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Sphingolipid metabolism, Sit4p and TORC1 in the yeast model of Niemann-Pick type C1 disease

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Sphingolipid metabolism, Sit4p and TORC1 in the yeast model of Niemann-Pick type C1 disease

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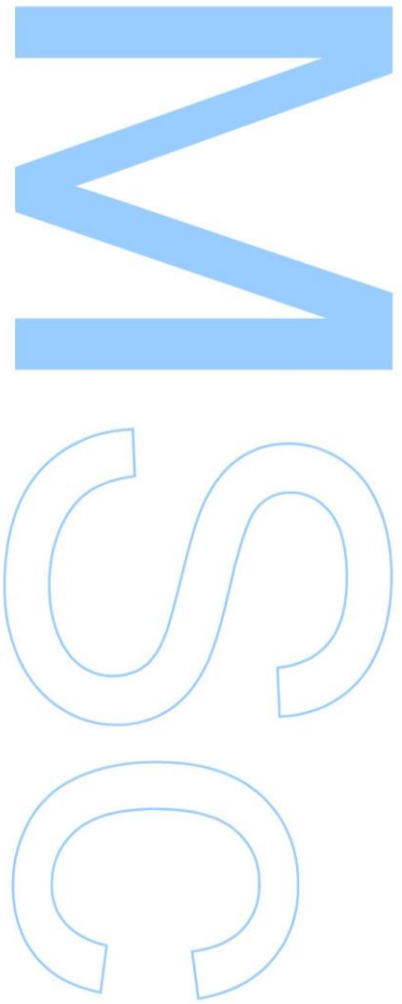

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Resumo

A Niemann-Pick tipo C (NPC) é uma doença metabólica rara com um envolvimento neurológico e visceral, caracterizada por uma acumulação anormal de lípidos (colesterol e esfingolípidos) no sistema endossomal e lisossomal. Mutações pontuais nas proteínas NPC1 ou NPC2, envolvidas no transporte de lípidos na via endocitica, parecem ser fundamentais para o aparecimento desta patologia. Células de *Saccharomyces cerevisiae* sem a proteína Ncr1p, o homólogo em levedura da proteína NPC1 de mamíferos, apresentam uma maior sensibilidade a peróxido de hidrogénio, um tempo de vida mais curto e disfunções mitocondriais associadas com acumulação de bases esfingóides de cadeia longa. Os mutantes *ncr1*Δ também acumulam diversas espécies de ceramida, levando à ativação da proteína serina/treonina fosfatase Sit4p, homologa da proteína fosfatase 6 humana. Consistentemente, verificou-se que a atividade desta fosfatase aumenta no modelo de levedura de NPC1 e a sua deleção suprime os defeitos mitocondriais e tempo de vida reduzido de células *ncr1*Δ.

Este trabalho teve como objetivo desvendar o papel do Sit4p na regulação do metabolismo de esfingolípidos e nas disfunções mitocondriais de células *ncr1*Δ. Para isso avaliámos alterações na transcrição de genes que codificam Lac1p e Lag1p, componentes do complexo ceramida sintetase, e Ydc1p e Ypc1p, duas ceramidases, em células transformadas com repórteres lacZ. Os nossos resultados mostram que a expressão destes genes está aumentada em células *ncr1*Δ por mecanismos dependentes da Sit4p. No entanto, a deleção do *LAG1* ou *YDC1* nestas células não restaurou a função mitocondrial, visto que os duplos mutantes são incapazes de crescer em fontes de carbono não fermentativas. O tratamento de células *ncr1*Δ com miriocina, um inibidor da serina-palmitoiltransferase, também não suprimiu as disfunções mitocondriais.

A autofagia é crucial para a homeostasia celular e função mitocondrial, mas está alterada em células de NPC1. Estas células também apresentam valores aumentados de catepsina D, uma protease lisossomal. Estes fenótipos estão conservados no modelo de levedura, já que células deficientes em Ncr1p apresentam um aumento basal da autofagia e da atividade da Pep4p, um homólogo da catepsina D, dependentes da Sit4p. Estes resultados sugerem que a ativação do Sit4p medeia alterações no metabolismo de esfingolípidos, autofagia e longevidade em células *ncr1*Δ. A Sit4p também funciona abaixo do TORC1 (Target of rapamycin complex 1),

um regulador do metabolismo de esfingolipidos, da autofagia e do envelhecimento em levedura. A deleção do gene *TOR1* aumentou a longevidade e a resistência a peróxido de hidrogénio de células *ncr1*Δ. Estes resultados implicam a Sit4p e o TORC1 nas disfunções mitocondriais e envelhecimento prematuro do modelo de levedura da doença NPC1.

Palavras chave: Niemann-Pick tipo C1, Ncr1p, esfingolípidos, autofagia, TORC1, Sit4p, função mitocondrial

Abstract

The Niemann-Pick Type C (NPC) is a rare metabolic disease with neurological and visceral involvement, characterized by an abnormal accumulation of lipids (cholesterol and sphingolipids) in the late endosomal/lysosomal network. Point mutations in either NPC1 or NPC2, which are involved in lipid transport through the endocytic pathway, seem to be instrumental for the onset of this pathology. *Saccharomyces cerevisiae* cells lacking Ncr1p, the yeast homolog of mammalian NPC1, present higher sensitivity to hydrogen peroxide, shortened chronological lifespan and mitochondrial dysfunctions associated with the accumulation of long chain sphingoid bases. The *ncr1*Δ mutants also accumulate several ceramide species, leading to activation of the serine threonine protein phosphatase Sit4, an homolog of human protein phosphatase 6. Consistently, the activity of this phosphatase was also shown to be increased in the yeast model of NPC1, and its deletion suppressed the mitochondrial defects and shortened lifespan of *ncr1*Δ cells.

This work aimed to disclose the role of Sit4p is the regulation of sphingolipid metabolism and mitochondrial dysfunctions of *ncr1*Δ cells. For that we assessed changes in the transcription of genes encoding Lac1p and Lag1p, components of the ceramide synthase complex, and of Ydc1 and Ypc1, two paralogue ceramidases, in cells transformed with lacZ reporters. Our results show that expression of these genes was increased in *ncr1*Δ cells by mechanisms dependent on Sit4p. However, the deletion of *LAG1* or *YPC1* in *ncr1*Δ cells did not restore mitochondrial function as double mutants were unable to grow on a non-fermentable carbon source. Treatment of *ncr1*Δ cells with myriocin, an inhibitor of serine palmitoyltransferase, also did not suppress mitochondrial dysfunctions.

Autophagy is crucial for cell homeostasis and mitochondrial function but is altered in NPC1 cells. These cells also show increased levels of cathepsin D, a lysosomal protease. This is conserved in the yeast model, since Ncr1p deficient cells exhibited Sit4-dependent increases in autophagy and in the activity of Pep4p, a homolog of cathepsin D. These results suggest that Sit4p activation mediates changes in sphingolipid metabolism, autophagy and lifespan in *ncr1*Δ cells. Sit4p also functions downstream of TORC1 (Target of Rapamycin Complex 1), a regulator of sphingolipid metabolism, autophagy and aging in yeast. The deletion of *TOR1* increased the chronological lifespan and hydrogen peroxide resistance of *ncr1*Δ cells. The overall results implicate Sit4p and TORC1 in the mitochondrial dysfunctions and premature aging of the yeast model of NPC1.

Key words: Niemann-Pick type C1, Ncr1p, sphingolipids, ceramide, LCBs, autophagy, TORC1, Sit4p, mitochondrial function

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List of abbreviations

AKT/PKB	Protein kinase B
CLS	Chronological lifespan
CNS	Central nervous system
DHC	Dihydroceramide
DHS	Dihydrosphingosine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
IPC	Inositol-phosphorylceramide
KDS	2-Keto dihydrosphingosine
LAC1	Longevity assurance gene cognate
LAG1	Longevity assurance gene 1
LCB	Long chain bases
LSDs	Lysosomal storage diseases
MIPC	Mannose-inositol-phosphorylceramide
M(IP)2C	Mannose-diinositol-phosphorylceramide
MM	Minimal medium
NCR1	NPC-related gene 1
NPC1	Niemann-Pick type C1
NTD	N-terminal domain
OD	Optical density
PCR	Polymerase chain reaction

PDK1	Phosphoinositide-dependent kinase-1
PDS	Post diauxic shift phase
PHC	Phytoceramide
PHS	Phytosphingosine
PKC	Protein kinase C
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SAPs	Sit4 associated proteins
SP	Stationary phase
SPT	Serine palmitoyltransferase
SSD	Sterol sensing domain
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TE	Tris EDTA
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
TTBS	Tris-buffered saline plus Tween
YDC	Yeast dihydroceramidase
YPC	Yeast phyto-ceramidase
YPD	Yeast peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 Lysosomal storage diseases

The late-endosome/lysosomal system comprehends a group of organelles and pathways required by cells in order to process and recycle various exogenous metabolites and macromolecules, which can then be transported to their respective cellular compartments where they will have a specific function (Winchester, Vellodi et al. 2000). An impairment of this pathway, caused by a genetic defect in a luminal or membrane protein for instance, can many times result in the accumulation of molecules in lysosomes and other vesicles. These can be lipids or proteins and their excessive storage can be highly detrimental to the cell, leading to deficiencies in any human organ or tissue. Diseases associated with these phenotypes can lead to a class of rare, inherited heterogeneous group of disorders called lysosomal storage diseases (LSDs) (Winchester, Vellodi et al. 2000). This group of diseases, which affects mostly children of a young age, has a significant incidence on the Northern Portuguese population (25 per 100 000 live births) when compared to other countries, so the study of the molecular and genetic mechanisms underlying them are of high importance (Pinto, Caseiro et al. 2004). Examples of this disease's include Farber's disease, Hunter syndrome and, under focus on this thesis, Niemann-Pick type C.

1.2 Niemann-Pick type C

The Niemann-Pick disease, a condition first described in the late nineteen twenties by Albert Niemann and Ludwig Pick, comprehends a group of autosomal recessive disorders caused by lipid storage abnormalities in lysosomes (Vanier 2010). Although initially defined as a single pathology, it quickly became evident that its clinic expression was highly variable in patients, particularly in what regarded age of onset, lifespan and affected organs – while some presented mostly neurological symptoms, others had a more visceral (spleen, liver and sometimes the lung) involvement. These observations led Allan C. Crocker to first divide it into four main types (Crocker 1961): type A, related to an early acute central nervous system (CNS) impairment and a massive visceral accumulation of sphingomyelin; Type B, with a chronic course and no nervous system involvement; types C and D with moderate and slower course and minor visceral involvement. In terms of biochemical and molecular criteria, it became clear there was either a primary deficiency in acid sphingomyelinase (types A and B) or a defective transport and processing of low density lipoprotein-derived cholesterol (type C) (Vanier 2010). The latter is, therefore, a lipid trafficking disorder where unesterified cholesterol and other lipids accumulate in the endocytic pathway, particularly endosomes and lysosomes.

Niemann-Pick type C (NPC) quickly became a significant disease model, not only to develop therapeutic applications for it but also as a mean to better understand the metabolic impact of lipid accumulation in cells. It is an autosomal recessive, neurovisceral condition, caused by accumulation of cholesterol in the liver, spleen and brain (Pentchev, Comly et al. 1985, Pentchev, Brady et al. 1994). It has an estimated prevalence of 1:150,000 individuals and a birth prevalence that ranges from 0.66 to 0.88 per 100,000 in countries like France, UK or Germany (Vanier and Millat 2003, Vanier 2010). At the cellular level, unesterified cholesterol accumulates in the late endosomal/lysosomal system, due to a trafficking defect of this sterol to other subcellular compartments. This disease also has a wide clinical spectrum at several levels: although most of the affected individuals are young children that end up dying very young, it also affects adults, and it has a great variety of symptoms. It involves not only an accumulation of unesterified cholesterol but also sphingolipids, gangliosides (mostly GM1 and GM2) and phospholipids within the cell's endosome/lysosomal system (Chang, Reid et al. 2005). In Portugal, the incidence of this disease appears to be much higher, with 9 cases from 1986 to 2005, when compared to other countries (Pinto, Caseiro et al. 2004, Vanier 2010).

1.2.1 Symptomatology

There is a great variety of symptoms and phenotypes that usually affect patients with the disease, that include hepatic, neurological or psychiatric manifestations (Vanier and Millat 2003). Typical symptoms generally include cerebellar ataxia, dysarthria, dysphagia, and progressive dementia. Cataplexy, seizures and dystonia are other quite common features, and psychiatric disturbances are frequent in late-onset patients. However, the symptomatology of the affected subject varies according to the age of onset, which is wide. Infantile manifestations of the disease include hepatosplenomegaly, which consists in the enlargement of the liver and spleen, and respiratory failure. Young children with NPC may also show a deficient development of motor skills, many never learning to walk. Older children (6-15 years) show learning disabilities and clumsiness. In adults, the manifestations are mostly neurological and psychiatric. Psychosis is not uncommon, mostly through paranoid delusions or auditory or visual hallucinations. Patients also show depressive syndrome, behavioral problems, aggressiveness, and bipolar disorders, among others (Vanier 2010).

1.2.2. Molecular genetics and the NPC proteins

The onset of NPC is thought to be caused by point mutations in two genes: *NPC1*, which is involved in the majority of cases (95%), and *NPC2*, in only 5% of the

cases (Steinberg, Ward et al. 1994, Vanier, Duthel et al. 1996). The discovery of these mutations led to a subdivision between Niemann-Pick C1 and C2, according to the gene involved. The loss of function of either one of these proteins seems to be the underlying cause behind the disease and its phenotypes (Yu, Jiang et al. 2014). The *NPC1* gene is localized in chromosome 18q11-12 and encodes a large integral glycoprotein found predominantly in the limiting membrane of late endosomes and lysosomes (Higgins, Davies et al. 1999, Ioannou 2005). Topological models have revealed that NPC1 has thirteen transmembrane helices and three large, glycosylated loops projecting into the lumen of the lysosome (Figure 1), which seems to be responsible for the correct recycling of cholesterol in vesicles (Yu, Jiang et al. 2014). The first of these domains is designated N-terminal domain (NTD) and appears to be the site for cholesterol binding, (Infante, Radhakrishnan et al. 2008).

A cluster of five membrane spanning sequences of NPC1 share homology with the sterol-sensing domain (SSD), which is capable of binding sterols and is present in several key players of cholesterol homeostasis, namely 3-hydroxy-3-methyl-glutaryl-CoA reductase and sterol regulatory element binding protein cleavage-activating protein (SCAP) (Davies and Ioannou 2000). This domain was later found to be required for NPC1 to bind several sterols, with point mutations in SSD diminishing this ability (Ohgami, Ko et al. 2004). NPC1 also contains a cysteine rich loop that may be important for protein-protein interactions. The defective efflux phenotype from late endosomes and lysosomes apparently drives an upregulation in cholesterol biosynthesis, which led some investigators to propose that this could also be the case for other membrane lipids like sphingolipids and gangliosides (Yiannis a Ioannou 2005).

The *NPC2* gene is localized in chromosome 14q24.3. It encodes a small, soluble, secreted and recaptured lysossomal glycoprotein that binds cholesterol with high affinity in a hydrophobic pocket (Vanier and Millat 2004). Mutations in either NPC1 or NPC2 seem to lead to the same pattern of lipid accumulation (Sleat, Wiseman et al.2004), which indicates that these proteins seem to act in a cooperative fashion to take cholesterol out of the lysosomes to other cellular compartments. A hypothetical model proposes that cholesterol binds NPC2 in the lumen with its hydroxyl group exposed, and then it is transferred to the N-terminal domain of NPC1 by reversing its orientation (Kwon, Abi-Mosleh et al. 2009). NPC1 seems therefore to be important for cholesterol to “enter” the membrane.

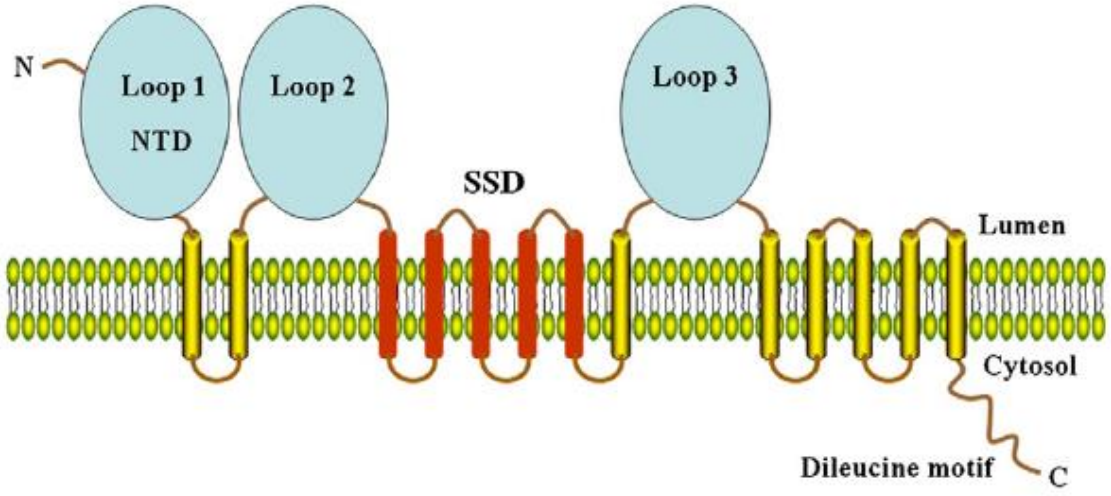

Figure 1 - Model of NPC1 structure. This protein has thirteen transmembrane domains, five of which share homology to the SSD of other sterol metabolism enzymes, three large luminal loops, and a dileucine C-terminal motif (Yu, Jiang et al. 2014).

1.2.3. Lipid involvement

The accumulation of unesterified cholesterol in the late endosome/lysosomal system was one of the first cellular hallmarks described for the NPC disease, observed in cultured skin fibroblasts of NPC1 patients (Pentchev, Comly et al. 1985). Usually, after endocytosis and hydrolysis of esterified cholesterol in the lysosomes, cholesterol is delivered to other cellular compartments, like the endoplasmic reticulum (ER), Golgi apparatus, and the plasma membrane. In cells lacking NPC1, this process is apparently impaired, probably due to a disruption in the transport of free cholesterol from the lysosomes to other organelles. As consequence, although cholesterol is being accumulated, the cell is not able to sense it and therefore induces the synthesis of more cholesterol. The accumulation of cholesterol can lead to altered levels of other lipids – NPC cells also accumulate sphingomyelin, glycosphingolipids and bis-(monoacylglycerol) phosphate. The accumulation of sphingomyelin seems to be due to a decreased activity of acid sphingomyelinase (Devlin, Pipalia et al. 2010). Another important phenotype is the accumulation of sphingosine in the liver and spleen of NPC patients (te Vruchte, Lloyd-Evans et al. 2004). It has also been reported that sphingosine accumulates in the acidic compartment of drug induced models of NPC1, leading to reduced levels of sphingosine-1-phosphate and low lysosomal calcium concentrations, the latter as a consequence of sphingosine accumulation (Lloyd-Evans, Morgan et al. 2008). Sphingosine is the most simple of sphingolipids, playing important roles in cell signaling (see point 1.3 below).

The pattern of lipid accumulation appears to be different in the brain and visceral organs (Vanier 2010). In liver and spleen there is no predominant compound

and these organs accumulate unesterified cholesterol, glycolipids, sphingomyelin, sphingosine and sphinganine (Vanier 2010). In the brain significant alterations occur in glycosphingolipids, particularly gangliosides GM2 and GM3, glucosylceramide, lactosylceramide and GA2, all in gray matter (Vanier 1999). In fact, some studies have emphasized that brain lipid alterations occur essentially in this part of the brain, with the exception of a severe loss of galactosylceramide and other myelin lipids in the white matter of patients with infantile and late-infantile form of the disease (Vanier 1999).

1.2.4. The yeast model of Niemann-Pick type C

The budding yeast *Saccharomyces cerevisiae* is a unicellular fungus, commonly known as baker's yeast, which has been widely used as a eukaryotic model to characterize cellular processes conserved in evolution. Yeast is genetically well defined, is easy to manipulate and has a relatively short generation time, comparing with mammalian cells. Furthermore, the publication of its complete genome sequence in April 1996 has turned yeast into a powerful tool for gene manipulation, like gene disruption, gene marking, mutation or gene dosage effects (Mager and Winderickx 2005). This has facilitated the characterization of protein function, localization and interactions as well as the biological consequences of their loss of function. Notably, 30% of human genes involved in disease have yeast orthologues, most of them being key components in metabolic pathways (Mager and Winderickx 2005). Thus, studies using yeast can contribute to our understanding of the molecular basis of diseases, including NPC.

The *NCR1* (*NPC1-related gene 1*) gene is the yeast orthologue of human *NPC1* (Berger, Hanson et al. 2005). Ncr1p shares 35% sequence identity with mammalian NPC1 (Malathi, Higaki et al. 2004) and is localized in the limiting membrane of the vacuole (Zhang, Ren et al. 2004), the equivalent of mammalian lysosome. Furthermore, this protein seems to transit through the biosynthetic vacuolar proteins sorting pathway. Importantly, Ncr1p was shown to be capable of suppressing ganglioside and cholesterol accumulation in mammalian NPC1 cells, which proves the functional equivalence of Ncr1p and NPC1. The SSD, NPC and cysteine rich domains are also conserved in yeast Ncr1p and the pleckstrin homology domain has several regions of similarity (Malathi, Higaki et al. 2004). A study revealed several pathways involved in NPC1 phenotypes. Among them, there is an up-regulation of histone deacetylase genes in cells lacking this protein (Munkacsi, Chen et al. 2011). Recently, Ncr1p-deficient cells have been used as a model of NPC1 in yeast. These cells present higher sensitivity to hydrogen peroxide and reduced chronological lifespan, associated with

increased levels of oxidative stress markers, decreased anti-oxidant defenses and mitochondrial dysfunctions (Vilaça, Silva et al. 2014). Namely, they have a compromised mitochondrial network in post diauxic shift (PDS) phase, and reduced mitochondrial membrane potential, oxygen consumption and cytochrome *c* oxidase activity (COX). These seem to be associated with alterations in sphingolipid levels, particularly ceramides and long chain bases, a class of bioactive lipids discussed below in section 1.3.

1.3. Sphingolipids

Although lipids were initially seen as being only involved in membrane structure and energy metabolism, the discovery that they could modulate cellular responses and play a role in signalling changed the way they were seen by biologists and biochemists. Sphingolipids are bioactive membrane lipids that play important signaling roles in eukaryotes, regulating cellular processes such as apoptosis, senescence, cell growth and proliferation (Dickson 2008, Hannun and Obeid 2008). Ceramide, sphingosine and sphingosine-1-phosphate (S1P) are the most studied sphingolipids, but their intricate metabolism and the several modifications they can endure gives rise to many different forms (see 1.3.1). They are also involved in certain inflammatory processes and many pathologies, like cancer (Ogretmen and Hannun 2004).

Their structure is rather particular - they are composed of a long chain sphingoid base (LCB), usually a sphingosine with 18 carbons, which can be amidated with a fatty acid to generate ceramide (Figure 2). Ceramide is the precursor of complex sphingolipids such as sphingomyelin and glycosphingolipids, which are formed by addition of a polar head group (phosphocholine) or a sugar moiety, respectively (Malagarie-Cazenave, Andrieu-Abadie et al. 2004). Ceramides can also be hydroxylated and exist in saturated and non-saturated forms.

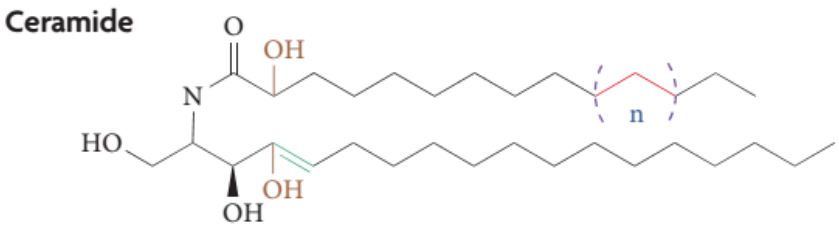

Figure 2 - General structure of ceramide. Ceramide, one of the core sphingolipids in mammals and yeast, is composed of sphingosine amidated to a fatty acid of variable length. This sphingolipid is actually a family of more than 50 species (Hannun and Obeid 2008).

1.3.1. Metabolism of sphingolipids in yeast

The de novo synthesis of LCBs and ceramide

The *de novo* synthesis of sphingolipids begins in the ER with the condensation of palmitoyl-CoA with serine, in a reaction catalyzed by serine palmitoyltransferase (SPT), a membrane associated enzyme, to yield 3-keto-dihydrosphingosine (KDS) (Hanada 2003) (Figure 3). This enzyme is encoded by *LCB1* and *LCB2* in yeast (Lee, Lee et al. 2014, Martin, Flandez et al. 2005). Besides Lcb1p and Lcb2p, SPT also requires a third subunit, Tsc3p, for optimal activity (Gable, Slife et al. 2000). This step represents the only entry point and the first rate-limiting step in this pathway. Regulation of SPT controls the rate of sphingolipid synthesis and it can be pharmacologically inhibited by myriocin (Wadsworth, Clarke et al. 2013).

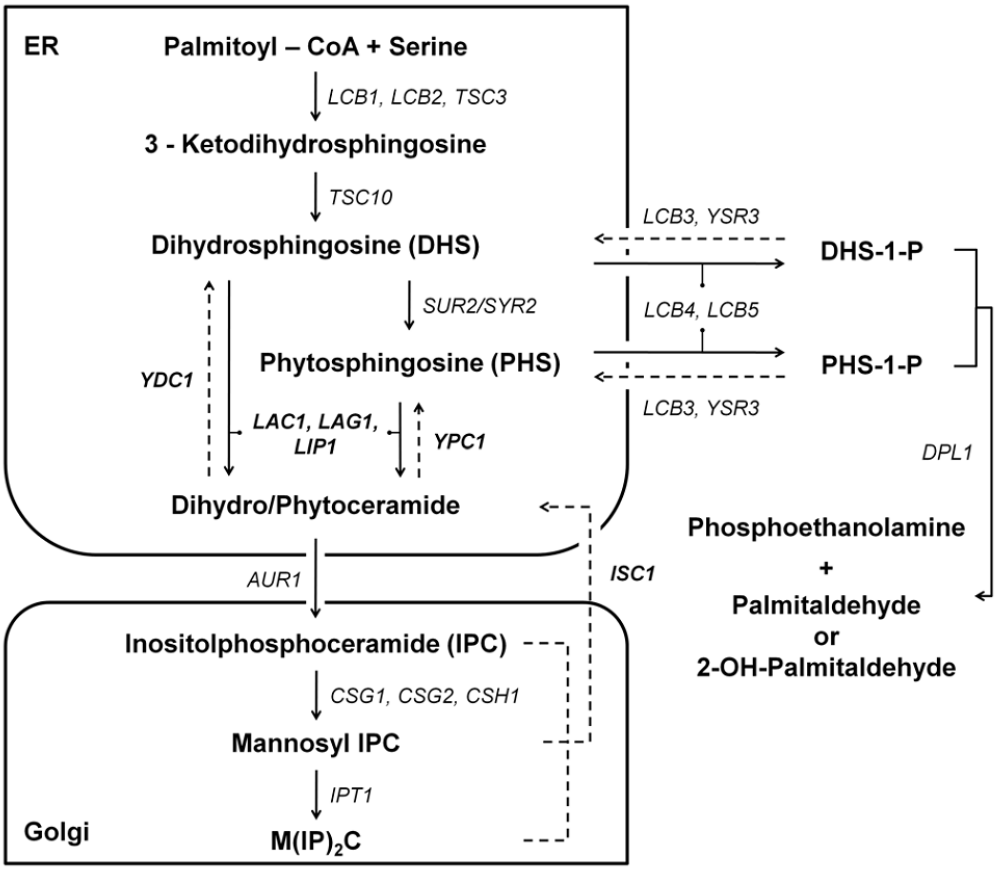

Figure 3 - Sphingolipid metabolism in yeast. In *Saccharomyces cerevisiae*, sphingolipid metabolism is spatially divided between the Endoplasmic Reticulum (ER) and the Golgi apparatus (for complex sphingolipids). The first entry point is through SPT, and the only exit point is catalyzed by Dpl1p (Rego, Trindade et al. 2012).

In *S. cerevisiae* KDS is then reduced to dihydrosphingosine (DHS) by Tsc10p, in a reaction that requires NADPH as a reducing agent. DHS in turn can be hydroxylated by

Sur2p or Syr2p to yield phytosphingosine (PHS). Dihydrosphingosine and phytosphingosine are the long chain bases present in yeast and they can be either phosphorylated or used to generate ceramide species. Dihydrosphingosine and phytosphingosine can be amide linked to a C_{26} fatty acid to generate dihydroceramide (DHC) and phytoceramide (PHC), respectively. This reaction is catalyzed by the ceramide synthases Lac1p (longevity assurance gene 1 cognate) and Lag1p (longevity assurance gene 1) (Guillas, Kirchman et al. 2001, Schorling, Vallée et al. 2001). Lac1p and Lag1p form a heteromeric complex with Lip1p which is required for optimal ceramide synthase activity (Vallée and Riezman 2005). Phytoceramide can also be generated by the hydroxylation of dihydroceramide catalyzed by Sur2p (Haak, Gable et al. 1997).

At this point, phytoceramide and dihydroceramide can be hydrolyzed back to LCBs and a free fatty acid in a reaction catalyzed by Ypc1p or Ydc1p, respectively (Mao 2000, Mao, Xu et al. 2000). *YDC1* and *YPC1* are homologous genes that encode alkaline ceramidases in yeast. Ypc1p even has reverse ceramide synthase activity (Mao 2000). The LCBs here generated can then be phosphorylated by Lcb4p or Lcb5p to yield their phosphorylated analogous, DHS-1-phosphate or PHS-1-phosphate. These species can be converted to non lipidic species (hexadecenal or phosphoethanolamine) through the only exit point in sphingolipid metabolism in yeast, catalyzed by dihydrosphingosine-1-phosphate lyase (DPL1p) (Saba, Nara et al. 1997).

Synthesis and hydrolysis of complex sphingolipids

Ceramides can also be transported to the Golgi apparatus where they are used for the synthesis of complex sphingolipids, through the addition of different polar head groups (Dickson 2008). The inositol phosphoryl ceramide synthase, encoded by *AUR1,* adds *myo*-inositol phosphate to the C_1 hydroxyl group of ceramide, to yield inositol phosphorylceramide (IPC). Then, IPC can be mannosylated by the transfer of mannose from GDP-mannose to form mannose-inositol-phosphorylceramide (MIPC), in a reaction catalyzed by the inositol phosphoceramide mannosyl transferase. This enzyme can be present in two protein complexes that contain a regulatory subunit (Csg2p) and one catalytic subunit (Csg1p or Csh1p). The last and most abundant of the complex sphingolipids in yeast, mannose-diinositol-phosphorylceramide ($M(IP)_2C$), is generated through the addition of another inositol-phosphate moiety to MIPC, catalyzed by the enzyme inositol-phosphotransferase (Ipt1p), (Dickson, Nagiec et al. 1997). These complex sphingolipids are present in the Golgi apparatus, vacuole and

plasma membrane (Hechtberger and Daum 1995), but their function is yet to be disclosed.

The hydrolysis of these complex sphingolipids is catalyzed by inositol phosphosphingolipid phospholipase C (Isc1p), the yeast orthologue of mammalian neutral sphingomyelinase-2 (Saway, Okamoto et al. 2000). It has phospholipase type C activity and cleaves the polar head groups of these lipids, releasing dihydroceramide and phytoceramide. Isc1p is localized in the ER during exponential growth on media containing glucose (fermentative metabolism), but then it is translocated to the mitochondrial membrane during PDS phase when yeast shift to respiratory growth (Vaena de Avalos, Okamoto et al. 2004). This suggests that Isc1p plays a role in respiration/mitochondrial function. Consistently, yeast cells lacking Isc1p exhibit mitochondrial dysfunctions, a shortened chronological lifespan and hydrogen peroxide sensitivity (Almeida, Marques et al. 2008).

1.3.2. Sphingolipid signaling

As previously mentioned, different sphingolipids have different functions in the regulation of cell metabolism. While ceramides and LCBs are commonly associated with apoptosis and cell-cycle arrest, sphingosine-1-phosphate and other phosphorylated analogs induce proliferation and cell survival. The cellular effects of these lipids in mammals are mostly mediated by protein kinases and phosphatases (Figure 4). The interplay between these different types of sphingolipids is rather intricate and changes in the activity of enzymes involved in their synthesis or degradation may have a huge impact on their levels and ultimately on cell fate (Hannun and Obeid 2008).

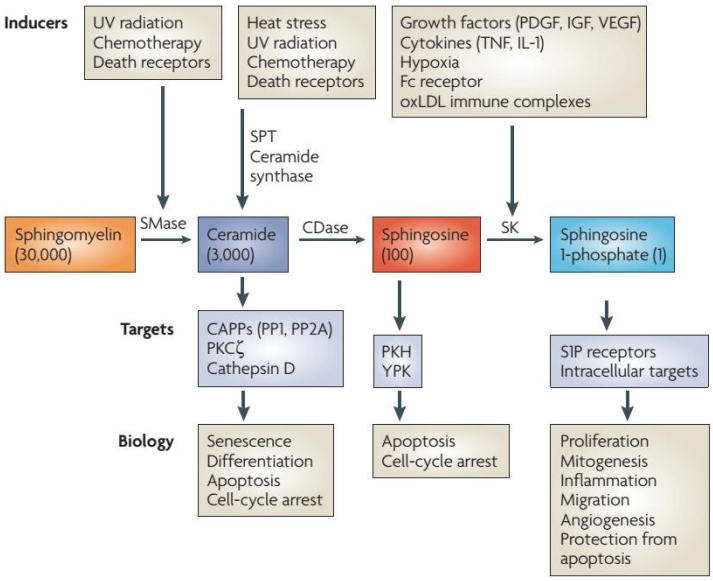

Figure 4 - Cell signaling by sphingolipids in mammals. Ceramides and sphingosine, whose generation is associated with different types of stress, induce cell cycle arrest and cell death, while sphingosine-1-phosphate, e.g. generated in response to growth factors, has protective and proliferative effects (Hannun and Obeid 2008).

Ceramide Signaling

Ceramide occupies a core position in sphingolipid metabolism. It can be produced either through the *de novo* pathway or by the hydrolysis of complex sphingolipids. It is also known to activate several protein kinases and phosphatases (Ruvolo 2002) (Figure 5). The accumulation of ceramide due to alterations in these pathways has been implicated in many diseases. For example, in Farber's disease a deficiency in acid ceramidase leads to an intralysosomal accumulation of ceramide with pathological consequences (Park and Schuchman 2006). In what concerns its activity as a second messenger, ceramide activates Ser/Thr phosphatases (CAPPs), such as protein phosphase 1 (PP1) and protein phosphatase 2A (PP2A) (Chalfant, Szulc et al. 2004), enhancing cytotoxic and apoptotic effects. The activation of PP2A is responsible for the down-regulation of insulin signaling, and by the dephosphorylation and consequent inhibition of the AKT/PKB pathway (Zhout, Summers et al. 1998). Ceramide also activates protein kinases, like PKCζ, and cathepsin D, a protease homolog to yeast Pep4p (Heinrich, Neumeyer et al. 2000). Ceramide also affects mitochondrial function: when accumulated in this organelle, it inhibits components of the respiratory chain favoring the generation of reactive oxygen species (ROS) and ultimately the induction of apoptotic events (Siskind 2005). In fact, mitochondrial apoptotic factors can be released through ceramide channels in mitochondria. Beclin-1, a tumor suppressor

protein that induces autophagosome formation (discussed in section 1.4.3), is also activated by ceramide (Scarlatti, Bauvy, et al. 2004). Dephosphorylation and inhibition of anti-apoptotic Bcl2 proteins is also induced by ceramide through activation of a PP2A (Ruvolo, Deng et al. 1999)

In yeast, there is a ceramide-activated protein phosphatase containing the regulatory subunits Cdc55p and Tpd3p and a catalytic subunit encoded by *SIT4* (Joseph T. Nickels 1996). Sit4p is a type 2A-related Ser/Thr phosphatase, sharing high homology with human protein phosphatase 6. It regulates G1/S transition of the mitotic cycle, ER to Golgi traffic, cell wall and actin cytoskeleton organization and mitochondrial function (Sutton, Immanuel et al. 1991). Sit4p deficiency is known to increase chronological lifespan (CLS) as well as hydrogen peroxide resistance (Barbosa, Osorio et al. 2011). CLS represents the amount of time a cell survives in nutrient depletion conditions, after entering stationary phase (Fabrizio and Longo 2003). *SIT4* deletion also confers resistance to yeast strains lacking mitochondrial DNA, increasing proliferation and mitochondrial membrane potential (Garipler, Mutlu et al.2013). Sit4p-deficient cells also show an increased respiratory rate at exponential phase, which probably explains the upregulation of antioxidant defenses later on and consequent increase in lifespan. However, they fail to grow on respiratory substrates such as galactose, ethanol or glycerol (Jablonka, Guzman et al. 2006). In fact, *SIT4* mutants have high glycogen content since they redirect carbohydrate flux to glycogenesis. Consistently, *sit4*Δ cells show a low fermentative capacity (de Assis, Zingali et al. 2013). Interestingly, Sit4p can associate with different subunits giving rise to complexes involved in distinct pathways. The target of rapamycin complex 1 (TORC1) down regulates this phosphatase activity through the phosphorylation of Tap42p, which then interacts with Sit4p and inhibits it (Di Como and Arndt 1996). Sit4p can also associate with the Sit4p associated proteins (SAPs) (Luke, Seta et al. 1996). In yeast, ceramide is also involved in the activation of Hog1p, a mitogen-activated protein kinase (MAPK) of the high osmotic glycerol pathway (HOG). Hog1p is functionally related to mammalian p38 and JNK, both involved in stress-induced apoptosis through mitochondrial damage and caspase activation (Hohmann, Krantz et al. 2007, Kumar, McLaughlin et al. 2006). Hog1p activation and ceramide have been implicated in the reduced lifespan and hydrogen peroxide sensitivity of Isc1p-deficient cells (Barbosa, Graça et al. 2012). Consistently, Hog1p phosphorylation increases in these mutants by a mechanism dependent of the protein kinase a Sch9p, which acts upstream of Hog1p in response to ceramide (Teixeira, Medeiros et al. 2014).

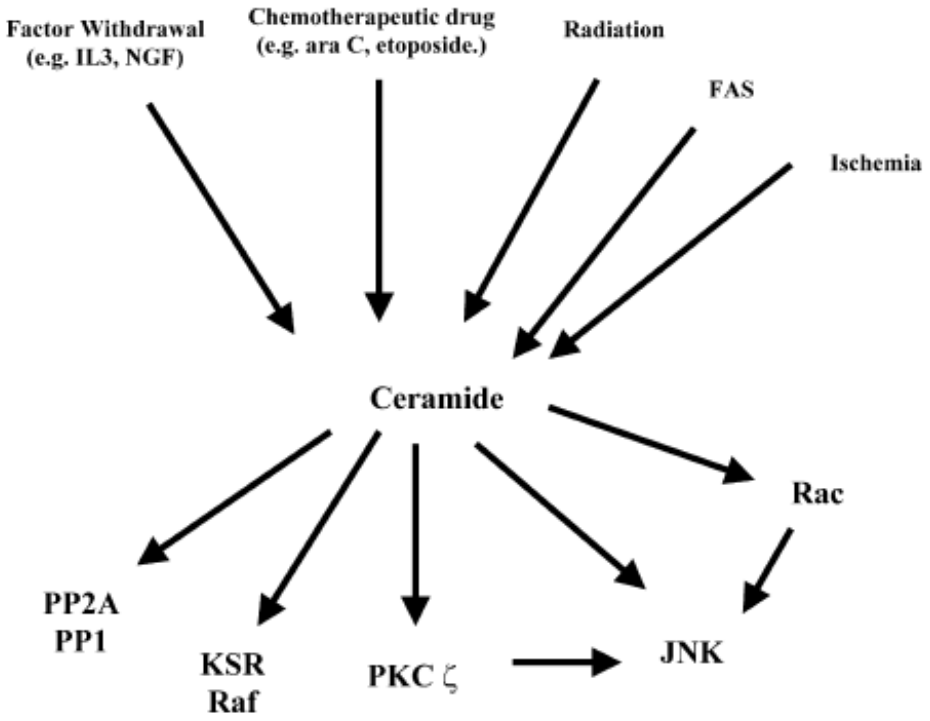

Figure 5 - Ceramide activates several protein kinases and phosphatases. Considered to be at the core of sphingolipid metabolism, ceramide, produced in response to several stimuli, activates metabolic pathways that ultimately result in apoptose in mammalian cells (Ruvolo 2002).

Sphingosine and LCBs signalling

Sphingosines are mostly involved in apoptotic events, cell cycle arrest and the regulation of actin cytoskeleton and endocytosis (Hannun and Obeid 2008). They are known inhibitors of protein kinase C (PKC) (Smith, Merril et al. 2000), mitogen-activated protein kinases (MAPK) and activators of stress-activated protein kinases (SAPK) such as p38 and JNK (Olivier 2002). On the other hand, phosphorylated sphingosines like sphingosine-1-phosphate have protective effects in cells, playing roles in survival, proliferation. They function in an autocrine way, activating G-protein coupled receptors (Strub, Maceyka et al. 2010). In yeast, one of the first observations suggesting that LCBs could have a significant role as second messengers was the fact that their concentration increase transiently when cells were submitted to heat stress (Jenkins, Richards et al. 1997, Dickson 2008). It was later found that these sphingolipids are activators of the redundant protein kinases Pkh1p and Pkh2p, homologous to mammalian 3-phosphoinositide-dependent protein kinase-1 (PDK1). In mammals, PDK1 is involved in the activation of enzymes, including AKT/PKB in the response to growth factors and hormones like insulin (Mora, Komander et al. 2004). After activation by LCBs, the Pkh1/2p kinases phosphorylate and activate Ypk1/2p,

Pkc1p and also Sch9p, the latter in a T570 residue (Dickson 2008). Sch9p is a protein kinase belonging to the AGC family that also requires phosphorylation by TORC1 in its C-terminal in order to be fully activated (Urban, Soulard et al. 2007). Sch9p, which shares high homology to mammalian AKT/Protein kinase B, is involved in the regulation of osmostress-responsive genes, mitochondrial function, cell aging, oxidative stress and sphingolipid biosynthesis (Pascual-Ahuir and Proft 2007, Lavoie and Whiteway 2008, Pan and Shadel 2009, Swinnen, Wilms et al. 2014). In fact, the downregulation of Tor1p or Sch9p was shown to increase CLS in yeast (Pan and Shadel 2009). Consistently, *sch9*Δ cells show an upregulation of genes encoding subunits of the mitochondrial respiratory chain and an increased respiration rate (Lavoi and Whiteway 2008). Sch9p is also involved in the regulation of sphingolipid metabolism as recently discovered, by regulating the expression of *YPC1* and *YDC1* (Swinnen, Wilms et al. 2014). The deletion of *SCH9* leads to an increase in the expression of these ceramidases, leading to the turnover of ceramides into LCBs and consequently increasing the levels of LCBs and of their phosphorylated forms. Also, the downregulation of sphingolipid synthesis by lowering SPT activity is known to increase CLS in yeast by reducing TORC1 and, consequently, Sch9p activity (Huang, Liu et al. 2012, Liu, Huang et al. 2013).

1.4. The Target of Rapamycin pathway

1.4.1. General aspects

The purification of the macrocyclic lactone rapamycin in the 1970s, and the later observation that it was capable of suppressing mammalian cell proliferation, led to the identification and characterization of the target of rapamycin (TOR) pathway. TOR is a Ser/Thr protein kinase highly conserved in eukaryotic organisms. Indeed, it is present in organisms such as *S. cerevisiae* (where most of the studies have been performed), *C. elegans*, *Drosophila* and mammals (Raught, Gingras et al. 2001, Rohde, Heitman et al. 2001, Schmelzle and Hall 2000). TOR belongs to the family of phosphatidylinositol kinase-related kinases (PIKK) and it senses the cell's nutritional state in order to regulate processes such as cellular growth, stress response, autophagy and aging (De Virgilio and Loewith 2006, Wullschleger, Loewith et al. 2006). Rapamycin forms a complex with FKBP12 that binds and inhibits TOR, mimicking nutrient starvation (Rohde, Heitman et al. 2001). Genetic studies in *Saccharomyces cerevisiae* led to the discovery that TOR signaling involves two different complexes, the TOR complex 1 (TORC1) that is sensitive to rapamycin and the TOR complex 2 (TORC2) that is insensitive to rapamycin. These complexes have different functions in cell growth

control (Loewith, Jacinto et al. 2002, De Virgilio and Loewith 2006, Wullschleger, Loewith et al. 2006) and are conserved in other organisms. In yeast, TORC1 is composed of Tor1p or Tor2p, Kog1p, Lst8p and Tco89p, while TORC2 has only Torp2, Avo1p, Avo2p, Avo3p, Lst8p and Bit61p. These complexes seem to regulate cell growth in different ways: while TORC1 controls temporal aspects of cell growth, like ribosome biogenesis, protein biosynthesis and tRNA gene expression, nutrient uptake and metabolism, stress responses, mitochondrial function and autophagy, TORC2 regulates actin cytoskeleton organization and spatial growth (Figure 6).

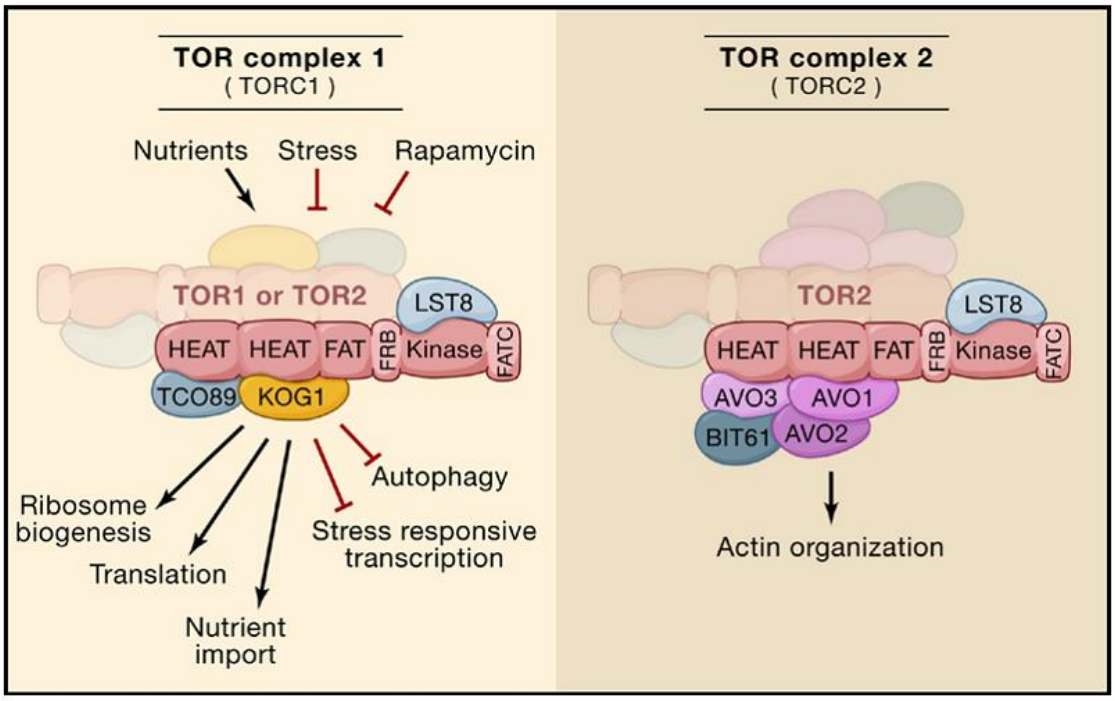

Figure 6 - The two different TOR complexes in *Saccharomyces cerevisiae*. TOR signaling is divided in two different macromolecular protein complexes, which in yeast are called TORC1 and TORC2. While rapamycin sensitive TORC1 regulates stress response and temporal aspects of cellular growth, TORC2 controls spatial growth through regulation of actin organization (Wullschleger, Loewith et al. 2006).

TORC1 integrates nutritional cues from nitrogen and carbon sources in order to regulate cellular growth (Loewith and Hall 2011). Nutrients are activators of TORC1, inducing anabolic processes such as ribosome biogenesis, translation, protein synthesis and nutrient import. Nutrient depletion leads to the inactivation of this pathway, and the consequent downregulation of transcription and translation (Swinnen, Ghillebert et al. 2014). This is mimicked by rapamycin treatment, as cells exit the cell cycle and enter into G0 quiescent state. In both cases, the expression of a specific set of genes involved in stress response and survival are induced, as well as catabolic processes like autophagy (Loewith and Hall 2011).

1.4.2. Regulation of mitochondrial function and longevity

TORC1 is also involved in stress response, mitochondrial function and aging – treating mice with rapamycin, even if at later stages, was shown to extend the lifespan in mice. In yeast, TORC1 is active during logarithmic phase and inhibits Rim15p, a protein kinase required for the activation of the transcription factors Gis1p and Msn2p/4p, which upregulate genes associated with stress response and CLS (Wanke, Pedruzzi et al. 2005, Wei, Fabrizio et al. 2008). In fact, the deletion of *TOR1* increases CLS in yeast (Powers III, Kaeberlein et al. 2006, Pan, Schroeder et al. 2011). In early stages of growth, Tor1p deficient cells have an increased mitochondrial oxygen consumption that increases the production of superoxide radicals. These ROS induce an adaptive response that allows cells to maintain ROS levels at low levels in stationary phase, increasing the CLS of *tor1*Δ mutants (Bonawitz, Chatenay-Lapointe et al. 2007). TORC1 regulates lifespan and oxidative stress resistance in part through modulation of Sch9p. Indeed, the deletion of *SCH9* also improves mitochondrial function, oxidative resistance and lifespan in yeast (Pan and Shadel 2009).

1.4.3. Regulation of autophagy

Autophagy is a highly regulated cellular process in eukaryotes, conserved from yeast to mammals, that plays essential roles in the regulation of normal development and cellular homeostasis. In this process, cellular proteins and whole organelles are engulfed by cytoplasmic double-membrane vesicles, designated as autophagosomes, which then direct the cargo to lysosomes where they are degraded (Klionsky and Emr 2000). In *S. cerevisiae*, this degradation step occurs in the vacuole, the yeast equivalent to lysosomes in mammalian cells. This process also represents an adaptive catabolic response of the cell in order to acquire nutrients and energy from more complex molecules, in response to nitrogen starvation or other types of cellular stress (Nakatogawa, Suzuki et al. 2009, Yang and Klionsky 2010). Its impairment is known to be an underlying cause behind several pathologies in humans, like cancer, immune disease and neurodegeneration, and has been implicated in aging (Levine and Kroemer 2008). It may lead to the accumulation of unfolded and toxic proteins, among other molecules. The autophagic flux seems to be defective in NPC1 cells, due to impaired degradation of autophagosomes (Sarkar, Carroll et al. 2013), and the restoration of this process may be a therapeutic approach to this problem (Sarkar, Maetzel et al. 2014).

Although initially seen as a non-selective process (macroautophagy), different types of autophagy are responsible for the degradation of different organelles in a more

selective fashion (Fimia, Kroemer et al. 2013, Kraft, Peter et al. 2010). In microautophagy, the lysosomal membrane directly invaginates the cargo to be degraded, independently of autophagosome formation. In mitophagy, only damaged or aged mitochondria are directed for degradation. This selective type of autophagy is of high importance for cells to maintain a good mitochondrial fitness, and represents a defense against oxidative stress and aging (Ding and Yin 2013). Pexophagy consists on the selective autophagic degradation of peroxisomes, in situations where the abundance of this organelle is not necessary anymore (Sakai, Oku et al. 2006). The cytoplasm to vacuole targeting (Cvt) pathway, specific to yeast, is responsible for delivering hydrolases like aminopeptidase 1 and α-mannosidase to the vacuole, and represents the only biosynthetic pathway that uses autophagy machinery (Lynch-Day and Klionsky 2010).

The molecular mechanisms underlying autophagy have been extensively studied in yeast where thirty-five autophagy related genes (*ATG*) have been identified. This complex machinery is essential for the autophagic pathways present in yeast: macroautophagy, Cvt, pexophagy and mitophagy. Only 15 *ATG* genes encode the core machinery essential for the all subtypes of autophagy (Nakatogawa, Suzuki et al. 2009). These are divided into 5 different subgroups, each with a specific function. One of these essential genes is *ATG8*. This gene encodes an ubiquitin-like protein with homology to mammalian microtubule-associated protein. It serves as a molecular marker for membrane dynamics during autophagy, since it is localized on the isolation membranes of autophagosomes (Kirisako, Baba et al. 1999). It is associated to phosphatidylethanolamine and it mediates tethering and hemifusion of liposomes in response to lipidation, driving the growth of autophagosomes (Nakatogawa, Suzuki et al. 2009).

TORC1 plays a key role in the regulation of autophagy in response to nutritional, hormonal and metabolic signals. In yeast, TORC1 negatively regulates macroautophagy through the hyperphosphorylation of Atg13p, which is involved in the first steps in the activation of this process (Figure 7) (Wullschleger, Loewith et al. 2006, Nakatogawa, Suzuki et al. 2009). In starvation like or stress conditions, the inhibition of TORC1 leads to the dephosphorylation of Atg13p, allowing its association with the Ser/Thr protein kinase Atg1p. This complex then interacts with other core Atg proteins, namely Atg17p, Atg29p and Atg31p, recruiting them to the pre-autophagosomal structure, necessary for the initiation of autophagy (Nakatogawa, Suzuki et al. 2009).

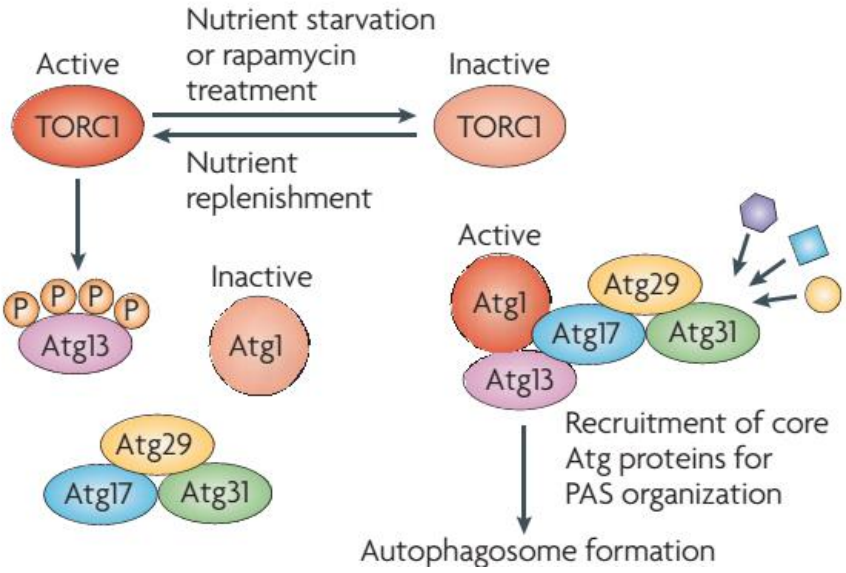

Figure 7 - The role of TORC1 in autophagy regulation in yeast. The inactivation of TORC1 leads to the dephosphorilation of Atg13p and consequent association with other Atg proteins, leading to the pre-autophagosomal structure organization.

1.4.4. TOR signaling and sphingolipid metabolism

The TOR pathway is also known to regulate sphingolipid synthesis at several levels. TORC1 inhibition stimulates the synthesis of complex sphingolipids downstream of SPT, through Npr1p-dependent phosphorylation of Orm1p and Orm2p (Shimobayashi, Oppliger et al. 2013). Rapamycin treatment leads to the activation of PP2A catalytic subunit Sit4p which in turn activates Npr1p, leading to Orm1/2p activation and synthesis of complex sphingolipids. The Orm1/2p also bind to and inhibit SPT, modulating the *de novo* sphingolipid biosynthesis (Breslow, Collins et al. 2010). In conditions of sphingolipid depletion (eg., upon treatment with myriocin), TORC2 activates Ypk1p which in turn phosphorylate and inhibit the Orm proteins, promoting SPT activation and sphingolipid synthesis (Roelants, Breslow et al. 2011).

TORC2 also controls ceramide synthase activity (Aronova, Wedaman et al. 2008, Dickson 2008). In response to growth signals, TORC2 phosphorylates the protein kinase Ypk2p, which if also phosphorylated by Pkh1/2p, activates ceramide synthase. This process is also enhanced by LCBs since these are known activators of Pkh1/2p. The protein phosphatase calcineurin has the opposite effect by downregulating ceramide synthase activity, in response to heat and other stresses (Aronova, Wedaman et al. 2008).

CHAPTER 2

AIM OF THE WORK

Recently, it was found that an increase in the levels LCBs in *ncr1*Δ cells leads to the hyperactivation of the Pkh1/2p-Sch9p pathway, resulting in the several mitochondrial dysfunctions and reduced lifespan of these mutants (Vilaça, Silva et al. 2014). TORC1 is a known activator of Sch9p through phosphorylation of its C-terminal moiety (Urban, Soulard et al. 2007), and this pathway is involved in aging in response to nutrients (Powers III, Kaeberlein et al. 2006, Pan, Schroeder et al. 2011). For this reason, we hypothesized genomic deletion of *TOR1* could lead to an increase in lifespan and resistance to hydrogen peroxide in Ncr1p-deficient cells. Moreover, the ceramide activated protein phosphatase Sit4p is activated in *ncr1*Δ cells, which. is correlated with the accumulation of specific ceramide species (Vilaça et al. unpublished). The autophagic flux and Pep4p activity are also increased in these cells (Vilaça et al. unpublished). However, the role of ceramide, Sit4p and Tor1p in *ncr1*Δ phenotypes is poorly characterized. This work aimed to assess the role of Sit4p in the regulation of sphingolipid metabolism, autophagy and vacuolar function in *ncr1*Δ cells, as well as how Tor1p modulates oxidative stress resistance and chronological lifespan in this model of NPC1 disease.

CHAPTER 3

MATERIALS AND METHODS

3.1. Yeast strains and growth conditions

Saccharomyces cerevisiae BY4741 was the parental strain of all haploid mutants used in this study, which are listed in table 1. Yeast cells were grown aerobically at 26ºC in a shaker at 140 rpm, in flasks with a 1:5 ratio of volume/medium, to either logarithmic (OD_{600} = 0.6) or post-diauxic shift (OD_{600} = 7-10) phase. Growth media used were yeast extract peptone dextrose (YPD - 2% (w/v) bacteriological peptone, 1% (w/v) yeast extract, 2% (w/v) glucose), yeast extract peptone glycerol [YPG - 2% (w/v) bacteriological peptone, 1% (w/v) yeast extract, 3% (w/v) glycerol), minimal medium [MM - 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, supplemented with 0.004% (w/v) histidine, 0.008% (w/v) leucine, 0.004% (w/v) methionine or 0.004% (w/v) uracil] and synthetic complete drop-out medium [SC - 2% (w/v) glucose, 3% (w/v) glycerol or 2% (w/v) galactose and 0.67% (w/v) yeast nitrogen base w/o amino acids, 0.14% drop out medium, supplemented with 0.008% (w/v) histidine, 0.04% (w/v) leucine, 0.008% (w/v) tryptophan and 0.008% (w/v) uracil].

Table 1. Yeast cells used in this work.

Strain	Genotype	Source
BY4741	Mata, *his3*Δ, *leu2*Δ, *met15*Δ, *ura3Δ*	EUROSCARF
*ncr1*Δ::*KanMX4*	BY4741 *ncr1*Δ::*KanMX4*	Vilaça *et al*., 2014
*ncr1*Δ::*URA3*	BY4741 *ncr1*Δ::*URA3*	Vilaça *et al*., 2014
*sit4*Δ	BY4741 *sit4*Δ::*HIS3*	Vilaça *et al*., unpublished
*ncr1*Δ*sit4*Δ	BY4741 *ncr1*Δ::*KanMX4 sit4*Δ::*HIS3*	Vilaça *et al*., unpublished
*tor1*Δ	BY4741 *tor1*Δ::*KanMX4*	Teixeira *et al*., 2014
*ncr1*Δ*tor1*Δ	BY4741 *ncr1*Δ::*URA3 tor1*Δ::*KanMX4*	This study
*ydc1*Δ	BY4741 *ypc1*Δ::*KanMX4*	EUROSCARF
*ypc1*Δ	BY4741 *ydc1*Δ::*KanMX4*	EUROSCARF
*lag1*Δ	BY4741 *lag1*Δ::*KanMX4*	EUROSCARF
*ncr1*Δ*ypc1*Δ	BY4741 *ncr1*Δ::*URA3 lag1*Δ::*KanMX4*	Vilaça *et al*., unpublished
*ncr1*Δ*ydc1*Δ	BY4741 *ncr1*Δ::*URA3 ydc1*Δ::*KanMX4*	This study
*ncr1*Δ*lag1*Δ	BY4741 *ncr1*Δ::*URA3* lag1Δ::*KanMX4*	This study

3.2. Genomic DNA extraction

Yeast cells were grown overnight in 10 ml YPD to stationary phase, pelleted and resuspended in 100 µl of lysis solution (2% (v/v) triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Cell lysis was performed by adding zirconium beads, 50 µl of phenol and 50 µl of chloroform/isomyl alcohol solution (48:1) followed by a subsequent 5 min vortexing step. After centrifugation (13,000 rpm, 5 min), the aqueous phase was transferred to a new tube containing 100 µl of chloroform and

100 µl of TE solution (100 mM Tris, 10 mM EDTA pH 8.0), which was then vortexed during 5 min. Afterwards, the aqueous phase was collected again to a new tube to which 1 mL of 100% ethanol was added. The mix was left at -20ºC for 5-10 min until formation of a precipitate and centrifuged at 14,000 rpm during 5 min. The pellet was resuspended in 400 µl of TE buffer and 30 µl of 1 mg/ml RNase A, and incubated at 37ºC during 7 min. Then 10 µl of 4 M ammonium acetate and 500 µl of 100% ethanol were added, and the solution mixed. After centrifugation, the pellet was washed once with 200 µl of 70% (v/v) ethanol, centrifuged again, dried and resuspended in 50 µl of dH_2O. Genomic DNA was then quantified using a Nano-drop spectrophotometer (ND-1000, Thermo Scientific).

3.3. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed with a reaction mix containing 1X Taq buffer (Promega), 0.2 mM dNTP's (Thermo Scientific), 0.2 µM forward primer, 0.2 µM reverse primer, 1.5 mM $MgCl_2$ (Promega) and 1 U Taq Polymerase (Promega), to a final volume of 20 µl. The primers used in this study are listed in table 2. For the amplification of the *KanMX4* cassette used in the disruption of *YDC1*, the annealing temperature was 53ºC and the elongation time 2 min. The *KanMX4* cassette also used for the disruption of *LAG1* was amplified using an annealing temperature of 50ºC and an elongation time of 2 min. For the amplification of the *HIS3* cassette used in the disruption of *YDC1*, the annealing temperature was 58ºC and the elongation time 1 minute. For the amplification of the *URA3* cassette used in the disruption of *NCR1*, the annealing temperature was 53ºC and the elongation time 2.5 min. All PCR products were analyzed by nucleic acid electrophoresis at 100V, using 1% (w/v) agarose gels and TAE buffer 1x (40mM Tris, 20mM acetic acid, 1mM EDTA). DNA bands were extracted from the gel using the Gel Band Purification Kit (GE Healthcare, Life Sciences).

Table 2. Primers used in this study

Primer	Sequence
YDC1_HIS3_Fw	5'TAGTGAATTTTTAAGAAAGTAAGATAAAGAAAAAAATAACCTCTGACACATGCAGCTCCCGGAG3'
YDC1_HIS3_Rv	5'TATATTTTGAAGATTCAAATGGATGGCACAAAATCACTCCCTTCAGTATCATACTGTTC3'
YDC1_Amp_Fw	5'TAGTGAATTTTTAAGAAAGTAAGATAAAGAAAAAAATCAA3'
YDC1_Amp_Rv	5'GGAGTGATTTTGTGCCATCCATTTGAATCTTCAAAATATA3'
NCR1_Amp_Fw	5'CCGTGGCTAATGTCACAACA3'
NCR1_Amp_Rv	5'TTACGACTGAAGCGTTGACC3'
NCR1_Conf_Rv	5'CCAGTGACACCATGAGCATTAG3'
Kan_Conf_Rv	5'AATCGAATGCAACCGGC3'
YDC1_Conf_Fw	5'CTGATTTTTACTGATCGTAGCCATT3'
LAG1_Amp_Fw	5'CGTCATCTTCCATTTGAAATCC3'
LAG1_Amp_Rv	5'TCTCGCCAAGACTCCTAGTAAG3'
LAG1_Conf_Fw	5'TGACAGATCTCAATGAATCATCG3'

3.4. Gene deletions and DNA manipulation

All mutants were generated by homologous recombination and yeast cells transformed according to the PEG/Lithium acetate protocol (Gietz and Schiestl 1997), as described in 3.5.

For *ncr1*Δ::*URA3 ydc1*Δ::*KanMX4* generation, a kanamycin resistance cassette containing the flanking regions (40 base pairs) of *YDC1* was amplified by PCR, using genomic DNA from *ydc1*Δ::*KanMX4* cells and primers YDC1_Amp_Fw and YDC1_Amp_Rv (Table 2). BY4741 *ncr1*Δ::*URA3* cells were then transformed with this cassette and *ncr1*Δ::*URA3 ydc1*Δ::*KanMX4* mutants were selected in YPD plates

containing 200 ng/ml geneticin (Sigma-Aldrich). For *ncr1*Δ::*URA3 lag1*Δ::*KanMX4* generation, a kanamycin resistance cassette containing the flanking regions of *LAG1* (about 300 base pairs) was amplified by PCR, using genomic DNA from *lag1*Δ::*KanMX4* cells and primers LAG1_Amp_Fw and LAG1_Amp_Rv. BY4741 *ncr1*Δ::*URA3* were transformed with this cassette and *ncr1*Δ::*URA3 lag1*Δ::*KanMX4* mutants were selected in YPD plates supplemented with 200 ng/ml geneticin. For *ncr1*Δ::*URA3 ypc1*Δ::*KanMX4 ydc1*Δ::*HIS3* generation, an *HIS3* cassette containing the flanking regions of *YDC1* (40 base pairs) was amplified by PCR from pRS313 plasmid using primers YDC1_HIS3_Fw and YDC1_HIS3_Rv. BY4741 *ncr1*Δ::*URA3 ypc1*Δ::*KanMX4* cells were transformed with this cassette and *ncr1*Δ::*URA3 ypc1*Δ::*KanMX4 ydc1*Δ::*HIS3* mutants were selected in minimal media plates lacking histidine. For *ncr1*Δ::*URA3 tor1*Δ::*KanMX4* generation, an *URA3* cassette containing the flanking regions of *NCR1* (about 300 base pairs) was amplified by PCR, using genomic DNA from BY4741 *ncr1*Δ::*URA3* cells and primers NCR1_Amp_Fw and NCR1_Amp_Rv. BY4741 *tor1*Δ::*KanMX4* cells were transformed with this cassette and *ncr1*Δ::*URA3 tor1*Δ::*KanMX4* mutants were selected on minimal medium plates lacking uracil. The correct insertion of the cassettes was confirmed by, PCR using a forward primer complementary to a region outside the recombination area and a reverse primer for a region inside the cassette.

For the β- galactosidase assays, yeast cells were transformed with a plasmid expressing the LacZ reporter (YEp357-*LacZ*) (Swinnen, Ghillebert et al. 2014) under the control of endogenous promoters of either *YPC1*, *YDC1*, *LAC1* or *LAG1*, and selected in minimal medium lacking uracil. For autophagic flux analyses, yeast cells were transformed with pRS416-*GFP-ATG8* and selected in minimal medium lacking uracil (Yorimitso, Zaman et al. 2007).

3.5. Yeast transformation

Yeast cells were grown overnight in YPD (20 ml, OD_{600} = 0.8), harvested at 4,000 rpm for 5 min, washed and resuspended in 100 µl of dH_2O. 50 µl of cells were used as negative control or for transformation. Afterwards, 240 µl of 50% (w/v) polyethylene glycol 3350 (PEG), 36 µl of 1 M lithium acetate and 25 µl of 5 mg/ml single stranded carrier DNA were added to the cell mix. An amount equivalent to 100 ng of plasmid DNA or 500 ng of the disruption cassette was added. For gene deletion the cell mix was initially incubated at 26ºC for 30 minutes and then another half hour at 42ºC, whereas for plasmid transformation only the latter step was carried out. After incubation, cells were pelleted, washed with dH_2O and plated on selective medium.

3.6. β galactosidase assay

Cells expressing LacZ reporters were grown in SC glucose medium lacking uracil to exponential phase (20 ml, OD_{600}=0.6). Cells were harvested by centrifugation (4,000 rpm, 5 min) and lysed in 100 µl of breaking buffer (100 mM Tris-HCl, 10% (v/v) glycerol) supplemented with a mix of protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boheringer, Manheim). Cell disruption was done using zirconium beads and by vortexing five times for one minute, with one minute intervals on ice. Cellular extracts were then centrifuged (14,000 rpm, 15 min) at 4º C and the supernatant was collected. Protein levels were quantified by the Lowry assay, using bovine serum albumin as a standard. A volume equivalent to 70 µg of protein was then diluted in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) to a final volume of 800 µl. The mix was briefly incubated at 30ºC and reactions initiated by adding 200 µl of 4 mg/ml 2-nitrophenyl-β-D-galactopyramidose (Sigma-Aldrich) for the appropriate time, until formation of a yellow color. Reactions were stopped using 400 µl of 1 M Na_2CO_3, and OD_{420} was measured.

3.7. Pep4 activity

Pep4 activity was determined by measuring the release of tyrosine containing acid-soluble peptides from acid denatured hemoglobin (Jones 1990). Cultures were grown overnight in YPD medium and harvested in exponential (55 ml, OD_{600} = 0.6) or PDS (OD_{600} = 6-10) phase. Cells were lysed in 100 µl Tris-HCl (pH 7.6) as previously described and protein levels quantified using the Lowry assay. A volume equivalent to 250 µg of protein was diluted in Tris-HCl to a final volume of 50 µl. Extracts were then incubated at 37º C with 1 ml of Hemoglobin-Glycine solution (2% (w/v) hemoglobin - 0.2 M glycine pH 3.2, in a 1:1 ratio) for 0 and 30 min, and the reactions stopped by adding 100 µl of 1 N perchloric acid on ice. Samples were centrifuged (30 min, 13,000 rpm), and 70 µl of supernatant were added to 700 µl of a solution of 2% (w/v) Na_2CO_3 : 1% (w/v) $CuSO_4$: 2% (w/v) Na^+K^+ Tartarate (100:1:1 ratio). Afterwards, 70 µl of 0.5 M NaOH were added to the samples and following 10 min of incubation, 70 µl of Folin reagent (Merck) diluted 1:1 in dH_2O were added. The OD_{750} was measured and results were expressed in µg $Tyr.min^{-1}$.

3.8 Oxidative stress resistance assay and chronological lifespan

For oxidative stress resistance, yeast cells were grown in SC-glucose medium to exponential phase (OD_{600}=0.6) and then treated with 1.5 mM H_2O_2 (Merck) for one hour. Afterwards, OD_{600} was measured and cells were diluted, plated on YPD medium containing 1.5% (w/v) agar, and grown for 3 days at 26ºC. Colonies were counted and cell viability was expressed as the percentage of colony forming units relative to untreated cells. To assess the effect of rapamycin on H_2O_2 resistance, cells were grown to OD_{600}=0.3 and pre-treated with 200 ng/ml rapamycin [Sigma-Aldrich; dissolved in dimethyl sulfoxide (DMSO)] or equal volume of the vehicle for three hours.

For chronological lifespan, overnight cultures were diluted in SC-glucose medium to an OD_{600} = 0.6, then grown for 24 h (to post-diauxic shift phase, t = 0) and kept in growth media overtime at 26ºC. Cells were subjected to standard dilutions and plated on YPD medium supplemented with 1.5% (w/v) agar. Viability was expressed as the percentage of colony forming units when compared to t = 0.

3.9. Autophagic flux analyses and Western Blotting

For the autophagic flux assay, cells expressing pRS416-*GFP-ATG8* were grown in SC-glucose medium lacking uracil to exponential phase and treated with either 200 ng/ml rapamycin (Sigma-Aldrich) or equal volume of vehicle (Sigma-Aldrich) for 3h. Cells were lysed in 100 µl of phosphate buffer (50 mM KH_2PO_4, 0.1 mM EDTA pH 7.0) supplemented with protease inhibitors and protein concentration was quantified by the Lowry assay. A volume equivalent to 30 µg was then diluted in an equal volume of sample buffer 2X (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue, 0.125 M Tris HCl). Samples were then heated for 5 min at 95ºC. Proteins were separated by SDS-PAGE in a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare, Life Sciences). The membrane was then blocked for 2 h in 5% (w/v) non-fat dry milk in TTBS (20 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween-20, pH 7.6) and incubated overnight at 4ºC with mouse anti-GFP antibody (Roche, Basel, Switzerland) diluted 1:5000 in TTBS containing 5% milk, or mouse anti-Pgk (1 : 50000). After washing with TTBS, the membrane was incubated for 1 h with goat anti-mouse IgG-peroxidase antibody

(Invitrogen) diluted 1:10000 in TTBS containing 5% milk. Immunodetection was performed by chemiluminescence using WesterBright^{tm} ECL reagent (Advansta) and exposing the membranes to an X-ray film.

For stripping, membranes were washed in TTBS and incubated with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10 mM 2-mercaptoethanol) for 30 min at 50ºC. Membranes where washed again for complete removal of β-mercaptoethanol.

3.10. Growth in glycerol plates

In order to evaluate respiratory capacity, yeast cells were grown overnight in SC-glucose to exponential phase (OD_{600}= 0.6). Cultures were then diluted to an OD_{600}=0.1 and fivefold serial dilutions were plated on SC-glucose and SC-glycerol medium containing 1.5 % agar (w/v). Cells were grown for 3-5 days at 26ºC.

3.11. Statistical analyses

Results were analyzed using GraphPad Prism software v6.02, and by mean and standard deviation of at least three independent experiments. Values were compared by one-way ANOVA with Bonferroni test.

CHAPTER 4

RESULTS

4.1. Sphingolipid metabolism in ncr1Δ cells

4.1.1. Sit4p regulates sphingolipid metabolism in the yeast model of NPC1

Aiming to characterize how sphingolipid metabolism is affected in *ncr1*Δ cells and the role of Sit4p in those changes, the expression of genes encoding for ceramide synthases (*LAC1* and *LAG1)* or ceramidases (*YPC1* and *YDC1*) was measured in BY4741, *ncr1*Δ, *sit4*Δ and *ncr1*Δ*sit4*Δ cells transformed with a LacZ reporter under the control of the respective promoters. The analysis of β-galactosidase activity showed that the expression of the genes encoding for the ceramide synthases Lac1p and Lag1p increased in *ncr1*Δ cells when compared to parental cells (Figure 8). This increase was mostly noted for *LAG1*, which presented a 10-fold upregulation while *LAC1* expression only increased 2-fold. Importantly, the deletion of *SIT4* in *ncr1*Δ cells decreased the expression of these genes to levels similar to those observed in parental cells.

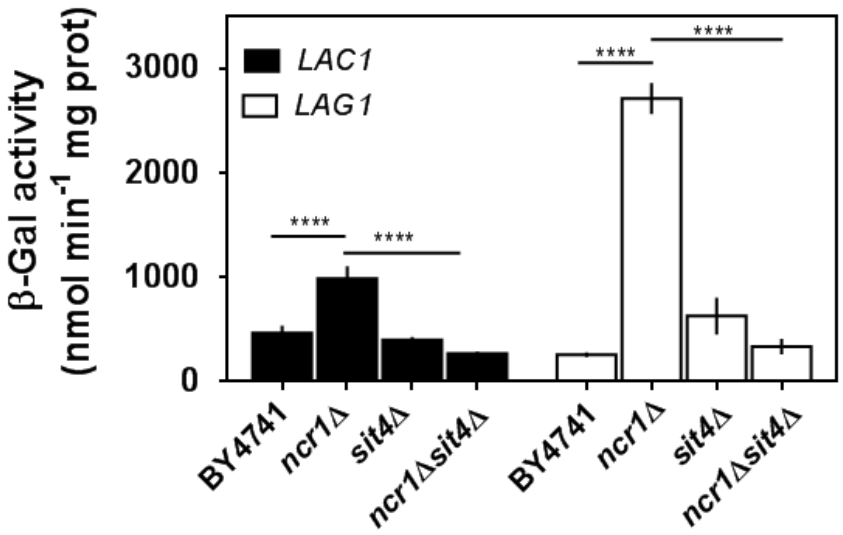

Figure 8 - Expression of ceramide synthase genes are increased in *ncr1*Δ cells by a Sit4p-dependent mechanism. *S. cerevisiae* BY4741, *ncr1*Δ, *sit4*Δ and *ncr1*Δ*sit4*Δ cells carrying LacZ reporter fusions with the promoters of *LAG1* or *LAC1* in the multicopy plasmid YEp357 were grown to log phase (OD_{600}=0.6) in SC-medium lacking uracil. β-Galactosidase activity was measured spectophotometrically, as described in methods. Results are mean ± SD values of at least three independent experiments. ****p*<0.0001

The expression of the genes encoding for the ceramidases Ypc1p and Ydc1p also increased in Ncr1p deficient cells (Figure 9), although not so significantly as for *LAG1* gene. Indeed, *YPC1* and *YDC1* were upregulated 5- and 4-fold, respectively, in

*ncr1*Δ cells when compared to parental cells. The deletion of *SIT4* in *ncr1*Δ cells also suppressed these changes as β-galactosidase activity in *ncr1*Δ*sit4*Δ double mutants was similar or even lower to wild type levels.

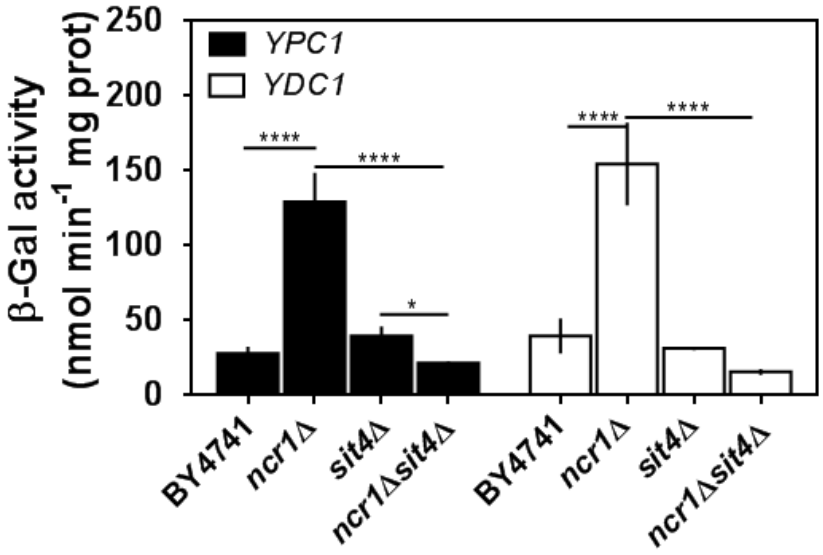

Figure 9 - Expression of ceramidase genes are increased in *ncr1*Δ cells by a Sit4p-dependent mechanism. *S. cerevisiae* BY4741, *ncr1*Δ, *sit4*Δ and *ncr1*Δ*sit4*Δ cells carrying LacZ reporter fusions with the promoters of *YPC1* or *YDC1* in the multicopy plasmid YEp357 were grown to log phase (OD_{600}=0.6) in SC-medium lacking uracil. B-Galactosidase activity was measured spectophotometrically, as described in the methods section. Results are mean ± SD values of at least three independent experiments. *p<0.05, ***p<0.0001

Taken together, these results suggest an increase in the expression of both ceramidases and ceramide synthases in *ncr1*Δ cells, in a Sit4p dependent manner. This indicates that sphingolipid dynamics are altered in these cells. A higher activity of these enzymes may very well be the underlying cause behind the altered sphingolipid levels in the yeast model of NPC1.

4.1.2. Modulation of sphingolipid metabolism in *ncr1*Δ cells

The upregulation of ceramide synthase and ceramidase genes prompted us to investigate the role of these enzymes in the phenotypes presented by *ncr1*Δ cells. For that, we deleted those genes in Ncr1p deficient cells and assessed its impact on mitochondrial function by assessing its capacity to grow on media containing glycerol (non-fermentable carbon source). We first deleted *LAG1*, since the expression of this gene presented the most notorious increase in *ncr1*Δ cells and, therefore, Lag1p could have a major contribution to the accumulation of ceramides in this mutant (Vilaça et al. unpublished). Also, the deletion of this gene is known to increase chronological life-span in yeast cells, which is closely related to mitochondrial function (D'mello,

Childress et al. 1994). To monitor respiratory capacity, BY4741, *ncr1*Δ, *lag1*Δ and *ncr1*Δ*lag1*Δ cells were grown in SC-glucose medium to exponential phase, diluted to and OD_{600}=0.1 and fivefold dilutions were plated on SC-Glucose and SC-Glycerol plates. BY4741 and *lag1*Δ cells, but not *ncr1*Δ cells, were able to grow in glycerol plates (Figure 10). However, *ncr1*Δ*lag1*Δ cells did not grown in this medium, suggesting that the deletion of *LAG1* does not restore the respiratory capacity in cells lacking Ncr1p. Similar results were obtained with *ncr1*Δ*ydc1*Δ cells (Figure 11) as well as *ncr1*Δ*ydc1*Δ*ypc1*Δ triple mutants (data not shown).

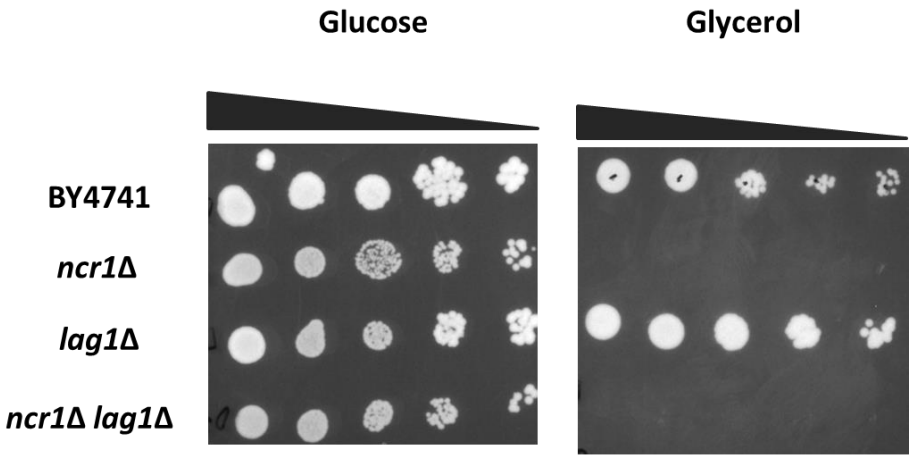

Figure 10 - Deletion of *LAG1* in *ncr1*Δ cells does not restore mitochondrial function. Yeast cells were grown overnight to log phase in SC-glucose medium, then diluted to an OD_{600}=0.1 and fivefold serial dilutions were spotted in SC-glucose or SC-glycerol plates.

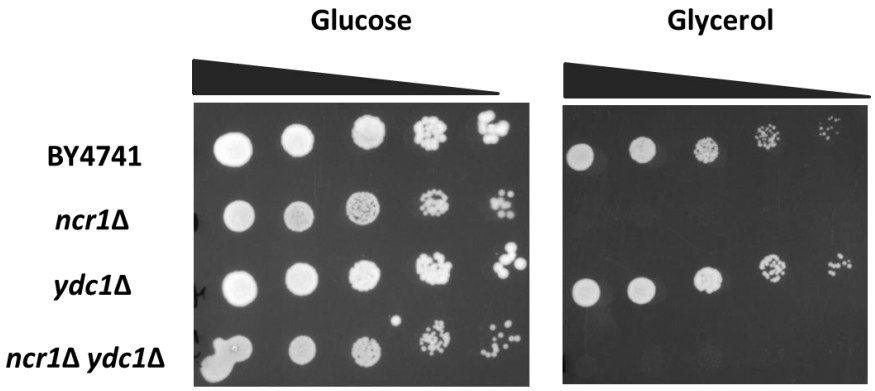

Figure 11 - Deletion of *YDC1* in *ncr1*Δ cells does not restore mitochondrial function. Yeast cells were grown overnight to log phase in SC-glucose medium, then diluted to an OD_{600}=0.1 and fivefold serial dilutions were spotted in SC-glucose or SC-glycerol plates.

Myriocin is an inhibitor of serine-palmitoyl transferase, an enzyme that catalyzes the first step in the *de novo* synthesis of sphingolipids (Miyake, Kozutsumi et al. 1995). It was recently reported that myriocin was capable of increasing yeast chronological lifespan, through the inhibition of sphingolipid synthesis and consequent down regulation of the Pkh1/2p-Sch9p pathway (Huang, Liu et al. 2012, Liu, Huang et al. 2013). This prompted us to investigate the effect of myriocin on the mitochondrial function of *ncr1*Δ cells. For that, cells were grown on SC-glucose and SC-glycerol plates supplemented with 350 ng/ml myriocin. The results show that the inhibition of SPT and consequent lowering of sphingolipid burden in *ncr1*Δ cells was not capable of restoring mitochondrial function (Figure 12).

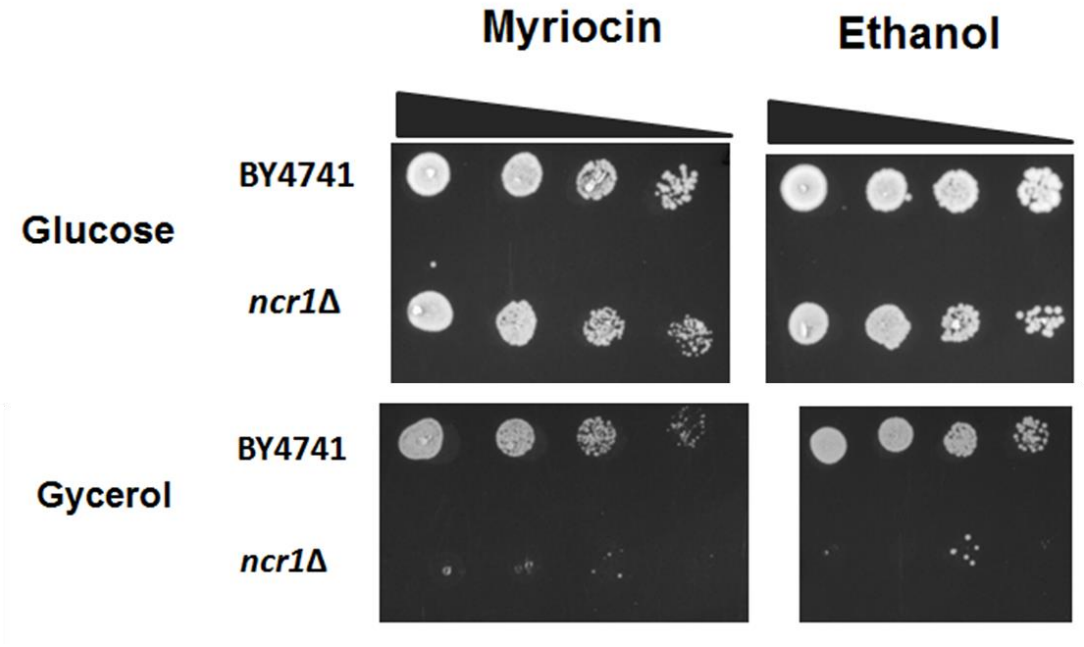

Figure 12 - Inhibition of SPT does not improve the mitochondrial function of *ncr1*Δ cells. Yeast cells were grown overnight to log phase in SC-glucose medium, then diluted to an OD_{600}=0.1 and fivefold serial dilutions were spotted in SC-glucose or SC-glycerol plates containing 350 ng/ml myriocin or equal volume of ethanol (vehicle).

4.2. Involvement of Sit4p in autophagy and Pep4 activity in the yeast model of NPC1

4.2.1. The deletion of *SIT4* in *ncr1*Δ cells restores the autophagic flux

In mammals, NPC1 deficient cells show increased basal autophagy as evidenced by the increased levels of LC3-II (Pacheco, Kunkel et al. 2007). However, there is an impaired degradation of autophagosomes (Sarkar, Carroll et al. 2013). In yeast, *ncr1*Δ cells also present an increased basal autophagic flux as well as an increase in the activity of the vacuolar protease Pep4p (Vilaça et al. unpublished). To investigate whether Sit4p is implicated in the modulation of autophagy in *ncr1*Δ cells, we analysed the processing of GFP-Atg8p in basal conditions and upon treatment with rapamycin to induce autophagy (Yorimitsu, Zaman et al. 2007). Atg8p is an ubiquitin like protein, homolog to mammalian LC3-II, and involved in the formation of autophagosomes. When autophagy is induced, GFP-Atg8p recruited to the phagophore and lipidated with phosphatidylethanolamine to drive autophagosome biogenesis (Kirisako et al., 1999). It is then delivered to the vacuole where Atg8p is degraded by resident vacuolar hydrolases. However, the GFP moiety is relatively resistant to proteolysis, whereby the appearance of free GFP signal is indicative of autophagic flux induction (Shintani and Klionsky, 2004). As previously observed, the basal autophagic flux was increased in *ncr1*Δ cells when compared to parental or *sit4*Δ cells (Figure 13). Treating cells with rapamycin was capable of inducing the autophagic flux in all strains, including *ncr1*Δ cells. In the double mutant *ncr1*Δ*sit4*Δ, the autophagic flux was restored to levels similar to those observed for *sit4*Δ cells. This result implicates Sit4p in the increased autophagic flux of Ncr1p deficient cells.

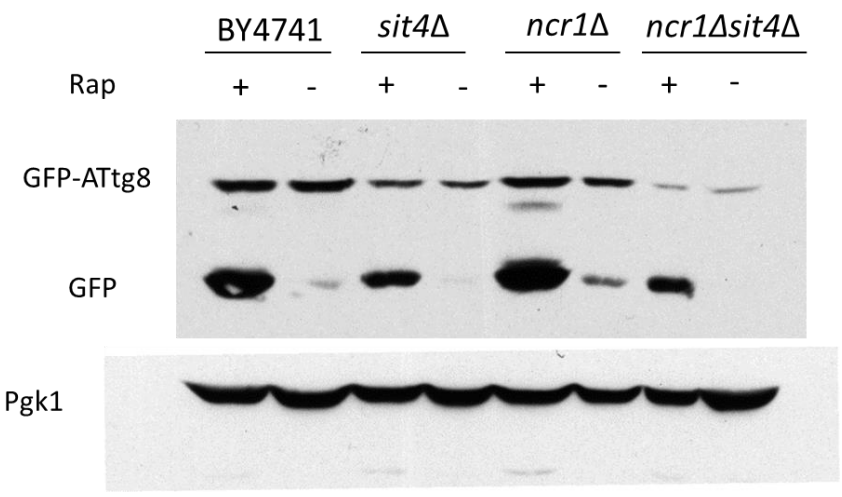

Figure 13 - Analysis of the autophagic flux in BY4741, *ncr1*Δ, *sit4*Δ and *ncr1*Δ*sit4* cells. Cells carrying pRS416-GFP-Atg8 were grown to exponential phase in SC-Glucose medium without uracil and treated with 200 ng/ml rapamycin (Rap) or equal volume of DMSO (vehicle) for three hours. Protein extracts were analyzed by western blot using an anti GFP antibody. One out of three independent experiments is shown.

4.2.2. The deletion of *SIT4* in *ncr1*Δ cells restores normal Pep4p activity

Pep4p is the major vacuole protease in yeast (Woolford, Daniels et al. 1986). It is responsible for the posttranslational activation of percursors of vacuolar hydrolazes and degradation of cargo during autophagy. Cathepsin D, a closely related protease in mammals, is increased in NPC cells (German, Liang et al. 2002), a feature found to be conserved in the yeast model for this disease (Vilaça et al. unpublished). To assess the involvement of Sit4p in the deregulation of Pep4 in *ncr1*Δ cells, we measured its activity in *ncr1*Δ, *sit4*Δ and *ncr1*Δ*sit4*Δ cells grown to exponential and PDS phase.

In all strains, Pep4p activity increased during growth from exponential to PDS phase (Figure 14). As previously reported, *ncr1*Δ cells presented an increased Pep4p activity when compared to parental cells, particularly at the exponential phase (3-fold). Notably, the deletion of *SIT4* in *ncr1*Δ cells decreased significantly Pep4p activity to the levels observed in parental and *sit4*Δ cells. The overall results implicate the ceramide activated protein phosphatase Sit4p in basal autophagy induction and Pep4p activation in Ncr1p deficient cells.

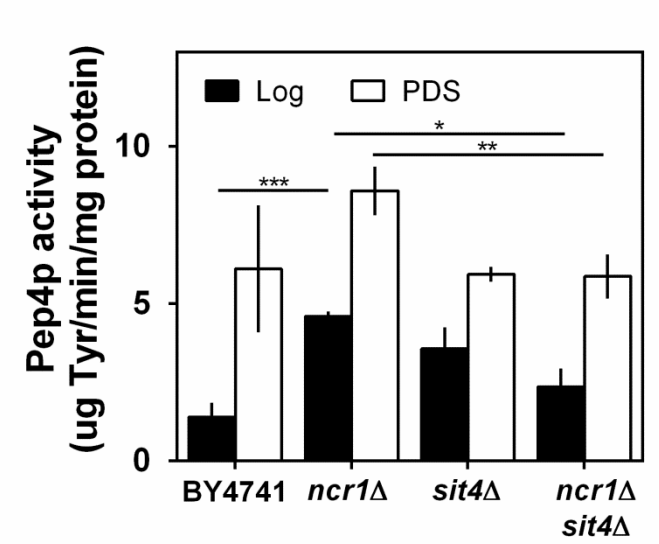

Figure 14 - Pep4p activity is increased in *ncr1*Δ cells by a Sit4p-dependent mechanism. Yeast cells were grown on YPD medium to exponential (log) or post-diauxic shift (PDS) phase. Pep4p activity was measured spectrophometrically as described in methods. Results are mean ± SD values of at least three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001

4.3. Role of Tor1p in the oxidative stress resistance and chronological lifespan of *ncr1*Δ cells.

4.3.1. TORC1 downregulation suppresses the hydrogen peroxide sensitivity of *ncr1*Δ cells

TORC1 is a Ser/Thr protein kinase in yeast and conserved in many other eukaryotic organisms. It senses nutritional cues and regulates cellular growth as well as anabolic processes, like protein synthesis, ribosome biogenesis, among others (Loewith and Hall 2011). It also regulates transcription factors involved in the expression of stress response genes, like Gis1p and Msn2/4p, through the protein kinase Rim15p (Wanke, Pedruzzi et al. 2005, Wei, Fabrizio et al. 2008). In fact, the modulation of this pathway is known to increase longevity not only in mammals but also in yeast (Bonawitz, Chatenay-Lapointe et al. 2007, Pan, Schroeder et al. 2011, Pan and Shadel 2009). In the regulation of aging and mitochondrial function in yeast, TORC1 functions upstream of Sch9p, activating it through the phosphorylation of its C-terminal (Urban, Soulard et al. 2007). It also regulates Sit4p, by phosphorylating Tip41p/Tap42p which then interact with Sit4p and inhibit its function (Di Como and Arndt 1996). TOR is also an established inhibitor of autophagy (Wullschleger, Loewith

et al. 2006, Nakatogawa, Suzuki et al. 2009), which is deregulated in *ncr1*Δ cells. Moreover, previous studies implicate Sch9p in the mitochondrial dysfunction, oxidative stress sensitivity and shortened lifespan of *ncr1*Δ cells (Vilaça et al., 2014). Since TORC1 is required for full activation of Sch9p (Urban, Soulard et al. 2007), we assessed how TORC1 downregulation affects *ncr1*Δ phenotypes.

First, we analysed hydrogen peroxide resistance in BY4741, *ncr1*Δ, *tor1*Δ and *ncr1*Δ*tor1*Δ cells, as well as in BY4741 and *ncr1*Δ cells treated with rapamycin for the pharmacological inhibition of TORC1. The results show that, as previously reported (Vilaça, Silva et al. 2014), *ncr1*Δ cells were more sensitive to hydrogen peroxide than parental cells (Figure 15). Notably, the deletion of *TOR1* in *ncr1*Δ cells increased cellular viability (4-fold) to the levels observed in parental and *tor1*Δ cells. Consistently, the pharmacological inhibition of TORC1 using rapamycin had a protective effect similar to the observed upon *TOR1* deletion. However, the oxidative stress resistance of *ncr1*Δ cells treated with rapamycin was significantly lower to that of parental cells treated with this drug.

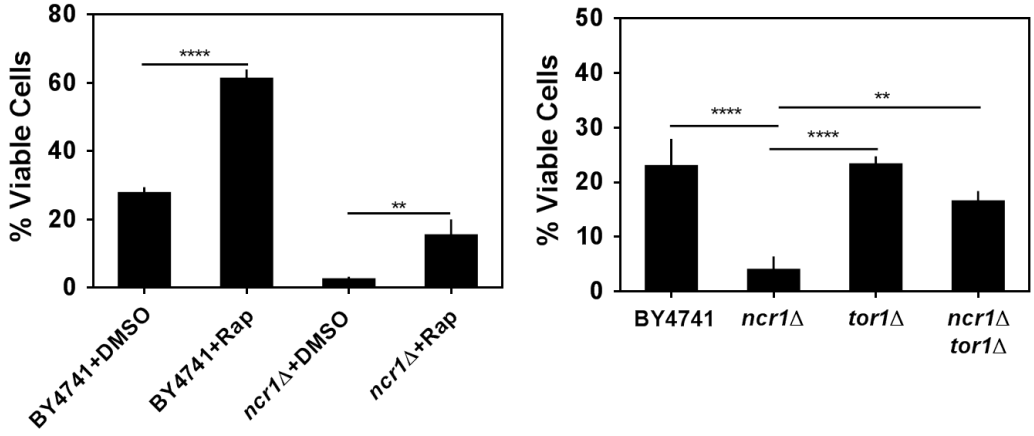

Figure 15 - Deletion of *TOR1* or rapamycin treatment increases hydrogen peroxide resistance in *ncr1*Δ cells. (A) Cells were grown in SC-Glucose medium to earl-exponential (OD_{600}=0.3), treated with 200 ng/ml rapamycin or equal volume of DMSO (vehicle) for three hours, and then for one hour with 1.5 mM H_2O_2. (B) Cells were grown to exponential phase (OD=0.6) and treated with 1.5 mM H_2O_2 for one hour. Results are mean ± SD values of at least three independent experiments. ***p*<0.01, *****p*<0.0001

4.3.2. Deletion of TOR1 restores chronological lifespan in *ncr1*Δ cells

Yeast is a well established model for studying both replicative and chronological aging (Longo and Shadel 2012). In yeast, CLS represents the time a nondividing cell survives in stationary phase. Nutrient sensing pathways, including the Tor1p-Sch9p

pathway, are known to be involved in the regulation of yeast lifespan through the modulation of mitochondrial function. In fact, the downregulation of the TOR pathway in yeast has been reported to increase CLS (Bonawitz, Chatenay-Lapointe et al. 2007, Pan and Shadel 2009, Pan, Schroeder et al. 2011).

Previously, it was reported that the yeast model of NPC1 presents a reduced CLS when compared to parental cells (Vilaça et al, 2014). The deletion of *TOR1* in *ncr1*Δ cells suppresses mitochondrial dysfunction (Vilaça et al. unpublished) and the oxidative stress sensitivity (this study) of this mutant. To investigate the role of Tor1p in aging of *ncr1*Δ cells, we assessed the CLS of BY4741, *ncr1*Δ, *tor1*Δ and *ncr1*Δ*tor1*Δ cells. Consistent with published data, CLS increased in *tor1*Δ cells comparing with parental cells. Importantly, the results show that *TOR1* deletion also increased life span in Ncr1p deficient cells (Figure 16). In fact, the double mutant cells had a lifespan similar to the observed in *tor1*Δ cells and, therefore, higher to that of parental cells. This result implicate TORC1 in the premature aging of *ncr1*Δ cells.

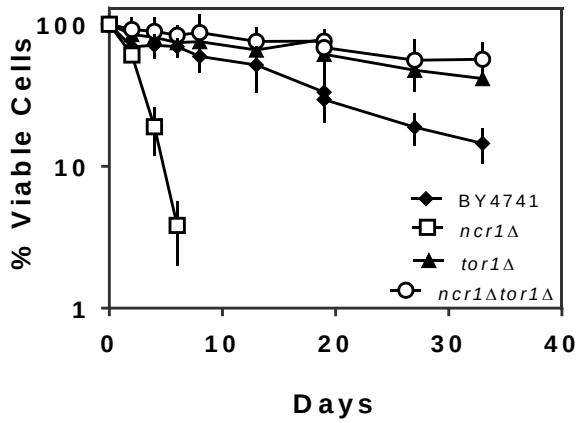

Figure 16 - Deletion of *TOR1* in *ncr1*Δ cells increases chronological lifespan. Overnight cultures were diluted to an OD_{600}=0.6 in SC-glucose, grown for 24 hours until PDS phase and then kept in the medium overtime. Cellular viability was expressed as the percentage of colony forming units, in relation to t0. Results are mean ± SD values of at least three independent experiments.

CHAPTER 5

DISCUSSION

Niemann-Pick type C is an autosomal hereditary disease, characterized by an acute accumulation of low density lipoprotein derived cholesterol, in late endosomes/lysosomes. This affects both the central nervous system and systemic organs like spleen and liver in patients with a wide range of ages. Cells of these patients also accumulate other classes of lipids, such as phospholipids, gangliosides and sphingolipids (Vanier 2010). Sphingolipids are bioactive compounds that function as signaling molecules, besides their structural roles in biological membranes. They regulate apoptosis, cell senescence but also proliferation and cell cycle (Hannun and Obeid 2008). NPC1 cells accumulate sphingosine, which causes alterations in calcium homeostasis and in the storage of other lipids (Loyd, Morgan et al. 2008). NPC1 has an orthologue in yeast, namely Ncr1p, a protein localized in the yeast vacuole, the equivalent to lysosomes in mammalian cells (Zhang, Ren et al. 2004). The deletion of *NCR1* in yeast also causes the accumulation of LCBs that activate the Pkh1/2p-Sch9p pathway, leading to mitochondrial dysfunction, oxidative stress sensitivity and a decrease in lifespan (Vilaça, et al 2014).

Our lab has previously shown that specific ceramide species, in particular C_{14}- to C_{20}-phytoceramides, also accumulate in *ncr1*Δ cells in both exponential and post diauxic shift phase (Vilaça et al, unpublished). A *Toxoplasma* model of NPC1, where the gene encoding for TgNCR1 is deleted, also shows increased sphingolipid levels, particularly ceramides (Lige, Romano et al. 2011). Increased ceramide levels can induce cell death in yeast (Nagiec, Nagiec et al. 1997). Ceramides are activators of Sit4p, a protein phosphatase related to type 2A family of protein phosphatases, causing mitochondrial dysfunction and a shortened lifespan (Barbosa, Osorio et al. 2011). This complex consists also of Cdc55p and Tpd3p (Joseph T. Nickels 1996). Notably, Sit4p is activated in *ncr1*Δ cells and *SIT4* deletion leads to restoration of mitochondrial function and chronological lifespan in Ncr1p-deficient cells (Vilaça et al. unpublished). These results suggest that sphingolipid metabolism is altered in these cells.

In this study, we used a β-galactosidase reporter gene to analyze promoter activity of genes encoding for ceramide synthases (*LAC1* and *LAG1*) and ceramidases (*YPC1* and *YDC1*). Our results indicate that all these genes are upregulated in *ncr1*Δ cells, with *LAG1* having the most noticeable increase (10-fold). For all cases, Sit4p was apparently involved since *SIT4* deletion abolished the induction of these genes in *ncr1*Δ cells. These results suggest that Sit4p, besides being activated by ceramide, regulates the metabolism of sphingolipids. This is supported by a recent study showing that *SIT4* deletion leads to a downregulation of ceramide synthesis (Woodacre, Lone et

al. 2013). Whether changes in the expression of the ceramide synthases and ceramidase genes are associated with a higher activity of these enzymes remains to be determined.

The changes in the sphingolipid dynamics in *ncr1*Δ cells led us to postulate that an increase in the synthesis of ceramide species by ceramide synthases, leading to Sit4p activation, and its turnover by ceramidases, ultimately leading to LCBs accumulation and Pkh1/2p activation, may contribute to *ncr1*Δ phenotypes (Figure 17). Indeed, changes in sphingolipid metabolism are known to affect oxidative stress and lifespan in yeast (Jiang, Kirchman et al. 2004, Barbosa, Osorio et al. 2011). Genes involved in sphingolipid metabolism have been shown to modulate of lifespan, namely *LAG1*, since its deletion appears to extend CLS in yeast (D'mello, Childress et al. 1994). However, downregulation of these enzymes did not improve the mitochondrial function in *ncr1*Δ cells. Since the accumulation of both LCBs and ceramides can be toxic to cells, the deletion of ceramide synthase and ceramidase genes may not be sufficient to suppress *ncr1*Δ phenotypes as it may further increase LCBs and ceramide levels, respectively. Regarding ceramide, its accumulation may also be due to a higher activity of Isc1p, an orthologue of mammalian sphingomyelinase that catalyzes the degradation of inositol phosphoinositolsphingolipids, releasing ceramide (Saway, Okamoto et al. 2000). More studies are required to assess the role of Isc1p in *ncr1*Δ phenotypes.

The levels of LCBs are a balance between its formation (by de novo biosynthesis or through the action of ceramidases) and its phosphorylation mediated by LCB kinases such as Lcb4p, the major kinase responsible for the synthesis of LCB-1-phosphates in yeast (Nagiec, Skrzypek et al. 1998). This protein is activated by phosphorylation, a process regulated by the sterol composition (Nagiec, Skrzypek et al. 1998). Since Ncr1p deficient cells present alterations in ergosterol homeostasis (Vilaça et al, 2014), Lcb4p activity may be compromised in these cells. Also, LCB-1-phosphates are known to counteract the pro-apoptotic effects of sphingosine and ceramide (Strub, Maceyka et al. 2010), although its accumulation can also be toxic in yeast (Kim, Fyrst et al. 2000). Thus, the overexpression of Lcb4p may have protective effects in *ncr1*Δ cells, by decreasing the “pool” of toxic LCBs. Whether Lcb4p is deregulated in this mutant remain to be clarified.

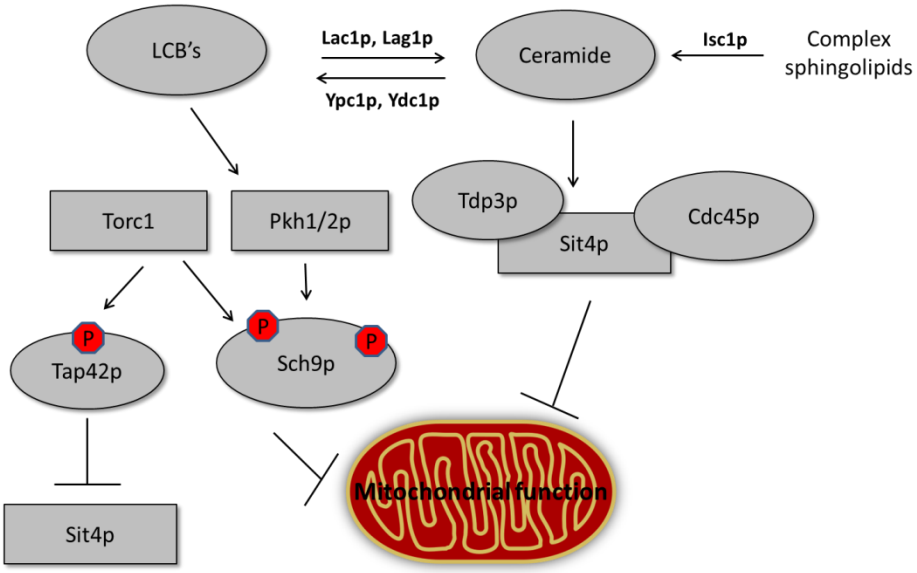

Figure 17 - Interplay between ceramide species and LCBs in yeast. In *ncr1*Δ cells, both LCBs and ceramide species may induce mitochondrial dysfunctions, through activation of the Pkh1/2p-Sch9p pathway and Sit4p, respectively.

The modulation of cell signaling pathways that regulate overall sphingolipid metabolism may be required to properly restore the sphingolipid rheostat and mitochondrial function in *ncr1*Δ cells. Ceramide synthases are regulated by TORC2 and calcineurin (Dickson 2008). Sensing growth signals, TORC2 phosphorylates the AGC kinase Ypk2p (Kamada, Fujioka et al. 2005), which then activates ceramide synthase (Aronova, Wedaman et al. 2008). Ypk2p activation also requires its phosphorylation by Pkh1/2p (Dickson 2008), which is known to be activated in *ncr1*Δ cells (Vilaça et al, 2014). It would be interesting in the future to analyze the phosphorylation status of Ypk2p and its role in the modulation of ceramide synthesis in *ncr1*Δ cells.

In mammals, sphingolipids also play a role in autophagy regulation (Li, Li et al. 2014). This is a conserved process in eukaryotic cells used to recycle damaged organelles or long-lived proteins (Klionsky and Emr 2000). Ceramide for example induces macroautophagy via upregulation of Beclin 1, an homolog to yeast Atg6p, and inhibition of protein kinase B (Scarlatti, Bauvy et al. 2004). Basal autophagy, as well as lysosomal proteases like cathepsin D, are known to be increased in NPC1 cells (Amritraj, Wang et al. 2013, German, Liang et al. 2002). However, Cathepsin B activity is lower and autolysosome turnover is impaired due to lipid accumulation (Elrick, Yu et al. 2012). In *ncr1*Δ cells, both autophagic flux and Pep4p activity are increased (Vilaça et al. unpublished). However, the mechanisms underlying autophagy induction were still elusive. Our data implicate Sit4p in the increased basal autophagic flux in *ncr1*Δ cells. Consistently, *SIT4* deletion in *ncr1*Δ cells also diminished Pep4p activity in cells

grown to both exponential and PDS phase. However, there is still a lot to uncover regarding the mechanisms involved in the deregulation of autophagy in these mutants. It is known that subunits of protein phosphatases type 2A regulate positively the transcription of autophagy related genes, like *ATG14*, through modulation of transcription factors like Gln3p (Cebollero and Reggiori 2009). This may explain the downregulation of autophagic flux in cells lacking Sit4p, the catalytic subunit of PP2A in yeast. Myriocin is also a known inducer of autophagy through the down regulation of TORC1 activity (Liu, Huang et al. 2013) and in mammalian cells, autophagy induction leads to an accumulation of lipids in the lysosomes (Elrick, Liu et al. 2012). Thus, the treatment with myriocin may not have a beneficial effect by further enhancing the autophagic flux and lipid accumulation.

TORC1 is a negative regulator of autophagy (Wullschleger, Loewith et al. 2006, Nakatogawa, Suzuki et al. 2009) and of Sit4p (Di Como and Arndt 1996), and an activator of Sch9p (Urban, Soulard et al. 2007). In yeast, this large protein complex functions as a controller of cellular growth, stress response and ribosome biogenesis (Wullschleger, Loewith et al. 2006). TOR is also an established key player in the aging process in several organisms. Treatment of mice with rapamycin, a known inhibitor of the TORC1 pathway, extends lifespan even when the animals are treated at later stages in life (Harrison, Strong et al. 2009). In *Saccharomyces cerevisiae*, deletion of *TOR1* leads to a higher chronological lifespan due to an upregulation of genes encoding mitochondrial proteins, and increased respiration rate (Bonawitz, Chatenay-Lapointe et al. 2007). These mutants also induce an adaptive response to an increase of ROS production during growth at the exponential phase that leads to an enhanced lifespan in stationary phase (Pan, Schroeder et al. 2011). TORC1 downregulation leads to activation of the protein kinase Rim15p, which promotes the translocation of the transcription factors Msn2/4p and Gis1p into the nucleus. As consequence, the expression of stress response genes increases and cells accumulate trehalose and glycogen, increasing cell survival under nutrient starvation and during aging in yeast (Wullschleger, Loewith et al. 2006).

In this study, we analyzed hydrogen peroxide resistance of *ncr1*Δ cells deleted for *TOR1* or treated with rapamycin. Our results show that both suppressed the sensitivity of *ncr1*Δ cells to hydrogen peroxide. Consistent with this, we also observed a significant increase in chronological lifespan in *ncr1*Δ*tor1*Δ cells. The mitochondrial function is also restored in these double mutants, as shown by their capacity to grow on non-fermentable substrates and by the increase of oxygen consumption rate to parental levels (Vilaça et al, unpublished). The overall data implicate the TOR1

pathway in the defective stress response and mitochondrial function of *ncr1*Δ cells, leading to a shortened lifespan. The mechanism underlying the protective effect of *TOR1* deletion remains to be elucidated. TORC1 signaling is, in part, mediated by phosphorylation of Sch9p in its C-terminal domain (Urban, Soulard et al. 2007). Previous studies have shown that Sch9p-Thr570 phosphorylation in its activation loop increases in *ncr1*Δ cells due to activation of Pkh1p in response to the accumulation of LCBs (Vilaça et al, 2014). Thus, it could be that a higher Tor1p activity contributes to the phenotypes presented by this mutant or TORC1 downregulation attenuates the deleterious effects of Sch9p activation, including mitochondrial dysfunction (Figure 17). Since TORC1 is a negative regulator of the expression of subunits of the respiratory chain in mitochondria (Bonawitz, Chatenay-Lapointe et al. 2007), its activation may impair the capacity of *ncr1*Δ cells to respire. The analysis of Sch9p phosphorylation in the C-terminal mediated by TORC1, as previously described (Urban, Soulard et al. 2007), could give us an indication of changes in the activity of this protein complex in *ncr1*Δ cells.

Taken together, these results implicate Sit4p and TORC1 in the mitochondrial dysfunctions and reduced lifespan presented by Ncr1p deficient cells. Also, this work gives further insight regarding the molecular mechanisms behind sphingolipid homeostasis, autophagy, stress resistance and lifespan in the yeast model of NPC1. Being yeast a valuable scientific tool, with metabolic pathways conserved in mammals, data from this work can further help to understand the NPC1 pathology

CHAPTER 6

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