC1 and YDC1) and ceramide synthases (LAG1 and LAC1), BY4741 and ncr1Δ cells were transformed with lacZ reporters under the control of those gene promoters. The ncr1Δ cells displayed an increase in reporter activity for YPC1 and YDC1 (Fig. 3A) as well as for LAC1 and LAG1 (Fig. 3B). However, the induction of LAG1 (10-fold) was much higher than those
observed for the other genes (3-4-fold). We also evaluated changes in Lag1 and Ypc1 levels in BY4741 and ncrΔ cells expressing 9Myc tagged LAG1 and YPC1. Consistent with the induction of LAG1 expression, the levels of Lag1-9Myc increased significantly in ncrΔ cells (Fig. 3C), but the increase of Ypc1-9Myc levels was mild (Fig. 3D). These results suggest that upregulation of ceramide synthases in exponential phase may contribute to the accumulation of phytoCer in ncrΔ cells in log and PDS phase. The concomitant induction of ceramidases, which hydrolyze ceramides, probably explains the accumulation of LCBs in this mutant [17].

3.2. Activation of CAPP is associated with oxidative stress sensitivity, premature ageing and mitochondrial dysfunctions of Ncr1 deficient cells

Besides structural functions, ceramide is known to regulate cellular processes through modulation of signaling pathways [1]. Yeast cells present a CAPP composed of two regulatory subunits, Tpd3 and Cdc55, and the catalytic subunit Sit4 that is stimulated by ceramide [37, 38, 56]. Thus, we postulated that accumulation of phytoceramide species in ncrΔ cells could mediate cell responses by activation of the Sit4 phosphatase [38]. Sit4 regulates the dephosphorylation of Gln3 transcription factor, leading to its translocation to the nucleus [64]. To assess Sit4 activation, we measured the Gln3-dependent MEP2 expression in BY4741, ncrΔ, sit4Δ, ncrΔsit4Δ, cdc55Δ, and ncrΔcdc55Δ cells transformed with a MEP2-lacZ reporter [58]. In addition, Sit4 levels were analyzed by Western blotting. At PDS phase, β-galactosidase activity increased 2.8-fold in ncrΔ cells (relative to BY4741), and this effect was suppressed by SIT4 or CDC55 deletion (Fig. 4A). The levels of Sit4 were similar in ncrΔ cells and decreased by deletion of CDC55 (Fig. 4B). We also assessed β-galactosidase activity in cells depleted for Sur2, a sphinganine C4-hydroxylase responsible for conversion of dihydrosphingosine to phytosphingosine and dihydroceramide to phytoceramide. Notably, deletion of SUR2 led to a decrease of β-galactosidase in ncrΔ cells (Fig. 4A) but this decrease was not as marked as that associated with SIT4 deletion. These results suggest that CAPP activation increases in Ncr1-deficient cells, in part due to an increase of phytoceramide levels, with no major changes in Sit4 expression.
Next, we assessed if Sit4 activation by phytoceramides contributes to the oxidative stress sensitivity and shortened CLS of ncr1Δ cells. As previously described [52], hydrogen peroxide resistance and CLS were higher in sit4Δ as well as cdc55Δ single mutants in comparison with parental cells. Notably, deletion of SIT4 or CDC55 suppressed the hydrogen peroxide sensitivity and reversed the shortened CLS of ncr1Δ cells (Fig. 4C-D).

Sit4 negatively regulates mitochondrial function [42, 52] and ncr1Δ cells also present severe mitochondrial defects [17]. Thus, we hypothesized that an increase of CAPP activity could be associated with mitochondrial dysfunctions displayed by this mutant. To test this hypothesis, we analyzed mitochondrial functionality in BY4741, ncr1Δ, sit4Δ, ncr1Δsit4Δ, cdc55Δ and ncr1Δcdc55Δ cells. Our results show that deletion of SIT4 or CDC55 led to a reversal of the low oxygen consumption rate, low cytochrome c oxidase (COX) activity and growth defect on a non-fermentable carbon source (glycerol) exhibited by ncr1Δ cells (Fig. 5A-C). Notably, the deletion of SUR2 in ncr1Δ cells also restored oxygen consumption and growth on glycerol plates and increased CLS (Fig. S2). This is consistent with the hypothesis that CAPP activation by phytoceramides contributes to mitochondrial dysfunction in ncr1Δ cells.

To get further insights on mitochondrial function, we also assessed the integrity of the mitochondrial network by fluorescence microscopy using cells expressing a mitochondria matrix-targeted peptide fused to DsRed (Fig 5D). Our results show that the punctate pattern exhibited by ncr1Δ cells, indicative of mitochondrial network fragmentation, was suppressed by SIT4 or CDC55 deletion, with double mutant cells displaying a normal tubular mitochondrial network. The overall findings indicate that CAPP activation in ncr1Δ cells contributes to an impairment of mitochondrial dynamics and functionality, leading to a decrease in oxidative stress resistance and chronological lifespan.

### 3.3. Levels of sphingolipids in sit4Δ and ncr1Δsit4Δ cells

The regulation of sphingolipid metabolism is highly complex, and it may involve feedback mechanisms controlled by signaling proteins modulated by bioactive sphingolipids. Thus, we postulated that the suppression of ncr1Δ phenotypes upon SIT4 deletion could be associated with
the modulation of sphingolipid homeostasis. To test this hypothesis, ceramide levels were measured in \textit{sit4}Δ and \textit{ncr1Δsit4}Δ cells grown to PDS phase. Our results show that the levels of LC dhCer decreased about 50% in \textit{sit4}Δ cells when compared to parental cells (Fig. 2A). Although there was an increase of C\textsubscript{16}-dhCer species, the levels of C\textsubscript{18:1}-, C\textsubscript{20}-, C\textsubscript{20:1}-, C\textsubscript{22}- and C\textsubscript{22:1}-dhCer, which are the most abundant dhCer species, decreased about 2-fold in \textit{sit4}Δ cells (Fig. S1A). These results are consistent with a previous report showing that total dhCer levels decrease in \textit{sit4}Δ cells [56]. Interestingly, deletion of \textit{SIT4} in \textit{ncr1Δ} cells increased C\textsubscript{18}-dhCer content to parental levels. Concerning VLC dhCer, \textit{sit4}Δ cells displayed an increase in the levels of C\textsubscript{24}-dhCer (Fig. S1B) and \textit{SIT4} deletion suppressed the lower levels of VLC dhCer exhibited by \textit{ncr1Δ} cells (Fig. 2B).

Notably, \textit{SIT4} deletion had major effects on phytoCer. The levels of LC (C\textsubscript{14}- to C\textsubscript{22}-) phytoCer were significantly higher (more than 3-fold) in \textit{sit4}Δ and \textit{ncr1Δsit4}Δ cells compared to parental or \textit{ncr1Δ} cells (Fig. 2C; Fig. S1C). In contrast, the levels of C\textsubscript{26}- and C\textsubscript{26:1}-phytoCer (the most abundant species) decreased almost 3-fold in \textit{sit4}Δ cells (Fig. 2D; Fig. S1D), which is consistent with the reduction of total phytoCer previously described for this mutant [56]. Regarding αOH-phytoCer, similar changes were observed: the levels of most LC species increased in \textit{sit4}Δ and \textit{ncr1Δsit4}Δ cells whereas αOH-phyto-C\textsubscript{26}-Cer decreased (Supplementary Fig. S1E-F). These findings suggest that Sit4 plays a role in the regulation of sphingolipid metabolism. Since the accumulation of long chain sphingoid bases (LCBs) and the LCB-activated protein kinase Pkh1 have been implicated in \textit{ncr1Δ} phenotypes [17], we also investigated how \textit{SIT4} deletion affects the levels of dihydrosphingosine (DHS), phytosphingosine (PHS) and its phosphorylated forms (DHS-1-P and PHS-1-P). LCBs increased in \textit{sit4}Δ vs parental cells, due to the accumulation of DHS (in the exponential phase) or both DHS and PHS (in the PDS phase). In both growth phases, the levels of DHS-1-P and PHS-1-P also increased in \textit{sit4}Δ cells (the increase was even higher, comparing to LCBs). Notably, \textit{ncr1Δsit4}Δ cells exhibited similar changes (Table 3). Thus, \textit{SIT4} deletion seems to suppress \textit{ncr1Δ} phenotypes by means unrelated with a decrease in LCBs.
3.4. *SIT4* deletion suppresses the activation of Pkh1-Sch9 pathway in *ncr1Δ* cells

Since Pkh1 is activated by LCBs and the levels of DHS (in the exponential phase) or both DHS and PHS (in the PDS phase) further increased in *ncr1Δsit4Δ* double mutants, compared with *ncr1Δ* cells, we decided to investigate the effect of *SIT4* deletion on the activation of the Pkh1-Sch9 pathway in *ncr1Δ* cells. As previously shown [17], the levels of Sch9 and Sch9-phospho-T570 (Pkh1-dependent phosphorylation) increased in *ncr1Δ* cells. Notably, they greatly decreased in both *sit4Δ* and *ncr1Δsit4Δ* cells, when compared with parental and *ncr1Δ* cells (Fig. 6).

In *ncr1Δsit4Δ* double mutants, the increase of LCB-1-Ps was higher to the observed for LCBs. Thus, we postulated that LCB-1-Ps could mediate the cellular effects of *SIT4* deletion in *ncr1Δ* cells. To test this hypothesis, the *LCB4* gene, which encodes for the major LCB kinase in yeast, was deleted. Our results show that the levels of Sch9 and Sch9-phospho-T570 increased in *sit4Δlcb4Δ* and *ncr1Δsit4Δlcb4Δ* mutants, compared with *sit4Δ* or *ncr1Δsit4Δ* cells, although to levels significantly lower to the observed in parental or *ncr1Δ* cells (Fig. 6). Consistently, *ncr1Δsit4Δlcb4Δ* mutants were still able to grow on glycerol medium (data not shown), suggesting that *SIT4* deletion improves *ncr1Δ* phenotypes by a LCB-1-P-independent mechanism. Moreover, the deletion of *DPL1*, which encodes for the LCB-1-P lyase, did not suppress the mitochondrial dysfunction of *ncr1Δ* cells (data not shown), suggesting that the mitochondrial dysfunction of *ncr1Δ* cells is not related with the decrease of LCB-1-P levels. The overall results indicate that the protective effect of *SIT4* deletion in *ncr1Δ* cells is mainly associated with downregulation of the Pkh1-Sch9 pathway.

4. Discussion

The Niemann-Pick type C1 disease is a lysosomal storage disorder caused by mutations in the NPC1 protein, which seems to be involved in the transport of cholesterol through the endolysosomal system. Several studies have shown a high degree of complexity of lipids that accumulate in NPC1 cells, including cholesterol, complex lipids such as glycosphingolipids and sphingomyelin [65], as well as sphingosine, which was suggested to be the first lipid that accumulates in these cells [14].
We have previously reported that, similarly to NPC1 cells, yeast cells lacking Ncr1 accumulate LCBs. This may contribute to the activation of the Pkh1-Sch9 pathway that ultimately contributes to mitochondrial dysfunctions, oxidative stress sensitivity and a shortened CLS [17]. Here we show that yeast ncr1Δ cells grown to PDS (respiratory) phase also exhibit significant changes in ceramide levels. These include a decrease in LC dhCer and an increase in LC phytoCer. Our data also demonstrate that both ceramide synthases and ceramidases genes are upregulated in ncr1Δ cells, with the LAG1 ceramide synthase being highly induced. These changes seem to favor phytoCer accumulation in ncr1Δ cells and may also contribute to the increase of LCBs promoted by the ceramidases. However, the mitochondrial defects exhibited by this mutant was not suppressed by deletion of the LAG1 ceramide synthase or ceramidases (YPC1, YDC1 or both) genes (data not shown). It is to be expected that modulations in any of the sphingolipid enzymes cause ripple effects that alter the concentrations of many sphingolipids. So, it is possible that the downregulation of Lag1 or ceramidases increases the levels of LCBs and ceramides, respectively, leading to further activation of downstream signaling pathways, which are known to promote mitochondrial dysfunctions [17, 52]. The inhibition of SPT (catalyzes the first step in sphingolipid biosynthesis) with low doses of myriocin also did not alleviate ncr1Δ phenotypes [17]. However, myriocin induces autophagy in ncr1Δ cells, which is probably detrimental since this process is already increased in untreated cells (data not shown) as described in NPC1 cells [66].

Ceramide constitutes the metabolic hub in sphingolipid metabolism since it is the core lipid that drives the synthesis of more than 50 distinct molecular species. Ceramides with different fatty acids have distinct roles as signaling molecules, acting through modulation of specific downstream components of signaling pathways, including CAPP [1]. Here we show that Sit4, the catalytic subunit of the CAPP related to type 2A family of protein phosphatases, was more activated in Ncr1-deficient cells. Moreover, the deletion of genes encoding for the catalytic (Sit4) or regulatory subunit (Cdc55) of CAPP abolished the shortened CLS, H2O2 hypersensitivity and mitochondrial dysfunctions displayed by ncr1Δ cells. Also, deletion of SUR2, which encodes for C4-hydroxylase responsible for generation of phytosphingosine and phytoceramides (Fig.1), suppressed the shortened lifespan and mitochondrial defects of ncr1Δ cells. These findings support our hypothesis
that phytoceramides are key mediators of sphingolipid signaling in ncr1Δ cells, promoting, at least in part, CAPP/Sit4 activation.

Previous studies have shown that Sit4 is involved in catabolic repression and negatively regulates mitochondrial function [42, 43]. In addition, sit4Δ cells exhibit a higher resistance to oxidative stress and an increased lifespan [52]. Thus, we propose that Sit4 activation mediates the mitochondrial defects of ncr1Δ cells, decreasing hydrogen peroxide resistance and CLS. It is likely that Sit4 affects mitochondria function through modulation of mitochondrial proteins or other proteins related to mitochondrial function and dynamics. Consistent with this hypothesis, published work has shown that Sit4 interacts with Atp3 (subunit of the ATP synthase) and Nde1 (external NADH dehydrogenase), and a mutation in Atp3 or NDE1 deletion increase CLS [67, 68]. The identification of Sit4 direct targets will provide new hints to the elucidation of how Sit4 regulates mitochondria and lifespan.

Importantly, our results suggest that Sit4 may also function as a modulator of sphingolipid homeostasis and dynamics. The deletion of SIT4 gene suppressed the lower levels of dhCer in ncr1Δ cells. This effect may contribute to the improvement of mitochondrial fitness in ncr1Δsit4Δ cells, since dhCer can exert protective effects by inhibiting the assembly of ceramide channels in mitochondria [69]. In contrast, long chain phytoceramides species were significantly increased in sit4Δ and ncr1Δsit4Δ cells. Notably, similar effects were observed upon deletion of SCH9 (data not shown), which also suppresses ncr1Δ phenotypes [17]. More studies are needed to uncover downstream effects mediated by these specific ceramide species as well as its subcellular localization.

We have previously shown that SCH9 deletion suppresses the high levels of LCBs displayed by ncr1Δ cells [17]. Notably, sit4Δ and ncr1Δsit4Δ cells exhibited major changes in LCBs. The levels of PHS and DHS increased in these mutants concomitantly with a higher increase of their 1-phosphate forms, leading to an increase of DHS-1-P/DHS and PHS-1-P/PHS ratios. Although the increase of LCB-1-phosphates has been associated with inhibition of cell death pathways [2], our results suggest that it does not contribute to cellular effects of SIT4 deletion. LCBs are known activators of the Pkh1 and Pkh2 protein kinases [70] that activate the Sch9 kinase by
phosphorylating a T570 residue [71, 72]. Our results show that Sch9 and Sch9-phospho-T570 levels decrease in ncr1Δsit4Δ cells, suggesting that the protective effect of SIT4 deletion is, at least in part, mediated by downregulation of the Pkh1-Sch9 pathway. This crosstalk between Sit4 and Pkh1/2-Sch9 seems to be conserved in mammals. Indeed, mammalian Akt/PKB, a homologue of yeast Sch9, is inhibited by ceramide by a CAPP-dependent mechanism [73, 74]. More studies are needed to uncover the molecular mechanisms underlying the intricate regulation of these pathways and its relation with other protein kinases and protein phosphatases that control sphingolipid biosynthesis, e.g. through modulation of Orm1/2 by Ypk1 [75-77].

In summary, our results suggest that the accumulation of phytoceramide species leading to activation of the ceramide activated protein phosphatase Sit4 and the crosstalk between Sit4 and Sch9 mediate the mitochondrial defects, premature ageing and oxidative stress sensitivity displayed by the yeast model of Niemann-Pick type C1 disease (Fig. 7). To our knowledge, this is the first report implicating ceramide signaling in NPC1 phenotypes.
Competing interests

No competing interests declared.

Acknowledgements

We are grateful to Bruno André (Université Libre de Bruxelles, Belgium), Paula Ludovico (ICVS, Universidade do Minho, Portugal), Joris Winderickx (Inst. Botany and Microbiology, K.U. Leuven, Belgium), Robert Dickson (University of Kentucky College of Medicine, Lexington, Kentucky, USA), Robbie Loewith (University of Geneva, Switzerland) and Yu Jiang (University of Pittsburgh, USA) for generously providing plasmids and antibodies used in this study. We would like to thank Paula Sampaio (ALM, IBMC) for technical assistance and data treatment on fluorescence microscopy. This work was funded by the project Norte-01-0145-FEDER-000008 - Porto Neurosciences and Neurologic Disease Research Initiative at I3S, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER), and in part by National Institutes of Health Grant GM063265 (to Y.A.H.), the Lipidomics Shared Resource, Hollings Cancer Center, Medical University of South Carolina (P30 CA138313) and the Lipidomics Core in the SC Lipidomics and Pathobiology COBRE, Department Biochemistry, MUSC (P20 RR017677).
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FIGURE LEGENDS

Figure 1. Schematic representation of sphingolipid metabolism in yeast. Metabolic intermediates and genes encoding the most important enzymes involved in each step of sphingolipid synthesis and turnover are shown.

Figure 2. Levels of dihydroceramide and phytoceramide species. S. cerevisiae BY4741, ncr1Δ, sit4Δ and ncr1Δsit4Δ cells were grown in SC-glucose medium to post-diauxic shift (PDS) phase. Levels of long chain ceramides (C_{14} to C_{22}) and very long chain ceramides (C_{24} to C_{26}) were measured by HPLC-MS/MS as described in Material and Methods. Data are expressed as pmol of lipid per total cell number (1.9x10^{9}) and are mean ± variance of three independent experiments.

Figure 3. Expression of ceramidases and ceramide synthases in Ncr1-deficient cells. (A,B) S. cerevisiae BY4741 and ncr1Δ cells were transformed with lacZ reporter plasmids expressing the promotors of ceramidases genes YDC1 and YPC1 (A) and ceramide synthases genes LAG1 and LAC1 (B). Cells were grown in SC-glucose medium lacking uracil to log phase and the transcriptional activation of these genes were measured by β-galactosidase activity. ****p<0.0001 relative to BY4741; Two-way ANOVA and Bonferroni test. (C,D) Analysis of Lag1 and Ypc1 levels in BY4741 and ncr1Δ cells expressing 9Myc tagged LAG1 and YPC1. Protein extracts were separated by SDS–PAGE and blotted into a nitrocellulose membrane, and tagged proteins were detected using an anti-Myc antibody. Pgk1 was used as loading control. One representative out of three is shown for (C) Lag1-9Myc and (D) Ypc1-9Myc. The quantification of Myc band intensities (normalized for Pgk1) is shown.

Figure 4. Sit4 activation increases in Ncr1 deficient cells and mediates oxidative stress sensitivity and premature ageing. (A) S. cerevisiae BY4741, ncr1Δ, sit4Δ and ncr1Δsit4Δ, cdc55Δ and ncr1Δcdc55Δ cells expressing a Sit4-Gln3-dependent MEP2-LacZ reporter were grown to post-diauxic shift phase in SC-glucose medium lacking uracil. (A) β-galactosidase activity was measured as described in Material and Methods. ***p<0.001; One-way ANOVA and Bonferroni test. (B) Immunoblot analysis of Sit4 levels in cells grown to PDS. Pgk1 was used as loading control. A
representative experiment out of three is shown. (C) Cells were grown to exponential phase
(O.D. \textsubscript{600nm}=0.6) in SC-glucose medium and exposed to 1.5 mM H\textsubscript{2}O\textsubscript{2} for 1 h. Cellular viability was measured as the percentage of the colony-forming unit (treated cells vs non-stressed cells).

\*\*\*\(p<0.001\), relative to \textit{ncr1\Delta}; One-way ANOVA and Bonferroni test. (D) Cells were grown to PDS phase and maintained in the growth medium overtime. Cellular viability was measured at 2 to 3 days intervals and was expressed as % colony forming units (aged vs day 0).

Figure 5. \textit{SIT4} and \textit{CDC55} disruption suppresses mitochondrial dysfunctions exhibited by \textit{ncr1\Delta} cells. (A,B) \textit{S. cerevisiae} cells were grown in SC-glucose medium to post-diauxic shift phase. Oxygen consumption rates (A) and cytochrome \textit{c} oxidase (COX) specific activity (B) were measured. **\(p<0.01\) and \*\*\*\(p<0.001\), relative to \textit{ncr1\Delta}; One-way ANOVA and Bonferroni test. (C) Cells were grown to exponential phase and fivefold serial dilutions were plated in SC solid medium containing glucose or glycerol as carbon source. One representative experiment out of three is shown. (D) Yeast cells transformed with pYX222-mtDs Red (expressing mitochondrial DsRed) were visualized by fluorescence microscopy. One representative experiment out of three is shown. Scale bar: 5 µm.

Figure 6 – \textit{Sch9} and \textit{Sch9-phospho-T570} levels decrease drastically upon \textit{SIT4} deletion. Yeast cells were grown in SC-glucose medium to exponential phase. \textit{Sch9} and phospho-T570-\textit{Sch9} levels were analyzed by immunoblotting, as described in Material and Methods. Pgk1 levels was used as loading control. A representative experiment out of three is shown.

Figure 7. Activation and cross-talk of LCB and ceramide signaling pathways in the NPC1 yeast model. Deletion of yeast NPC1 related protein induces high levels of LCBs, particularly phytosphingosine (PHS) \cite{17}, and long chain phytoceramide species (this study). Ceramide signaling targets the ceramide-activated protein phosphatase (CAPP) \textit{Sit4} that mediates mitochondrial dysfunction and decreases cellular lifespan in \textit{ncr1\Delta} cells. \textit{Sit4} also regulates \textit{Sch9} levels and \textit{Pkh1}-dependent phosphorylation which also contributes to \textit{ncr1\Delta} phenotypes, by an unknown mechanism \cite{17}. 
Table 1. *S. cerevisiae* strains used in this work.

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<th>Source</th>
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</thead>
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*ncr1Δ::KanMX4 sit4Δ::KanMX4*
Table 2. Plasmids used in this work.

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<td>URA3</td>
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Table 3: LCBs and LCB-1-Ps levels in cells grown to exponential and PDS phase.

Yeast cells were grown to exponential or post-diauxic shift (PDS) phase and sphingolipid levels were measured as described in Methods. Data are expressed as pmol/total cell number (mean ± SD of three independent experiments). The quantification in BY4741 and \textit{ncr1Δ} cells [17] (reproduced here with permission) was paired with the quantification in \textit{sit4Δ} and \textit{ncr1Δsit4Δ} mutants (this study).

| Strain       | Exponential phase |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|--------------|-------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|          |
|              | DHS*   | DHS-1-P  | PHS   | PHS-1-P  |          | DHS*   | DHS-1-P  | PHS   | PHS-1-P  |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| **BY4741**   | 123.5± | 2.95±   | 17.08± | 0.63±   | 50.53±  | 0.73±   | 28.84±  | 1.35±   |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| **ncr1Δ**    | 153.35± | 11.51±  | 24.05± | 1.62±   | 129.03± | 1.01±   | 55.90±  | 2.01±   |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|              | 32.35   | 0.94    | 6.47   | 0.09    | 4.58    | 0.29    | 6.28    | 0.28    |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| **sit4Δ**    | 195.74± | 21.44±  | 18.96± | 1.87±   | 266.79± | 6.46±   | 101.34± | 5.19±   |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|              | 11.70   | 1.13    | 2.08   | 0.10    | 124.48  | 2.15    | 5.87    | 0.45    |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| **ncr1Δsit4Δ** | 228.47± | 11.85±  | 22.90± | 1.31±   | 261.11± | 7.50±   | 89.54±  | 6.58±   |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|              | 4.42    | 0.46    | 2.49   | 0.10    | 27.14   | 0.78    | 9.76    | 1.12    |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |

* \( p<0.05 \), One-way ANOVA, non parametric test.