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# The role of eisosomes and calcineurin in a yeast model of Niemann-Pick type C1

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

O Presidente do Júri,

Porto, _____/_____/_____

*“The meaning of life is to find your gift.*
*The purpose of life is to give it away”*

David Viscott

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To Carolina because people like her don’t come so often in life.

# Abstract

The Niemann-Pick type C1 (NP-C1) disease is a rare and recessively inherited neurodegenerative disease that onsets mostly in children from 2-15 years of age due to cholesterol traffic defects and sphingolipid accumulation in late endosomes and lysosomes. These alterations result from defects in endocytic traffic caused by loss-of-function point mutations in NPC1, an endolysosomal protein that is linked to the vesicular transport of cholesterol and other lipids out of lysosomes. The cellular mechanisms that result from the imbalance in lipid homeostasis and that promote cellular function decline in NP-C1 are undetermined. We questioned the importance of sphingolipids in the expression of the cellular phenotypes of Niemann-Pick type C1 due to their role in endocytosis, cell senescence and lysosomal calcium alterations.

*Saccharomyces cerevisiae* lacking *NCR1* (*ncr1*Δ), the yeast ortholog of *NPC1*, has been used as a model for NP-C1 and presents severe mitochondrial defects and a shortened life span by a mechanism dependent of Pkh1-Ypk1 signalling. Consistent with Ypk1 activation, the abundance of simple sphingolipids (sphingosines and ceramides) is increased in *ncr1*Δ cells similarly to NP-C1 cells. Moreover, previous results implicated calcineurin activation, a calcium-activated phosphatase involved in complex sphingolipid synthesis, in the sensitivity of *ncr1*Δ to calcium, which is consistent with lysosomal calcium alterations in this disease. The purpose of this thesis was to characterize how eisosomes, regulators of Pkh1-Ypk1 signalling, and Ypk1 impact the regulation of sphingolipid synthesis and in the cellular death phenotypes of NPC1. Moreover, we investigated the activation of calcineurin and its role in the mitochondrial function of ncr*1*Δ cells.

Our results demonstrate that Pil1, an eisosome subunit, is necessary for Pkh1-Ypk1 signalling in *ncr1*Δ cells. Moreover, *PIL1* deletion rescued mitochondrial respiration defects and the shortened chronological lifespan of *ncr1*Δ cells. Also, preliminary data suggest that the expression of the ceramide synthase subunit Lag1 is increased by an Ypk1-independent mechanism and that it localizes in punctuate structures in the endoplasmic reticulum of *ncr1*Δ. On the other hand, the calcineurin-Crz1 pathway was activated in *ncr1*Δ but had no contribution for the mitochondrial defects of the yeast model for NP-C1.

The overall results suggest that stability of eisosomes is an important upstream regulator of Pkh1-Ypk1 signalling that contribute to the mitochondrial and life span defects of *ncr1*Δ cells. With the generalized accumulation of sphingosine and ceramide species in a yeast model for NP-C1, the increased expression and the altered subcellular

localization of Lag1 goes hand in hand with the possible activation of calcineurin and its role in complex sphingolipid synthesis to suggest that the yeast model of NP-C1 has an alteration in the metabolic flux associated to sphingolipid synthesis.

**Keywords:** Niemann-Pick type C1, sphingolipid-mediated signalling pathways, *S. cerevisiae,* eisosomes, calcineurin, calcium, Crz1, mitochondrial disease, Ypk1, Pkh1, Lag1, Pil1,

# Resumo

A doença de Niemann-Pick tipo C1 (NP-C1) é uma doença neurodegenerativa rara que é herdada de forma recessiva e que afeta sobretudo crianças dos 2-15 anos de idade por causa de defeitos no transito de colesterol e de acumulação de esfingolípidos nos endossomas tardios e lisossomas de células humanas. Estas alterações na via endocítica são causadas por mutações pontuais de perda de função na proteína NPC1, uma proteína endolisossomal que está ligada ao transporte vesicular de colesterol e outros lípidos para fora dos lisossomas. Pouco se sabe sobre os mecanismos moleculares que contribuem para o declínio celular nesta doença. Neste sentido, questionamo-nos sobre o papel dos esfingolípidos na expressão dos fenótipos celulares da doença de NP-C1, devido ao facto destas moléculas estarem associadas a eventos de endocitose, envelhecimento celular e alterações da homeostasia do cálcio lisossomal.

A levedura *Saccharomyces cerevisiae* com o gene ortólogo *NCR1* deletado (*ncr1*Δ), tem sido usada como modelo para a doença de NP-C1, apresentando defeitos mitocondriais severos e uma redução na sua longevidade de vida celular por mecanismos dependentes da via de sinalização Pkh1-Ypk1. Consistente com a ativação da Ypk1, o modelo *ncr1*Δ apresenta uma acumulação de esfingolípidos simples (esfingosinas e ceramidas), evento que é conservado nas células dos doentes. Resultados anteriores demonstraram que a ativação da calcineurina, uma fosfatase envolvida na síntese de esfingolípidos complexos ativada por cálcio, estaria envolvida na sensibilidade do modelo *ncr1*Δ ao stress mediado por cálcio, consistente com possíveis alterações do cálcio lisossomal descrito na doença humana. O propósito desta tese será entender como os eisossomas, reguladores da via de sinalização Pkh1-Ypk1, e a Ypk1 afetam a regulação da síntese de esfingolípidos e os fenótipos de morte celular da doença NP-C1. Também será investigada a ativação da calcineurina e o seu papel na regulação da função mitocondrial no modelo *ncr1*Δ.

Os nossos resultados demonstram que o Pil1, uma subunidade dos eisossomas, é necessária para a ativação da via Pkh1-Ypk1 no modelo *ncr1*Δ. Consistentemente, a deleção do gene *PIL1* reverteu os defeitos na respiração mitocondrial e consequente redução da longevidade de vida celular do modelo *ncr1*Δ. Verificou-se ainda que a expressão da *LAG1*, subunidade da ceramida sintetase, estava aumentada por um mecanismo independente da Ypk1 e que esta se encontrava em estruturas pontuadas no retículo endoplasmático do *ncr1*Δ. Por outro lado, a via de sinalização calcineurina-

Crz1 está ativada no *ncr1*Δ, mas não parece mediar a função mitocondrial no modelo de levedura para a doença de NP-C1.

No geral, os resultados demonstram que os eisossomas são estruturas que regulam a ativação da via de sinalização Pkh1-Ypk1 no modelo de levedura para a doença de NP-C1. Com a acumulação generalizada de espécies de esfingosina e ceramida nestas células, o aumento da expressão da proteína Lag1 e a alteração da sua localização subcelular é consistente com uma possível ativação da calcineurina e o seu papel na síntese de esfingolípidos complexos para sugerir que este modelo tem uma alteração no fluxo metabólico associado à síntese de esfingolípidos.

**Palavras-chave:** Niemann-Pick tipo C1, sinalização mediada por esfingolípidos, *S. cerevisiae,* eisosomas, calcineurina, cálcio, Crz1, doença mitocondrial, Ypk1, Pkh1, Lag1, Pil1.

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| | |
|---|---|
| **3-KDHS** | 3-ketodehydrosphingosine |
| **(m)TORC2** | (mammalian) Target of Rapamycin Complex-2 |
| **ATP** | Adenosine triphosphate |
| **CDRE** | Calcineurin-dependent responsive element |
| **CerS** | Ceramide Synthase |
| **CK2** | Casein Kinase 2 |
| **CLS** | Chronological life span |
| **DHC** | Dihydroceramide |
| **DHS** | Dihydrosphingosine |
| **DHS-1-P** | Dihydrosphingosine-1-phosphate |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxy(Nucleotide) triphosphate, where nucleotide can be adenine, thymine, cytosine or guanine. |
| **ER** | Endoplasmic reticulum |
| **GFP** | Green fluorescent protein |
| **IPC** | Inositolphosphoceramide |
| **LCB** | Long chain base |
| **LSD** | Lysosomal Storage Disease |
| **M(IP)2C** | Mannosediinositolphosphoceramide |
| **MCT** | Membrane Compartments of TORC2 |
| **MIPC** | Mannoseinositolphosphoceramide |
| **MM** | Minimal medium |
| **mRNA** | Messenger Ribonucleic Acid |
| **Ncr1** | Niemann-Pick type C1-related protein 1 |
| **NP-C1/2** | Niemann-Pick type C1 or C2 disease |
| **OD** | Optical density |
| **PBS** | Phosphate buffer saline |
| **PCR** | Polymerase chain reaction |
| **PDS** | Post-diauxic shift |
| **PH** | Pleckstrin Homology |
| **PHC** | Dihydroceramide |
| **PHS** | Phytosphingosine |
| **PHS-1-P** | Phytosphingosine-1-phosphate |
| **PI3K** | Phosphoinositide-3-kinase |
| **PM** | Plasma membrane |

| | |
|---|---|
| **PtdIns4,5P2** | Phosphatydilinositol-4,5-biphosphate |
| **S-1-P** | Sphingosine-1-Phosphate |
| **SC-glucose** | Synthetic Complete drop-out medium with glucose |
| **SC-glycerol** | Synthetic Complete drop-out medium with glycerol |
| **SDS-PAGE** | sodium dodecyl sulfate polyacrilamide gel electrophoresis |
| **SPT** | Serine Palmitoyl-CoA transferase complex |
| **SSD** | Sterol sensing domain |
| **TFEB** | Transcription Factor EB |
| **YPD** | Yeast extract,Peptone and Dextrose |

# Chapter 1

# INTRODUCTION

# 1.1. Overview of the Niemann-pick type C disease – from genes to disease.

## 1.1.1. Definition, epidemiology and diagnosis

The Niemann-Pick type C (NP-C) diseases are progressive autosomal recessive disorders that are caused by mutations in either the *NPC1* (OMIM#257220) or *NPC2* (OMIM#607625) genes. Mutations in either gene present very similar alterations in endosomal-lysosomal trafficking and lead to the accumulation of a plethora of lipids like sphingosines, glycosphingolipids, sphingomyelins and low-density lipoprotein-derived cholesterol species [1-3]. As such, NP-C are considered Lysosomal Storage Diseases (LSD), a group of complex disorders that also include Gaucher, Tay-Sachs and Niemann-Pick type A and B diseases. Differences between the phenotypes of the *NPC1* mutant variants (NP-C1) and the *NPC2* mutant variants (NP-C2) have been difficult to find. It is widely accepted that both variants have very similar NP-C phenotypes because of the almost equal and superimposing clinical symptoms. In line with this, a biochemical marker to discern between variants is unknown.

NP-C is a rare disease with an estimated minimal incidence of 1 to 120.000 births in Europe[4]. Around 95% of cases are perpetrated by *NPC1* variants while the rest originate from *NPC2* variants (around 5%). Life expectancy can vary greatly per case but is dependent on age onset. This also defines the classification of NP-C, from infantile, juvenile and adult forms. Visceral-neurodegenerative forms tend to manifest in an early-infantile period (<2 years), neurodegenerative forms tend to manifest in late-infantile and juvenile periods (2-15 years) while psychiatric-neurodegenerative forms tend to express in periods thereafter. Visceral manifestations are mostly liver related and present themselves as hepatosplenomegaly, cholestatic jaundice or in some cases as acute liver failure[5]. Neurodegenerative signs are observed through progressive cognitive decline (learning disabilities, loss of language skills) and loss of muscle coordination and response (dysphagia,dysarthia,dystonia,ataxia). Psychiatric disorders include psychosis and depression. The most common onset happens between late-infantile and juvenile periods thus explaining why NP-C is commonly denominated as a neurovisceral disorder. Regarding life expectancy, early age onsets have a rapidly progressive and fatal prognosis, while adults tend to live longer with chronic neurodegeneration.

Cholesterol accumulation was one of the first markers identified for NP-C, which could be visualized by filipin staining of cultured patient fibroblasts[3, 6]. Today, this

staining is used in the final steps of the differential diagnosis of the disease but is proceeded after *NPC1* and *NPC2* sequencing and detection of plasma biomarkers like elevated cholestane-triol and lysosphingomyelin-509 with a normal or slightly elevated lysosphingomyelin (Figure 1)[5]. Other biomarkers like 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholen-24-oic acid and its glycine- and taurine-conjugates in urine or Bis(monoacylglycero)phosphate in liver and spleen tissues of NP-C have been detected and could be of clinically useful in the future[7, 8]. Yet, these biomarkers and the lipid accumulation are associated with the onset of the other Niemann-Pick diseases, namely Niemann-Pick type A and B, and are not suitable for a direct and conclusive clinical diagnosis by themselves.

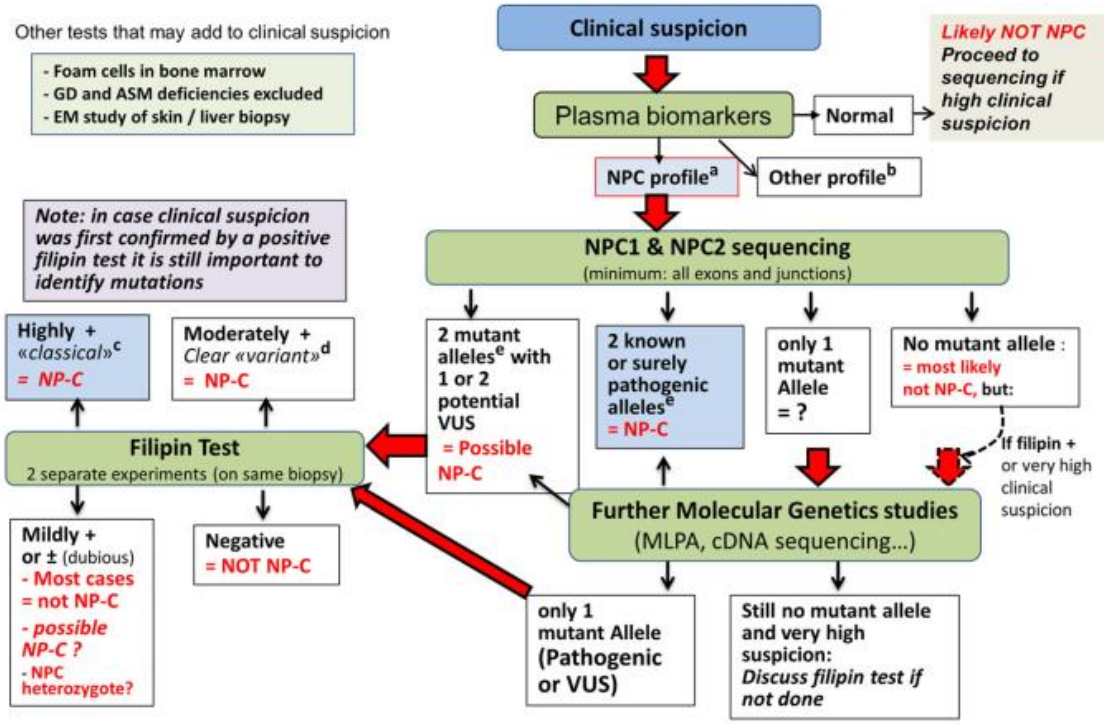


*Figure 1* – **A differential diagnosis diagram for NP-C detection.** As seen in [5]. Abbreviations: GD: Gaucher disease;ASMD: acid sphingomyelinase deficiency; EM: electron microscopy; VUS: variant of unknown significance; MLPA: Multiplex Ligation-dependent Probe Amplification (evaluates copy number changes, allows detection of large deletions or false homozygous status with a deletion on the other allele); lysoSM: lysosphingomyelin. **a** - Elevated cholestane-triol or bile acid derivative and/or lysoSM-509, with normal or slightly elevated lysoSM. **b** - Cholestane-triol also elevated in ASMD, acid lipase deficiency, cerebrotendinous xanthomatosis, certain neonatal cholestasis conditions. All lysoSM analogues and bile acid derivative are elevated in ASMD. **c**- I-cell disease (ML-II and -III) gives a false positive result (very different clinical features). **d** - ASMD can give a similar filipin pattern. **e** - Check allele segregation by parental study or other test.

## 1.1.2. NPC1 - Insights on protein structure and function

The exact function of NPC1 still remains elusive but some functional hints can be expected by looking at protein structure and homology with other similar proteins. NPC1 is a protein with 13 transmembrane segments and a small intramembrane domain homologous to the sterol sensing domain (SSD). The SSD domain is present on other

proteins like the sterol regulatory element-binding protein cleavage-activating protein (SCAP) and the 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR) protein in humans to allow binding to cholesterol. Similarly, The SSD-like domain in NPC1 allows binding to cholesterol and U18666A, a structural mimetic of cholesterol known for its role in the inhibition of intracellular cholesterol transport (Figure 2). Moreover, sorting of NPC1 to late-endosomes/lysosome structures has been shown to involve the SSD domain [9, 10].

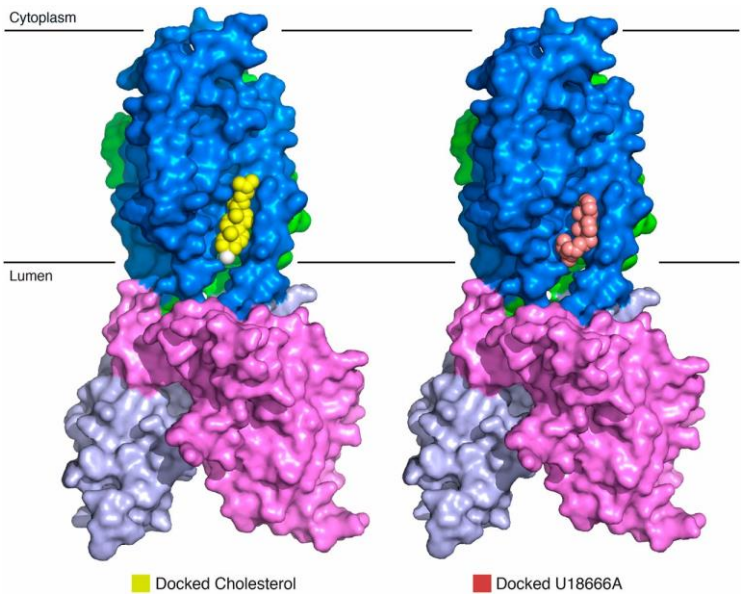


Figure 2 – **Surface representation of NPC1 bound to Cholesterol and U18666A.** . Image adapted from [11], supplemental material.

Besides the importance of the SSD domain in protein targeting, it is also hypothesised that this domain holds key functions in cholesterol egress out of the lysosomes. Cholesterol-bound NPC2 can hand-down cholesterol to NPC1 to promote its insertion into the lysosomal membrane[12, 13]. Interestingly, NPC1 is also capable of handing out cholesterol to NPC2 for its insertion into the lysosomal membrane, hinting for another pathway - redundant to NPC1 transport - for cholesterol insertion into the lysosomal membrane[12, 14-16]. Removal of the SSD domain leads to a cholesterol build up in lysosomes [17].

The NPC1 SSD domain also seems to play a role in subcellular sphingolipid distribution. An epidemiological study on NP-C1 identified that 19.4% of the missense mutations in *NPC1* that express NP-C1 are located in the SSD-like domain[18].Also, mutating the putative SSD domain of the yeast ortholog of NPC1 - *Ncr1* - led to the accumulation and redistribution of sphingolipids to the vacuole and other membrane compartments[19].

Curiously, there are indications that lysosomal cholesterol can regulate NPC1 subcellular location. NPC1 locates mostly to lysosomes but some localization in late-endosomes is observed[20]. In cholesterol laden cells, NPC1 localizes more abundantly in late endosomes through alterations in tubovesicular traffic[17, 21]. Indeed, the mode of action of NPC1 seems to revolve around the insertion on cholesterol into lysosomes and cholesterol surplus promotes the migration of NPC1 to late-endosomes to cope with cholesterol build-up and maintain lysosomal homeostasis. Consistent with a possible regulatory pathway for NPC1 function that is dependent on cholesterol, mRNA-*NPC1* levels increase and decrease when sterol metabolism is downregulated or upregulated, respectively[22, 23].

In a similar fashion to cholesterol-mediated alterations in the organellar location of NPC1, lysosomotrophic amines, like neutral red or imidazole, lead to lysosomal accumulation of NPC1, promoting the fusion of late-endosomes with lysosomes[20]. Efflux of these amines is NPC1-dependent, which suggests that NPC1 may also be important for vesicular endocytic traffic and resistance against toxic agents[17, 20]. In line with this, the human NPC1 amino acid sequence suggests that NPC1 belongs to the Resistance-Nodulation-Division (RND) superfamily of prokaryotic permeases, which are known for their unspecific efflux of various toxic compounds like antibiotics[9, 24, 25].

Resuming, NPC1 is required for proper cholesterol insertion into the lysosomal membrane and to deal with lysosomal accumulation of toxic compounds. With the insertion of cholesterol into the lysosomal membrane by NPC1, vesicular traffic is facilitated and should diffuse cholesterol into other organelles. To deal with lysosomal cholesterol accumulation, a partial result of NPC1 function, NPC1 location is shifted to late-endosomes and the expression of mRNA-*NPC1* levels is downregulated to reduce cholesterol insertion into lysosomes by NPC1 and to maintain cholesterol homeostasis. With toxic compounds, as seen with some lysosomotrophic amines, NPC1 accumulates in lysosomes to favour cholesterol accumulation in this organelle which will promote the diffusion and efflux of these toxic compounds. Loss of normal NPC1 function disables the cell of necessary coping mechanisms to deal with cholesterol accumulation and redistribution of not only toxic compounds but also other lipids like sphingolipids.

### 1.1.3. Role of sphingolipids and calcium signalling in NP-C1 expression

While cholesterol is in the centre of NPC1 function and regulation, its role as the sole metabolite in NP-C1 phenotypes has been challenged. There are other diseases

with cholesterol accumulation that still have a functional NPC1 and also the use of cholesterol reduction therapies has low clinical benefit for NP-C1 patients[26, 27]. Also, cholesterol is still transported out of lysosomes in *NPC1*-null fibroblasts[28, 29]. Thus, other metabolites should be involved in the disease. As referred earlier, an accumulation of simple and complex sphingolipids is observed in NP-C1[30]. Because these lipids are important membrane structure components and known signalling molecules, the role of sphingolipids should be considered in NP-C phenotypical expression (see section 1.2 for sphingolipid metabolism).

Glycosphingolipids, such as the monosialotetrahexosylganglioside (GM1) and disialotetrahexosylganglioside (GM2), are one the most abundant types of sphingolipids stored in the brain of NP-C1 patients[31, 32]. GM2 accumulation is also present in NP-C1 fibroblasts. In the latter case, accumulation results from a reduction of glucosylceramidase (GCase) activity due to a reduction in the mobility of various GCase lipid cofactors associated with alterations in endocytic traffic[33]. Glycosphingolipids are the most abundant class of sphingolipids in cells and they are known for their role in adhesion, mobility and growth through membrane glycosphingolipid-enriched microdomains[34]. Dealing with glycosphingolipid accumulation in NP-C1 patients treated with miglustat, a competitive substrate for Glucosylceramide synthetase, revealed a consistent improvement of several clinical manifestations relative to standard care[35-37]. This indicates that complex sphingolipid synthesis and abundance are important for the pathophysiological expression of this disease.

Sphingomyelin also accumulates in NP-C1 fibroblasts[27, 38, 39]. It seems that this is due to an alteration of the lysosomal acid sphingomyelinase location and activity, even though protein levels are normal[40]. Interestingly, transfection of NPC1-deficient Chinese ovary hamster (CHO) cells with wild type acid sphingomyelinase (SMase) or incubation with purified SMase decreases cholesterol accumulation as observed through filipin staining[40]. Other reports also showed that sphingomyelin accumulation happens in a similar time frame to cholesterol[38]. Thus, sphingomyelin accumulation and catabolism also play a role in cholesterol homeostasis and reiterates the importance of sphingolipids in NP-C phenotypical expression.

Sphingosine accumulation observed in liver, spleen and brain of NP-C1 patients is commonly attributed as a discerning factor between NP-C1 and other lysosomal storage diseases[30, 38, 41]. There are various indications that sphingosine can be one of the key metabolites in NP-C1. Some studies have showed that sphingosine mediates alterations in the lysosomal calcium concentration efflux[42]. Moreover, *NPC1*-null mice

present a reduction of calcium storage in lysosomes as a result of sphingosine accumulation in this organelle[38]. A defective calcium release seems to be responsible for the endocytic traffic defects observed in NP-C cells. Indeed, proteins from the soluble NSF attachment protein receptor (SNARE) family require calcium to mediate vesicular fusion events and treatment of *NPC1*-null mice with curcumin, a sarcoendoplasmic reticulum ATPase (SERCA) antagonist, to mimic calcium efflux from the lysosomes increased their survival and motor control (less tremors)[38]. Thus, sphingosine-related lysosomal calcium alterations are important for the vesicular traffic of NP-C1.

Lysosomal calcium signalling can regulate autophagy by the action of Transcription factor EB (TFEB)[43, 44]. TFEB activation is mediated by calcineurin, a calcium activated phosphatase[43]. Thus, sphingosine can induce autophagy in a lysosome-derived and calcium-dependent manner through activation of calcineurin and TFEB. Interestingly, autophagy is induced in NP-C1 cells[45-47]. Yet, progression of autophagy seems to be compromised due to failure of the SNARE machinery[45, 46, 48]. This leaves open ended questions regarding the role of calcium signalling and sphingosine (and sphingolipids in general) in NP-C1 phenotypes such as the endocytic defects which are an intracellular hallmark not only in NP-C1 but also in other LSDs.

## 1.2. Sphingolipid metabolism in yeast and mammals

The yeast *S. cerevisiae* has been extensively used as an eukaryotic model to unravel the molecular mechanisms underlying mammalian cell functions. The ease in manipulation and the amenability to genetic modifications of *S. cerevisiae* allows for large scale high throughput screenings of protein and study of gene interactions and functions[49-51]. Moreover, the conserved function and structure of yeast proteins with their human orthologs has yielded precious information about the human metabolism, including sphingolipid metabolism (Figure 3) and the molecular and cellular aspects of neurological pathologies [25, 52-54].

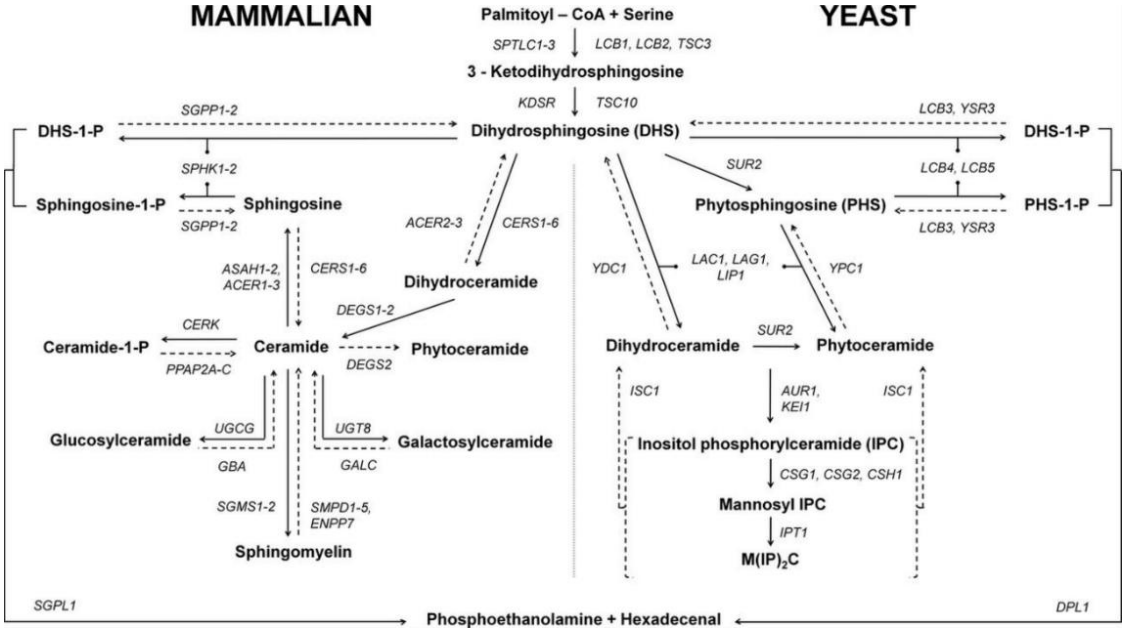


Figure 3 – **Comparison of sphingolipid biosynthesis and catabolism in mammals and yeast** Image adapted from [55].

An extensive homology between the sphingolipidome and the sphingolipid synthesis enzymes between yeast and mammals exists, specially at the first steps of sphingolipid synthesis (Figure 3). Sphingolipids are a type of lipids that contain an aliphatic amino alcohol backbone, normally referred as long-chain base (LCB, synonymous with sphingosine species or sphingoid bases) to which lipids, simple or complex sugars, phosphocoline or phosphoetanolamine can be linked. Sphingolipid synthesis starts with the formation of sphingoid bases in the endoplasmic reticulum with the condensation of a serine and one of various fatty acyl-CoA molecules, usually palmitoyl-CoA, to form 3-ketodihydrosphingosine (3-KDHS). This process is mediated by the serine palmitoyl-CoA transferase (SPT) complex, composed by Lcb1, Lcb2 and Tsc3 in yeast and SPT1, SPT2 and SPT3 in humans[56-58]. 3-KDHS is then reduced to dihydrosphingosine (DHS) by the reductase Tsc10 in yeast or by KDSR (also known as FVT1) in mammals[59, 60].

After the formation of sphingosine species, the biosynthesis of ceramides and more complex sphingolipids diverges between yeast and humans. In yeast, DHS can be hydroxylated by Sur2 to form phytosphingosine (PHS) or acylated by the (dihydro) ceramide synthase complex (CerS) composed by Lag1, Lac1 and Lip1 to form dihydroceramide (DHC)[61, 62]. Additionally, DHC can also be hydroxylated by Sur2 to form phytoceramides (PHC) or PHS can undergo acylation by CerS to form PHC[63] (Figure 3). In humans, DHS undergoes acylation by CerS1-6 to form DHC which then undergoes desaturation by the sphingolipid desaturases DEGS1/2 to form ceramides.

LCBs and ceramide species (simple sphingolipids) can be phosphorylated to form sphingosine-1-phosphate (DHS-1-P or PHS-1-P in yeast) or ceramide-1-phosphate (only in mammals).

Regardless of the organism, the formation of more complex sphingolipids occurs in the Golgi network, by modifications at the 1-OH position of the sphingosine backbone in ceramides (PHC, DHC or ceramide)(Figure 4)[64]. Humans use a vast set of carbohydrates for the synthesis of glycosphingolipids and gangliosides from ceramides[65, 66]. On the other hand, yeast only incorporate inositol and mannose into PHC or DHC. As such, the only species of complex sphingolipids present in yeast are inositolphosphoceramides (IPC), synthesized by the IPC synthase composed by Aur1 and Kei1, mannoseinositolphosphoceramides (MIPC), synthesized by the MIPC synthases Csg1, Csg2 and Csh1, and mannosediinositolphosphoceramides (M(IP)2C) which are synthesised by the inositolphosphotransferase Ipt1[67-69].

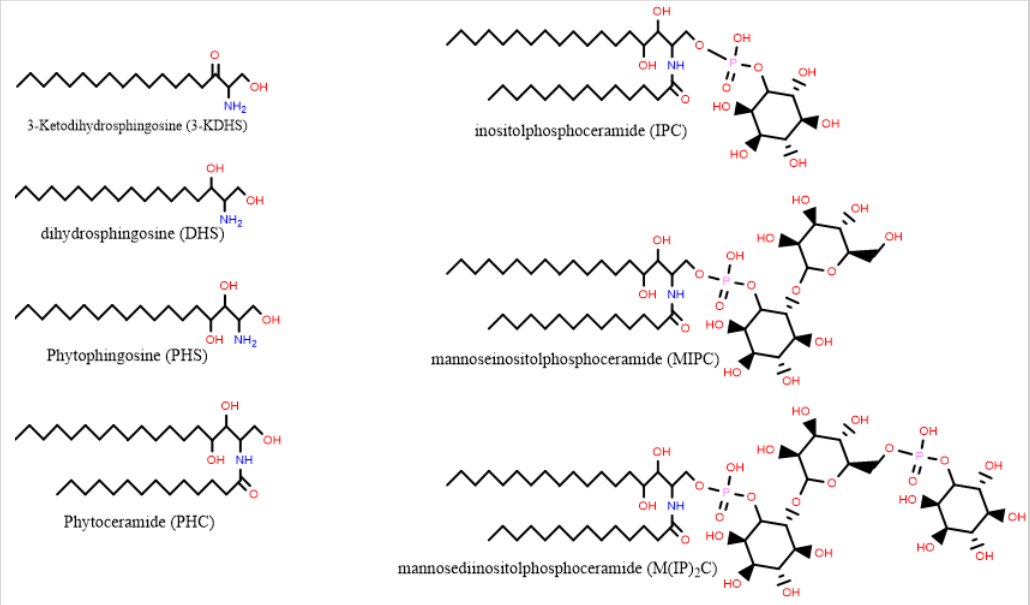


Figure 4 – **Sphingolipid species formed during sphingolipid biosynthesis.**

## 1.2.1. The roles of sphingolipids in *S. cerevisiae*

Sphingolipids constitute around 30% of the lipids observed in the plasma membrane of yeast cells[70]. Their importance in cellular function was first discovered by screening yeast mutants with abnormal lysosomal pH homeostasis for endocytosis defects[71]. One of these mutants possessed a thermosensitive allele for *Lcb1* (*lcb1-100*) which proposed an association between sphingolipid synthesis with endocytosis[72]. Moreover, these endocytic defects were later confirmed by others[73].

Beyond their role in endocytosis, sphingolipids are also involved in the transit of GPI-anchored proteins from the ER to the Golgi apparatus[72]. In fact, a genome wide visual screening of Mid1-GFP, a subunit of a Ca2+-permeable cation channel, transit to the plasma membrane (PM) in single knockout mutants identified that knocking out *SUR2*, a sphingolipid hydroxylase, caused the retention of Mid1-GFP protein in the vacuole and Golgi compartments [74]. The authors proposed that hydroxylation of sphingoid bases by *SUR2* could affect proper protein sorting.

Moreover, the size of the lipid moiety of sphingolipids was also shown to regulate protein sorting. In fact, Pma1, the main $H^+$-ATPase pump responsible for maintenance of cytosolic pH homeostasis, associates with membrane microdomains abundant in lipids with $C_{26}$ lipid moieties to be efficiently transported inside the cell[75]. These membrane microdomains containing Pma1 (called lipid rafts) are enriched in sterols and sphingolipids. Lipid rafts are also required for the proper activity and localization of Gas1p, a glucan transferase required for cell wall assembly[76, 77]. Moreover, they are required for the reorganization of proteins to the shmoo during the polarized growth of yeast cells when mating[78].

Sphingolipids function can also extend to the regulation of nutrient uptake. For example, PHS accumulation was found to block the uptake of tryptophan, leucine, histidine and uracil[79]. Mechanistically, it is not entirely clear how sphingolipids regulate nutrient uptake. Indeed, their role in vesicular transport and lipid rafts suggests an alteration in the cellular destination of related amino acid transporters. Somewhat in agreement with this, a heat-induced increase in PHS promotes the ubiquitin-dependent proteolysis of Fur4, a cell membrane uracil transporter[80]. Other studies showed that Can1, an arginine/$H^+$ symporter, is mistargeted from the PM by heat-stressing the *lcb1-100* mutant[81].

Sphingolipids are also involved in calcium signalling in *S. cerevisiae*. It was described that cytosolic levels of calcium increase in response to endogenous sphingosine-1-phosphate accumulation[82]. Moreover, the increase in calcium levels is responsible for calcineurin activation and Crz1-mediated gene expression[82] (discussed further in chapter 1.2.4, page 14).

Sphingolipids also participate in a homeostatic feedback loop for the regulation of their own abundance. For example, the first step of sphingolipid synthesis is downregulated by exogenous PHS treatment[83]. Moreover, when the first step of sphingolipid synthesis is chemically inhibited with myriocin, an inhibitor of the SPT

complex, the cell undergoes a coordinated response to activate the signalling pathways that induce sphingolipid synthesis [84, 85].

Overall, sphingolipids play an important role in the vesicular transport of the cell. Sphingolipids are required for proper transport of key proteins associated to amino acid uptake, mating, pH homeostasis and cell wall synthesis. Moreover, their lipid properties affect endocytosis and the homeostatic control of lysosomal calcium. Their overarching role in normal cell function is controlled by a homeostatic feedback mechanism that controls sphingolipid abundance and loss of proper sphingolipid synthesis regulation should, in principle, affect all cellular processes mentioned.

### 1.2.2. Regulation of sphingolipid biosynthesis by TORC2, Pkh1 and Ypk1 in *S. cerevisiae*

The biosynthetic portion of the sphingolipid metabolism is tightly regulated by upstream signalling pathways as a control mechanism. TORC2 and Pkh1/2 work together to regulate Ypk1/2 kinase activity (Figure 5). Both proteins transduce membrane-related stress events like heat stress, osmotic stress or possibly alterations in PM composition into Ypk1/2 regulation. Ypk1 is the key kinase that transduces the membrane signal into a physiological response, and its effect on SPT and CerS activity directly impact on sphingolipid synthesis[85, 86].

In *S. cerevisiae*, TORC2 is implicated in various cellular processes like cytoskeleton polarization, cell cycle progression, endocytosis and repression of calcineurin[87-92]. TORC2 is composed by Lst8, Avo1, Avo2, Avo3/Tsc11, Bit61, Slm1, Slm2 and Tor2 , the catalytic subunit (reviewed in [93]). The Avo1 subunit anchors the complex into the plasma membrane through a Pleckstrin Homology (PH) domain that is responsible for binding to phosphatydilinositol-4,5-biphosphate (PtdIns4,5P2)[93, 94].The PH-domain present in many proteins but it does not confer them specificity to the Membrane Compartments of TORC2 (MCTs) probably because of a necessity of certain protein-protein interactions. Curiously, Slm1 and Slm2 contain PH-domains and although they are important subunits of TORC2 they are trapped in membrane furrows distinct from MCTs called eisosomes[95]. Slm1/2 release from eisosomes is mediated by hypotonic stress and its association with TORC2 in MCTs induces the downstream activation of Ypk1, a direct target of TORC2. This release allows Slm1/2 to couple to TORC2 and promote phosphorylation of Ypk1 in the threonine residues 644 and 662, culminating in the induction of *de novo* sphingolipid biosynthesis [95].The fate of the recently synthesized sphingolipids is to intercalate back into the PM leading to a

reduction of membrane tension and, as a consequence, to TORC2-Ypk1 downregulation (reviewed in [96]).

Slm1/2 are important mediators of Ypk1 activation and downstream signalling because they recruit Ypk1 to the PM and allow for TORC2-mediated phosphorylation[95, 97]. This process requires Slm1/2 to first be recruited to the PM by binding to PtdIns4,5P2 species[97]. Moreover, Slm1/2 are also direct targets of TORC2[98]. Under heat stress, Slm1 becomes hypophosphorylated in a 15 min time window that coincides with Ypk1 hyperphosphorylation by TORC2[98, 99]. Treatment of yeast cells with myriocin, an inhibitor of the SPT complex, reduces Slm1 phosphorylation and increases Ypk1 phosphorylation in T662, which suggests that the hypophosphorylated state of Slm1 is key for TORC2-mediated phosphorylation of Ypk1[95, 99]. Moreover, Ypk1, Slm1 and Avo3 can be immunoprecipitated together under myriocin treatment suggesting that the interaction between these 3 proteins is dependent on the hypophosphorylated state of Slm1[95]. Under prolonged heat stress, Slm1/2 phosphorylation levels are recovered in a TORC2-dependent manner[98, 99]. While the dynamics of Slm1/2 phosphorylation are understood, the role of Slm1/2 phosphorylation in TORC2 signalling and sphingolipid synthesis is unclear[92, 95, 99-101].

Ypk1 activity is also regulated by Pkh1-mediated phosphorylation of threonine residue 504 that is responsible for the basal activity of the enzyme[102, 103]. The Pkh1 kinase is anchored to the PM through Pil1, a PH-domain protein that is essential for eisosome scaffolding and assembly[104]. However, the mechanism underlying activation and regulation of Pkh1 remains elusive. *In vitro* experiments showed that Ypk1 phosphorylation by Pkh1 can be regulated by PHS[105]. However, exposing yeast cells to PHS does not affect the phosphorylation of T504 in Ypk1[106]. The fact that Pil1 associates with Inp51, a PtdIns4,5P2 phosphatase, suggests that eisosomes intervene in PtdIns4,5P2 homeostasis as a possible regulatory pathway for Pkh1[107]. In fact, PtdIns4,5P2 synthesis and degradation is dependent on eisosome structure in a Pil1-dependent manner in *S. pombe*[108]. Also, loss of TORC2 function in *S. pombe* is reverted by blocking PtdIns4,5P2 catabolism[108].Thus, it is possible that PtdIns4,5P2 regulates sphingolipid biosynthesis via Pkh1 and Ypk1 in yeast cells.

Ypk1 is seen as the master kinase that regulates sphingolipid biosynthesis in yeast. Ypk1 controls SPT activity by phosphorylating and inactivating Orm1 and Orm2, two proteins known to inhibit SPT, the first enzymatic complex of sphingolipid biosynthesis[84, 86]. To a lesser degree Orm1/2 have also been found to interact with Lag1 and Lac1 but the exact role of this interaction is still unknown[109]. Ypk1 also

phosphorylates Lag1 and Lac1 stimulating the synthesis of ceramide species[85]. Lag1/Lac1 proteins can also undergo additional regulation by casein kinase 2 (CK2)-mediated formation of ER puncta[110]. In general, Ypk1 activity dictates the entrance of acyl-CoA into the sphingolipid biosynthetic pathway while at the same time promotes ceramide synthesis.

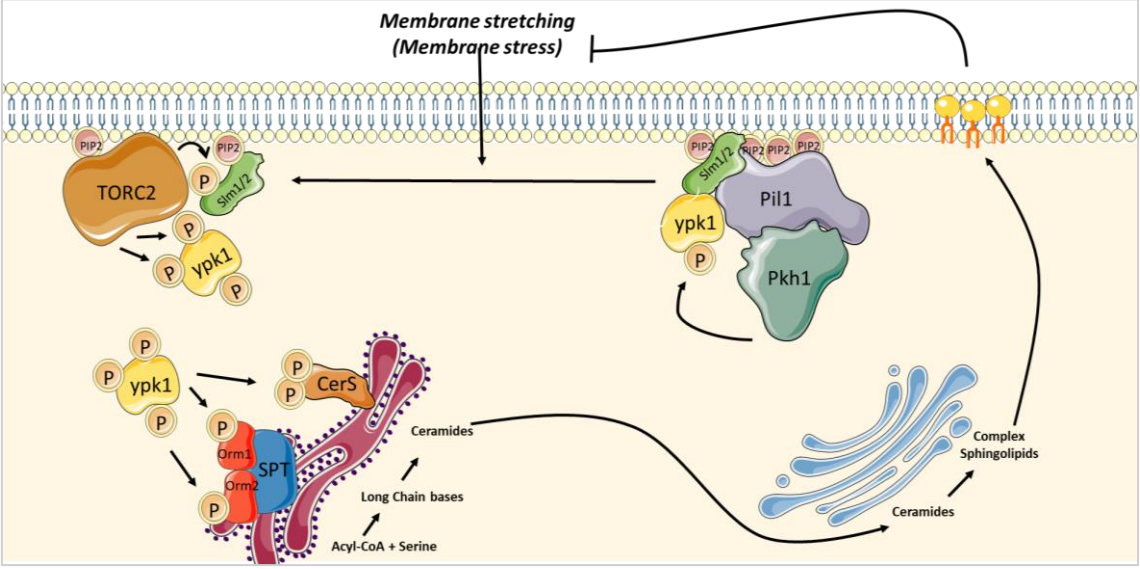


Figure 5 – **Illustration of the TORC2 and Pkh1-mediated regulation of sphingolipid biosynthesis via Ypk1 under membrane stress events.**

### 1.2.3. Homology between mammalian and yeast sphingolipid biosynthesis regulation

The homology of sphingolipid biosynthesis metabolism between yeast and mammals support the applicability of the discoveries made with *S. cerevisiae* to understand this process in mammals. The mammalian cells present structural and functional orthologs of Pkh1/2 (PDK1) and Ypk1/2 (SGK1). Casamayor showed that the lethality of the *pkh1*Δ*pkh2*Δ mutant can be rescued by expressing the human 3-phosphoinositide dependent kinase 1(PDK1)[102]. In a similar way to Pkh1, PDK1 can be activated by sphingosine and anchored to the membrane by phosphoinositide species[111]. Moreover, phosphoinositide-3-kinase (PI3K), which synthetize these phosphoinositol species, also plays a role in mammalian TORC2 activation[112]. This mimics the necessity of PtdIns4,5P2 for yeast TORC2 and Pkh1 anchoring to PM. In addition, *ypk1*Δ*ypk2*Δ lethality can be rescued by expression of the serum glucocorticoid kinase 1 (SGK1)[102] which is also regulated by mTORC2 and PDK1[113, 114]. In fact, mTORC2 regulates SGK1 under osmotic stress much like their yeast counterparts. Even

though the integrative signalling between mTORC2, PDK1 and SGK1 are conserved there is no indications that SGK1 may regulate the human ceramide synthase or even the Ormdl1/2/3 proteins, orthologs of Orm1/2. However, Ormdl1/2/3 have a similar function to their yeast counterpart and have been implicated in a feedback response to ceramide biosynthesis, so it is possible that some sphingolipid synthesis regulatory pathway must exist[86, 115, 116].

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### 1.2.4. Regulation of sphingolipid biosynthesis by calcium and calcineurin

Calcineurin is a protein phosphatase highly conserved between humans and yeast[117]. Calcineurin is activated by interaction with calmodulin and calcium. Calmodulin is a multifunctional calcium-binding protein that upon binding to calcium suffers a conformational alteration that allows for favourable interactions with calcineurin and other proteins to promote their activity (reviewed in [37, 49]). Moreover, the activation of calcineurin by calcium and calmodulin can be induced by other stimuli like sodium stress, membrane stress, pH and blue light[118, 119]. Calcineurin targets include Slm1/2, Crz1 - a transcription factor that mediates the expression of a wide array of genes - and Lag1(Figure 6)[120, 121].

The calcineurin-mediated dephosphorylation of Slm1/2 has been well characterized. Treating yeast cells with FK506, a calcineurin inhibitor , or calcium, which promote calcineurin activity, can abolish or promote Slm1/2 dephosphorylation, respectively[99]. However, the role of this regulation does not seem to affect TORC2 or Pkh1-Ypk1 signalling. In fact, inhibition of calcineurin with FK506 does not affect TORC2 phosphorylation of T662 and calcium stimuli does not alter Pkh1 phosphorylation of Ypk1[85, 95]. Thus, TORC2/Pkh1-Ypk1 signalling is independent of Slm1/2 phosphorylation even though Slm1/2 are necessary for TORC2/Pkh1-Ypk1 induction of sphingolipid synthesis.

Crz1 is a calcineurin-responsive zinc finger transcription factor that migrates into the nucleus under calcium stimulus. Crz1 exhibits 2-minute bursts of nuclear localization that increase in frequency proportionally to calcium concentration[122]. Of interest, exogenous calcium and sodium stimuli promote the expression of *SUR1* (an alias name for *CSG1*), a component of the MIPC synthase complex[123]. Moreover, the expression of *CSG2*, a component of the same complex, is regulated by Crz1 under calcium stimulus only. That way, the calcium stimulus leads to an increase in the levels of these proteins promoting the synthesis of MIPC and M(IP)2C [31, 101].

There are indications that calcineurin can also regulate Lag1 phosphorylation. Muir et al identified a calcium/calcineurin-dependent dephosphorylation of Lag1[85]. Moreover, the mechanism seems to be direct because the Pkh1-mediated phosphorylation state of Ypk1 remains constant under calcium stimuli, ruling out the possibility that calcineurin is acting indirectly over Lag1. Also, Lag1 has a possible binding site for calcineurin, at the C-terminal[124]. Importantly, calcineurin can revert Ypk1-mediated phosphorylation of Lag1, suggesting that Lag1 activity is a result of a dynamic regulation between TORC2/Pkh1-Ypk1 signalling and calcineurin signalling[85].

Overall, the coordinated downregulation of the ceramide synthase subunit Lag1 and the increase in abundance of the MIPC synthase suggest that calcineurin restricts ceramide synthesis to promote their conversion into more complex sphingolipid species.

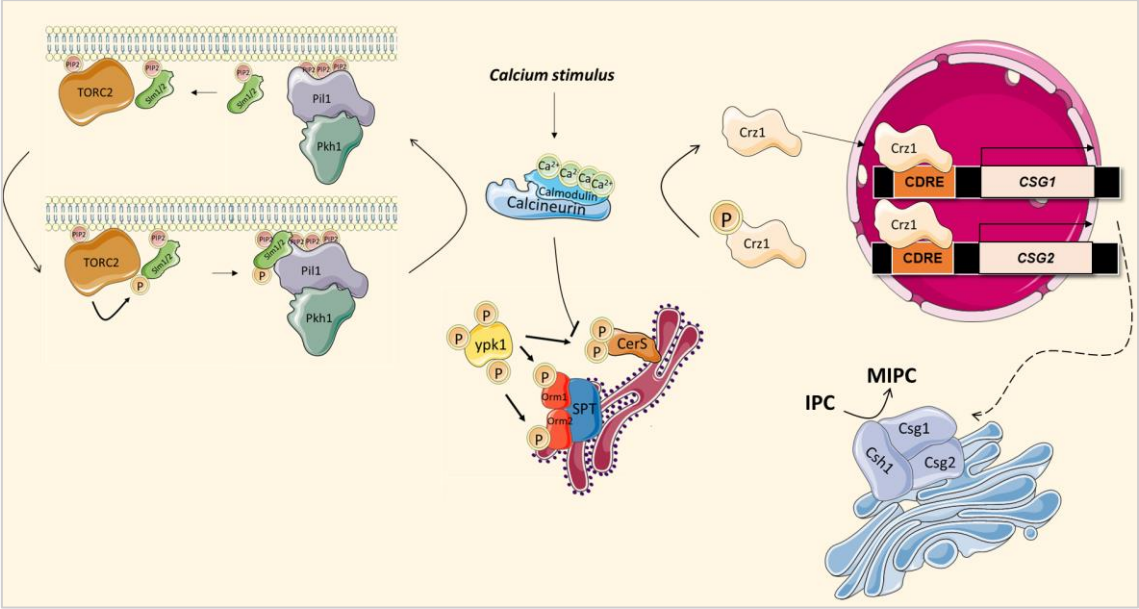


Figure 6 – **Illustration of the sphingolipid synthesis points that are regulated by calcineurin in *S. cerevisiae*.**

# 1.3. Yeast model for research in sphingolipid and calcium signalling in NP-C1

## 1.3.1. *S. cerevisiae* as a model organism for LSDs

*S. cerevisiae* has been used as a simple but reliable model to understand the molecular mechanisms underlying human neurodegenerative diseases[25]. The general approach consists in either disrupting yeast orthologs for human genes or expressing human genes that have no yeast orthologs with similar function (humanized yeast strains). Both approaches have been applied in understanding neurodegenerative

diseases such as the lysosomal disorder NP-C1[125, 126]. There are about 23 yeast orthologs of human genes with associations to lysosomal storage disorders which have been explored to establish yeast models for the comprehension of several LSDs like Battens disease, Friedreich's ataxia, Ataxia telangiectasia, Hereditary Spastic Paraplegia, NP-C1 and NP-C2[125, 127].

### 1.3.2. *S. cerevisiae ncr1*Δ cells as a model for NP-C1

The *S. cerevisiae* ortholog of the human NPC1 protein is the Niemann-Pick type C1 related-protein (Ncr1). It was shown that the expression of *NCR1* in *NPC1*-deficient Chinese hamster ovary cells restores normal lipid traffic[19]. This functional study opened the possibility for yeast to be used as a model for NP-C1. The current yeast model for NP-C1 was obtained by deletion of the entire open reading frame of *NCR1* (*ncr1*Δ) thus mimicking a total loss-of-function of the protein. Previous work showed that *ncr1*Δ cells present various phenotypes similar to the ones observed in patient cells or mammalian models of the disease. For example, *ncr1*Δ cells present significant alterations in the abundance of LCBs like PHS, PHS-1-P, DHS and DHS-1-P in logarithmic (fermentative) and post-diauxic shift (PDS)(respiratory) phase as well as of specific ceramides in PDS phase[128, 129]. Moreover, the *ncr1*Δ model also presents hypersensitivity to oxidative stress and a shortened lifespan[2, 128, 130, 131]. The shortened life span of *ncr1*Δ was associated with severe defects in mitochondrial respiratory function such as low oxygen consumption rates, absence of growth on non-fermentable media, mitochondrial depolarization and fragmentation of mitochondrial network but also increased levels of ROS and oxidation of proteins and lipids [128].Moreover, the yeast model also presents alterations in sterol levels and subcellular localization[128, 132].Various other defects were identified by a genome-wide screen of modifiers in *ncr1*Δ[133]. The study revealed a set ofproteins that were implicated in many different cell processes like histone deacetylation, cell cycle progression, glycerosphingolipid metabolism and sterol uptake[133]. Of interest, the histone deacetylation defects discovered in yeast were later investigated in human cells and studies have shown that vorinostat, a histone deacetylase inhibitor approved by the FDA to treat T-cell lymphoma, ameliorates the cholesterol and lipid defects in NP-C1 cells[133] [134]. This not only shows the homology in cellular function but also supports the use of a yeast model for drug testing and research.

### 1.3.3. Regulation of sphingolipid synthesis in *ncr1*Δ

Since much of the sphingolipid regulatory pathway is still to be discovered in mammals, the yeast counterpart gives a valuable opportunity to investigate how sphingolipid signalling mediate defects associated with neurologic disorders like NP-C1. Previous work revealed that Pkh1 is activated in *ncr1*Δ cells and that deletion of *PKH1* is sufficient to revert the mitochondrial respiratory defects and the shortened chronological life span (CLS) of *ncr1*Δ cells, suggesting that a Pkh1-regulated pathway promotes mitochondrial function alterations[128, 129]. Moreover, the increase in the phosphorylation of Sch9, a Pkh1 downstream target, was also associated with the cell death phenotypes of the yeast model of NPC1 and with the modulation of sphingolipid biosynthesis[128]. These events support a common regulatory pathway that mediates sphingolipid abundance changes and the mitochondrial defects observed in *ncr1*Δ cells. In agreement, sphingolipids have also been implicated in the mitochondrial defects of NP-C1 cells[48, 135, 136].

### 1.3.4. Calcium regulation in *ncr1*Δ

The possible role of calcium-mediated signalling in Niemann-Pick type C1 disease was recently described, as human cells have a large reduction in lysosomal calcium levels associated with sphingosine accumulation in this compartment[38, 137, 138]. We have previously found that *ncr1*Δ cells are more sensitive to calcium in a calcineurin-dependent manner. In fact, deletion of Cnb1, the regulatory subunit of calcineurin, supressed the sensitivity of *ncr1*Δ to high concentrations of calcium (unpublished data). As human cells have alterations in lysosomal calcium levels we postulate that the activation of calcineurin in the yeast model of NPC1 may result from calcium signalling.

## 1.4. Aim of this work

Sphingolipid and cholesterol accumulation in the late endosomes and lysosomes are two hallmarks of the NP-C1 disease. Although much is known about the type of lipids that accumulate in NP-C1, their role in the expression of the cellular phenotype of NP-C1 is uncharacterized.

In line with the previous reported data and unpublished results, this work aimed to characterize the possible role of eisosomes in the regulation of Pkh1 activity and its impact in the mitochondrial function and life span of a yeast model of NPC1. Moreover,

we aimed to characterize the activation of downstream targets of Ypk1, particularly in the expression of enzymes involved in sphingolipid synthesis. Given that calcium alterations have been implicated in the pathophysiology of the disease and the role of calcineurin in regulating sphingolipid synthesis in yeast, we also aimed to assess if the activation of calcineurin could mediate the mitochondrial and lifespan defects of *ncr1*Δ cells.

# Chapter 2

# MATERIALS AND METHODS

# 2.1. Yeast strains, plasmids and growth conditions

The *Saccharomyces cerevisiae* strains used in this work resulted from the BY4741 or YYA3 parental strains and are depicted accordingly in Table 1.

Table 1 – Strain, genotype and source of the *S. cerevisiae* strains used in this study.

| **Strain** | **Genotype** | **Source** |
|---|---|---|
| BY4741[a,c] | *MATa his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 | EUROSCARF |
| *ncr1*Δ | BY4741 *ncr1*Δ::*URA3* | [128] |
| *ncr1*Δ[a,c] | BY4741 *ncr1*Δ::*KanMX4* | [128] |
| *pil1*Δ | BY4741 *pil1*Δ::*KanMX4* | EUROSCARF |
| *pil1*Δ*ncr1*Δ | BY4741 *pil1*Δ::*KanMX4 ncr1*Δ::*URA3* | Lab collection |
| *ypk1*Δ | BY4741 y*pk1*Δ::*KanMX4* | EUROSCARF |
| *ypk1*Δ[c] | BY4741 y*pk*1Δ::MX*HIS3* | **This Study** |
| *ypk1*Δ*ncr1*Δ[c] | BY4741 y*pk*1Δ::MX*HIS3 ncr1*Δ::*KanMX4* | **This study** |
| *ypk1*Δ*ncr1*Δ | BY4741 y*pk1*Δ::*KanMX4 ncr1*Δ::*URA3* | Lab collection |
| *cnb1*Δ[a] | BY4741 *cnb1*Δ::*KanMX4* | Lab collection |
| *cnb1*Δ*ncr1*Δ[a] | BY4741 *cnb1*Δ::*MXHIS3 ncr1*Δ::*KanMX4* | Lab collection |
| BY4741 Lag1-GFP[b,*] | BY4741 *LAG1-GFP::KanMX4* | Lab collection |
| *ncr1*Δ Lag1-GFP[b,*] | BY4741 *LAG1-GFP::KanMX4 ncr1*Δ::*URA3* | **This study** |
| BY4741 Lag1-9Myc* | BY4741 *LAG1*-9Myc::*hphNT1* | Lab collection |
| *ncr1*Δ Lag1-9Myc* | BY4741 *LAG1*-9Myc::*hphNT1 ncr1*Δ::*URA3* | Lab collection |
| YYA3 | MATa his3::CRZ1-GFP-HIS3 leu2 met1 ura3 | [21] |
| YYA3 *ncr1*Δ | YYA3 *ncr1*Δ::*URA3* | Lab collection |
| YYA3 *cnb1*Δ | YYA3 *cnb1*Δ::KanMX4 | [21] |
| YYA3 *cnb1*Δ*ncr1*Δ | YYA3 *cnb1*Δ::KanMX4 *ncr1*Δ::*URA3* | **This study** |

[a] – Cells harbouring pCDRE-LacZ [b] – Cells harbouring FBp709 (see *Table 3*) [c]- Cells harbouring FBp701 *- strains were tagged in the C-terminal of the respective proteins

Yeast strains were grown aerobically at 26ºC on an orbital shaker at 140 r.p.m. using Erlenmeyer flasks. A 1:5 proportion of growth media to flask volume was used. Cells were grown to early logarithmic phase ($OD_{600}$ = 0.6-0.9) or to post-diauxic shift ($OD_{600}$ = 7-10). The liquid growth media used were: YPD - Yeast extract, Peptone and Dextrose [2% (w/v) bacteriological peptone, 1% (w/v) yeast extract and 2% (w/v) glucose], MM - Minimal Medium [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose] supplemented with the appropriate amino acids and nucleotides [0.004% (w/v) histidine, 0.008% (w/v) leucine, 0.004% (w/v) methionine, 0.004% (w/v)

uracil and 0.004% tryptophan), SC - Synthetic Complete drop-out medium with glucose [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose] supplemented with the appropriate amino acids and nucleotides [0.008% (w/v) histidine, 0.04% (w/v) leucine, 0.008% (w/v) tryptophan and 0.008% (w/v) uracil)] and SC with glycerol (SC-glycerol) where glucose was replaced by 3% (v/v) glycerol). Alternatively, 1.5% agar (w/v) was added to the broths to be used as solid media.

When indicated, strains were treated with 20 mM $CaCl_2$ for 15, 30 or 60 min or with 0.625 µM myriocin, 120 µM acetic acid or 50 µg/mL FK506 for 60 min.

## 2.2. Construction of yeast mutants

Yeast strains were transformed using a modified version of Polyethylene glycol (PEG)/Lithium acetate protocol[139]. Briefly, 20 mL of cells grown overnight in YPD until logarithmic phase ($OD_{600}$ = 0.8) were pelleted, washed and resuspended in 100 µL of water. 50 µL of cell suspension was transferred to an eppendorf and the remaining water was removed after centrifugation (12044g, 1 min). To the pellet, 240 µL of 50% (w/v) PEG 3350, 36 µL of 1M lithium acetate, 25 µL of 5 mg/mL single stranded DNA and 200 ng of plasmid or 1 µg of DNA cassette were added. The mixture was vortexed for 1 min, incubated at 26ºC for 30 min and then switched to 42ºC for another 30 min. The transformation mix was removed by centrifugation (4293g, 1 min) and the pelleted cells were washed with water and resuspended gently in selective media before being incubated for 4 to 6 hours at 26ºC. Finally, cells were pelleted again (4293g, 1 min), resuspended in 100 µL of selective media and spread in selective media plates. In the case of plasmids, the protocol was simplified: The incubation step at 26ºC was removed and cells were spread directly on selective plates after 42ºC incubation.

The following polymerase chain reaction (PCR) protocol was used to amplify the DNA cassettes used in the construction of the yeast mutants in this work. Polymerase chain reactions were performed in 20 µL reaction mixes containing 1x GoTaq buffer (Promega), 10 µM dNTPs (Thermofisher), 1.5 mM magnesium chloride (Promega), 0.5 µM of primers, 1 U of GoTaq polymerase and 200 ng of plasmid or 400 ng of DNA extract. DNA extraction was performed with alkaline lysis on single yeast colonies grown in YPD plates for 2-3 days. Briefly, yeast cells were resuspended in 30 µl of 20 mM NaOH and heated at 95ºC for 15 min followed by a subsequent 1 min vortexing step. The cell lysate was centrifuged (12044g, 1 min), and the supernatant was collected. PCR was performed in a T100 Thermo Cycler (Bio-Rad) with a heated lid using the following

conditions: Initial denaturation step at 95ºC for 3 min, secondary denaturation step at 95 ºC for 45s – where PCR cycling starts –, a primer annealing step with a temperature adjusted according to primer melting point for 45s, an elongation step at 72ºC for 1kb of PCR product/min – where PCR cycling ends – and a final elongation step of 10 min at 72ºC. The primers used in this work are described in Table 2.

Table 2 – Primers used in this study.

| **Primer** | **Sequence (5' -> 3')** |
|---|---|
| Amplif_*ncr1*-FW | CCGTGGCTAATGTCACAACA |
| Amplif_*ncr1*-RV | TTACGACTGAAGCGTTGACC |
| Conf_ext_*ncr1*-FW | AAGGTGCGAAATGACGGAAGA |
| Conf_int_*ncr1*-RV | CGTCGTCCACAATCATTGCCC |
| Conf_int_*ncr1*Δ::*KanMX4*-FW | ACGTTTCGAGGCCGCGATT |
| Conf_int_*ncr1*Δ::*URA3*-RV | CCCAGTGACACCATGAGCATTAG |
| Amplif_CassetteMX4-FW | TCCTTGACAGTCTTGACG |
| Amplif_CassetteMX4-RV | GAATGCTGGTCGCTATAC |

All PCR reaction products were analysed by nucleic acid electrophoresis at 100V, using 1% (w/v) agarose gels with GreenSafe premium 0.04 µL/mL (NZYtech) and TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). DNA bands were identified through UV fluorescence and extracted from the gel using the NZYgelpure kit (Nzytech) according to the manufacturer's instructions. DNA quantification was done using Nanodrop 1000 (Thermofisher).

To generate all *ncr1*Δ strains, *KanMX4* or *URA3* were amplified with flanking regions of *NCR1* to produce the *ncr1*Δ*::KanMX4* and *ncr1*Δ::*URA3* cassettes respectively, thus allowing for homologous recombination, gene deletion and selective growth of recombinant strains.

Amplification of *URA3* cassette was done with the Amplif_*ncr1*-FW and -RV primers, using genomic DNA from *ncr1*Δ::*MXURA3* with an annealing temperature of 53ºC and an elongation time of 2min 20s[128]. YYA3 *cnb1*Δ and BY4741 *LAG1-GFP* were transformed with the *URA3* cassette for deletion of *NCR1* and the resulting transformants were selected in MM lacking uracil. Insertion was confirmed by PCR using the Conf_ext_*ncr1*-FW and Conf_int_*ncr1*Δ::*URA3*-RV primers using an annealing temperature of 53ºC and an elongation time of 1 min 45s.

Amplification of the *KanMX4* cassette was done with the Amplif_*ncr1*-FW and -RV primers, using genomic DNA from *ncr1*Δ::*KanMX4* with an annealing temperature of

53ºC and an elongation time of 3min. *ypk1*Δ*::HIS3* cells were transformed with the *KanMX4* cassette for deletion of *NCR1* and the resulting transformants were selected in YPD with geneticin (200 µg/mL). Insertion was confirmed using the Conf_int_*ncr1*Δ::*KanMX4*_FW and amplif_*ncr1*_RV primers with an annealing temperature of 52ºC and an elongation time of 1 min and 45s.

Amplification of the *MXHIS3* cassette was done with the Amplif_CassetteMX4-FW and -RV primers with an annealing temperature of 42ºC and an elongation time of 1min 30s. The *ypk1*Δ::*KanMX4* strain was transformed with the *MXHIS3* cassette for *NCR1* deletion and transformants were selected in MM lacking histidine.

BY4741, *ncr1*Δ::*KanMX4, ypk1*Δ::*HIS3* and *ncr1*Δ::*KanMX4 ypk1*Δ::*HIS3* were transformed with the FBp701 plasmid (Table 3) using the the transformation protocol referred previously. Transformants were selected in MM lacking uracil.

BY4741 *LAG1-GFP* and *ncr1*Δ::*URA3 LAG1-GFP* were transformed with the FBp709 plasmid (Table 3) and selected in MM lacking leucine. Confirmation of plasmid expression was observed through fluorescence microscopy.

Table 3 – Plasmids used in this study.

| Name | Description | Marker | Source |
|---|---|---|---|
| pCDRE-LacZ | pAL300 with 4x*CDRE* insertion upstream of *LacZ* | *URA3* | [21] |
| FBp701 | YEp357 with a *LAG1* promoter insertion upstream of LacZ | *URA3* | [140] |
| FBp709 | pYX242 plasmid expressing $KAR2_{(1\rightarrow135)}$-mCherry-HDEL | *LEU2* | [140] |

# 2.3. Western blotting

For western blot analysis, cells were grown in 20 mL SC-glucose medium overnight until log phase. Cells were collected and proteins were extracted following the alkaline lysis protocol as in Vilaça R. et al[128]. Briefly, cells were resuspended in 100 mM NaOH, vortexed for 1 min, incubated at room temperature for 5 min, centrifuged and the pellet resuspended in gel lysis buffer (50 mM Tris-base pH 8.8, 2 % (w/v) SDS,10% (v/v) glycerol and 2 mM EDTA). After heating for 5 min at 95ºC in a dry bath, samples were centrifuged (12044g, 15 min) and the total protein content of the supernatant was quantified by using the Pierce BCA protein assay kit (Thermofisher) with a bovine serum

albumin standard. β-mercaptoethanol was added to a final concentration of 5% (v/v) before samples were applied in the gels. Proteins were resolved in a 9% SDS-PAGE gels then transferred to nitrocellulose membranes (Hybond-ECL GE Healthcare) in a semi-dry system at 0.8 mA/cm2 for 1 hour and 30 mins. The quality of protein separation and transfer to the nitrocellulose membranes was checked with Ponceau S staining prior to continuing with the protocol. Membranes were blocked with fresh 5% non-fat dry milk in TTBS (20 mM Tris-base, 140 mM NaCl, 0.05% (v/v) Tween-20 pH 7.6) for 1 hour. Afterwards, membranes were probed with primary antibodies: mouse anti-phospho-PKC (pan) (zeta Thr410) antibody (1:1000, Cell Signalling), goat anti-Ypk1 (1:500, Santa Cruz), mouse anti-cnb1 (1:3000, kindly provided by Maria Cardenas-Corona), mouse anti-*c-myc* (1:1000, Roche diagnostics) or mouse anti-yeast phosphoglycerate kinase antibody (1:5000, Molecular Probes). After washing with TTBS for 10-15 mins, membranes were re-probed with secondary antibodies: anti-mouse igG-peroxidase (1:5000, Molecular Probes), anti-rabbit igG-peroxidase (1:5000, Sigma) or anti-goat igG-peroxidase (1:1000, Sigma). Two consecutive steps of washing with TTBS and TBS for 15 min were done prior to immunodetection using Westbright ECL (Advansta) peroxidase reagents and Chemidoc xrs$^+$ (Bio-rad) chemiluminescence detector or LucentBlue X-ray films (Advansta).

## 2.4. β-Galactosidase activity essays

Cells harbouring the pCDRE-LacZ plasmid were grown in SC-glucose lacking uracil until logarithmic phase ($OD_{600}$ = 0.7) before being treated with 20 mM $CaCl_2$ for 15, 30 and 60 min. Cells were harvested by centrifugation, resuspended in breaking buffer (100 mM Tris, 1 mM DTT,10 % glycerol) and protease inhibitors (Complete mini EDTA-free Protease cocktail inhibitor tables) and mechanically lysed with zirconium beads for 5 min (1 min vortexing and 1 min on ice). Debris were pelleted at 12044g for 15 min at 4 ºC and the supernatant was collected for protein quantification. Total protein levels were quantified by the Lowry method using a bovine serum albumin standard curve[141]. Volumes with 30 ug of protein were diluted up to 800 µL with β-galactosidase buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). The enzymatic reaction was initiated by adding 200 µL of pre-warmed ONPG followed by short vortexing of the solutions. Samples were incubated at 30ºC for 30 min prior to stopping the reaction by basic pH shift using 400 µL of 1M $Na_2CO_3$. Ortho-nitrophenol absorbance was measured at 420 nm. β-galactosidase activity was calculated according to the following equation:

$$\beta - \text{Galactosidase activity} = \frac{\text{Abs}_{420nm} \times 1.4}{0.0045 \times \text{protein(mg)} \times \text{t(min)}}\ nmol.min^{-1}mg^{-1}$$

where: Abs420nm = orto-nitrophenol absorbance, 1.4 = volume correction factor, 0.0045 = Abs420nm of a 1nmol/mL solution of orto-nitrophenol, protein (mg) = mass of protein in mg per reaction, t(min) = reaction time in min.

## 2.5. Chronological lifespan

For chronological lifespan, cultures grown overnight were diluted in SC-glucose to an $OD_{600}$ = 0.6, then grown for 24 h (to PDS, t = 0) and kept at 26ºC on an orbital shaker at 140 rotations per minute. Cell viability was determined over time through the percentage of colony forming units that grew on YPD plates after standard dilution from t=0.

## 2.6. Oxygen consumption and respiratory capacity analysis

For oxygen consumption measurements, cells were grown to PDS in SC-glucose medium. $3x10^8$ cells was resuspended in 1 mL PBS buffer and transferred to an Oxygraph (Hansatech) oxygen electrode. Data was gathered for 6 min and average oxygen consumption rates were determined from oxygen levels in the container in the 2-4 min time window using the Oxyg32 v2.25 software.

To evaluate mitochondrial respiratory functions, cells were grown in a non-fermentable carbon source which restricts yeast survival to oxygen consuming and ATP producing mitochondria. Yeast cells were first cultured in liquid SC-glucose media at 26ºC to log phase ($OD_{600}$ = 0.7). Cultures were then diluted in PBS to an $OD_{600}$ = 0.3 and five-fold serially dilutions were spotted on solid SC-glucose and SC-glycerol and grown for 3-4 days at 26ºC.

## 2.7. Fluorescence microscopy

Cells harbouring the FBp709 plasmid and strains with the YYA3 parental background were grown in 10 mL SC-glucose to log phase or PDS phase. Live cells

were transferred to glass slides covered with lamella-thick agarose strips prior to fluorescence microscopy analysis using a Zeiss Axio Imager Z1. Image analysis and treatment was done with ImageJ (1.52a).

## 2.8. Statistical analysis

Data is represented by mean and standard deviation and analysis for statistical meaning was done with GraphPad Prism (v 6.01).

# Chapter 3

# RESULTS

# 3.1. The implications of the eisosome regulated Pkh1-Ypk1 pathway on the expression and location of Lag1 and mitochondrial defects in *ncr1*Δ cells.

## 3.1.1. Pkh1-Ypk1 signalling pathway is activated in *ncr1*Δ cells

The biosynthesis of sphingolipids is regulated by activation of Ypk1 through phosphorylation of T504 by Pkh1[84].Previous work showed that a yeast model for NP-C1 exhibit various alterations in sphingolipid abundance and sphingolipid-regulated cell signalling events[128]. Namely, Pkh1 was shown to be more activated in *ncr1*Δ cells as demonstrated by the analysis of the phosphorylation of Sch9, one of the downstream targets of Pkh1. Thus, other Pkh1-dependent pathways could be altered in *ncr1*Δ cells. To investigate if Ypk1, another target of Pkh1 and a master regulator of sphingolipid biosynthesis, was more activated in the yeast model of NPC1, we evaluated the phosphorylation of T504 residue in wild type (BY4741) and *ncr1*Δ cells. The deletion of *PIL1* or *YPK1* in *ncr1*Δ were used as controls for abolishing Pkh1 activity and for the identification of Ypk1 bands respectively. *PIL1* deletion was previously shown to downregulate Pkh1 activity[142]. We observed an increase in Ypk1-T504 phosphorylation in *ncr1*Δ cells relative to the wild type, consistent with previous reports (Figure 7)[143]. Moreover, the deletion of *PIL1* in *ncr1*Δ abolished the phosphorylation of Ypk1 suggesting that Pkh1 activity was downregulated. Consistently, we showed that *PIL1* deletion in *ncr1*Δ cells also led to a reduction of the phosphorylation of Sch9, another target of Pkh1 (data not shown). This result is consistent with the reduced phosphorylation of Sch9 in *ncr1*Δ*pkh1*Δ mutants[128].These results suggest that the Pkh1-Ypk1 signalling pathway is upregulated in *ncr1*Δ cells by a mechanism dependent of Pil1, an essential component of eisosome structure.

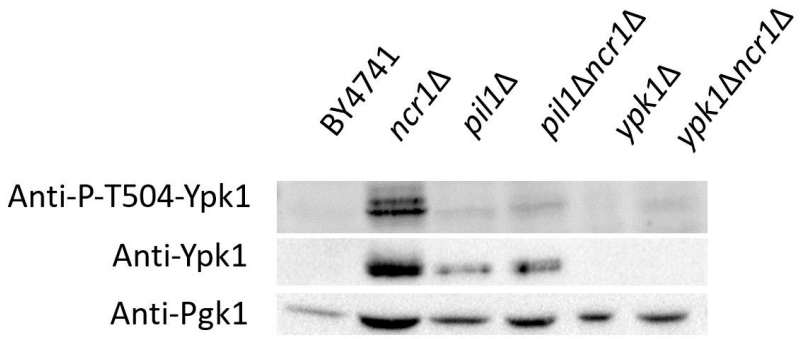


Figure 7 – **The Pkh1-Ypk1 pathway is hyperactivated in *ncr1*Δ cells.** Yeast cells were grown overnight to log-phase ($OD_{600}$=0.6-0.9) in SC-glucose medium. Total protein extracts were separated by SDS-PAGE and the phosphorylated and non-phosphorylated forms of Ypk1 were detected by western blotting using an anti-P-T504-Ypk1 and an anti-Ypk1 antibody respectively, as described in Material and Methods. Pgk1 protein levels were used as a loading control.

## 3.1.2. Eisosome organization mediates the mitochondrial respiratory defects in *ncr1*Δ cells.

Normal Pkh1 activity requires Pil1-dependent anchoring of Pkh1 to eisosomes [104, 142]. Moreover, Pkh1 activation mediates a plethora of mitochondrial respiratory defects in *ncr1*Δ cells[128]. Given that, we hypothesized that disrupting eisosome organization by deleting *PIL1* in *ncr1*Δ would recover mitochondrial respiration. As such, we measured oxygen consumption rate and cell growth in a non-fermentable carbon source in WT, *ncr1*Δ*, pil1*Δ *and pil1*Δ*ncr1*Δ. Indeed, oxygen consumption rate and growth in a non-fermentable media increased to parental levels when *PIL1* was deleted in *ncr1*Δ, suggesting that eisosome structure impacts on mitochondrial respiration through the Pkh1 signalling pathway (Figure 8).

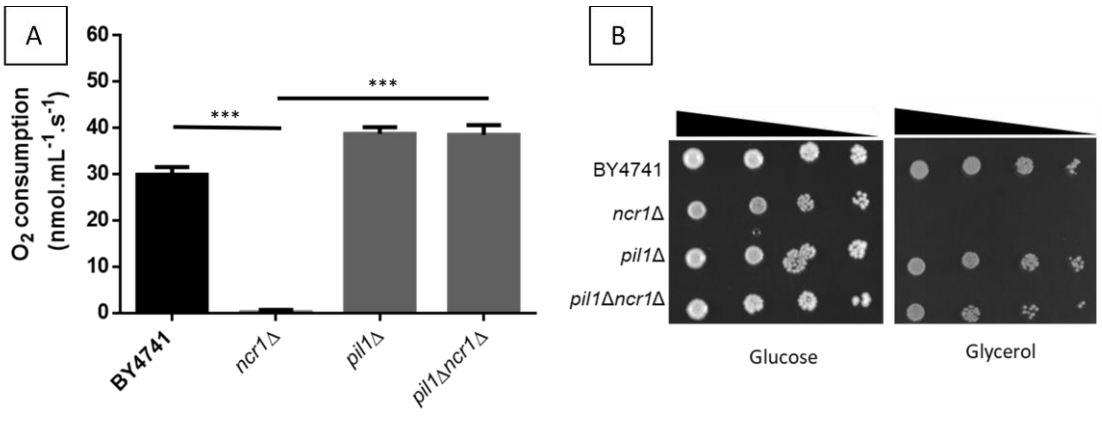


Figure 8 – **Eisosome disruption suppresses mitochondrial respiratory defects in *ncr1*Δ cells. A** - Yeast cells were grown in SC-glucose medium to Post-Diauxic Shift phase. Oxygen consumption measurements were done with $3x10^8$ cells resuspended in PBS buffer in a sealed chamber oxygen electrode at room temperature. Values are represented as a mean ± SD of three different experiments. *** - $p < 0.001$; One-way ANOVA and Bonferroni test **B** – Yeast cells were grown to log-phase in SC-glucose medium and 5-fold serial dilutions were plated in both SC-glucose and SC glycerol plates. One representative experiment is shown (out of six independent experiments).

Given the importance of Pil1 in the mitochondrial respiration defects, we also expected that the CLS of *ncr1*Δ would be ameliorated by *PIL1* deletion, similarly to the effect of *PKH1* and *YPK1* deletion in this model[128, 143]. As such, BY4741, *ncr1*Δ and the *PIL1*-null counterparts were grown for over 30 days and aliquots were periodically plated and counted for the number of colony forming units. Notably, we observed that disruption of eisosome organization led to the recovery of the CLS of *ncr1*Δ cells which is consistent with the previous results of mitochondrial function recovery (Figure 9).

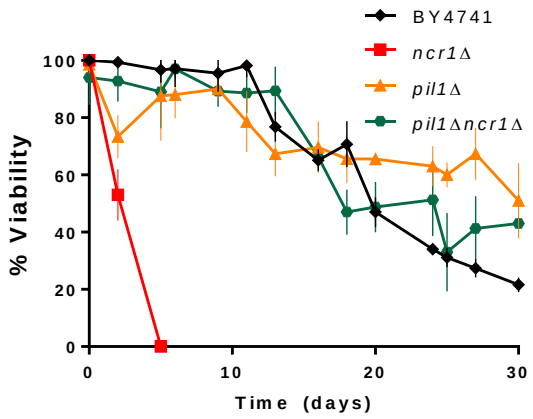


Figure 9 – **Eisosome-dependent signalling impacts cellular lifespan of *ncr1*Δ cells.** Yeast cells were grown overnight in SC-glucose, diluted to OD600=0.6 for over 30 days while aliquots were periodically collected and spotted in solid YPD media. Colony forming units were counted 24h after initial dilution (t=0). Values are represented as a mean ± SD of three different experiments.

### 3.1.3. Lag1 expression and subcellular localization is altered in *ncr1*Δ cells

As shown in the previous section, Pkh1-mediated phosphorylation of Ypk1 was increased in *ncr1*Δ. If such phosphorylation is consistent with Ypk1 activation, alterations in downstream targets of Ypk1 should be observed. We performed western blot analysis to examine alterations in the phosphorylation state of Lag1, a direct target of Ypk1. We fused a 9myc tag to the protein, as no commercial antibody is available to detect Lag1 and analysed the migration pattern in SDS-PAGE. The Lag1-9myc fusion protein was detected with an appropriate size (Figure 10). However, no observable differences in band mobility (shift to higher molecular weight) were detected in *ncr1*Δ cells compared with BY4741, even after treatment with acetic acid (positive control), an inducer of TORC2-Ypk1 activity[144]. Alternatively, we used cells expressing Lag1-GFP for the same purpose, but no differences were observed (data not shown). Other experimental conditions (regarding protein extraction or SDS-PAGE methodology) should be tested to improve the detection of the phosphorylated forms of the protein.

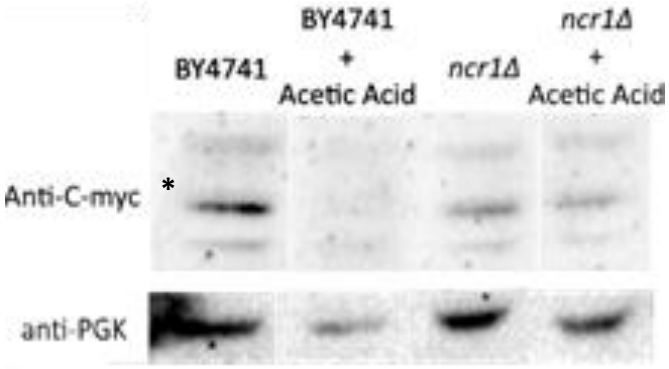


Figure 10 – **Analysis of Lag1 phosphorylation in *ncr1*Δ cells.** Yeast cells expressing Lag1-9myc were grown overnight in SC-glucose media to log phase were treated with 120 µM acetic acid for 60 min. A total protein extract was used for western blot analysis using an anti-c-myc antibody. Pgk1 was used as a loading control. * - Expected band for the Lag1-9myc protein (59kDa).

Regardless of the phosphorylation of Lag1, we searched for alterations in Lag1 subcellular location that could relate to the accumulation of sphingosine and ceramide species in *ncr1*Δ. As such, we examined the location of Lag1 in WT and *ncr1*Δ cells expressing a Lag1-GFP fusion protein. Cells were previously transformed with the FBp709 plasmid, which expresses a $KAR2_{(1\rightarrow135)}$-mCherry-HDEL, a red ER reporter. Surprisingly, we detected that Lag1-GFP forms punctate structures in the ER of *ncr1*Δ cells (Figure 11). Related to this result, another report showed that Lag1 forms punctate structures in the ER concomitantly with DHS and PHS accumulation[110]. Indeed, the results propose an alteration in the subcellular location of the ceramide synthase complex which could be affecting sphingolipid synthesis in NP-C1.

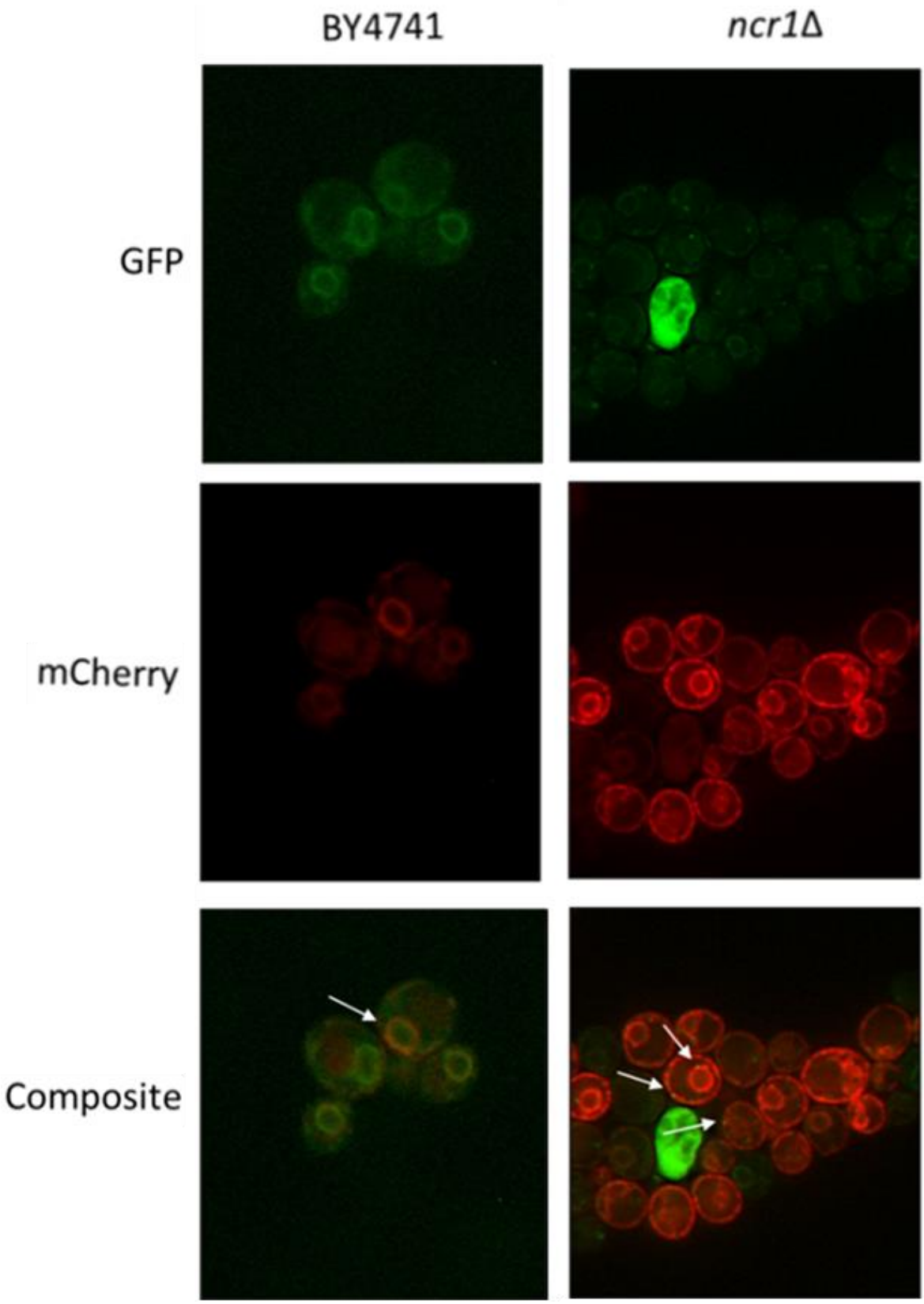


Figure 11 – **The localization of Lag1 is altered in *ncr1*Δ cells.** BY4741 and *ncr1*Δ cells with a genomically tagged Lag1-GFP and expressing a FBp709 plasmid (ER marker) were grown to PDS phase in SC-glucose media and observed with fluorescence microscopy. Removal of background fluorescence and composite images were done in ImageJ (1.52a).

Alterations in *LAG1* expression and protein levels in *ncr1*Δ have been previously described[129]. Yet, the mechanism underlying this upregulation is undetermined. We reasoned that the expression of enzymes related to the sphingolipid may be related with sphingolipid abundance. To access the role of Ypk1 in the regulation of *LAG1* expression the BY4741, *ncr1*Δ and their *YPK1*-null counterparts were transformed with a LacZ reporter under the promotor of LAG1 (FBp701 plasmid). The results show that *LAG1* expression increased 2-fold in *ncr1*Δ cells relative to the parental strain (Figure 12), consistent with previous reports[128]. *YPK1* deletion in the parental strain also upregulated *LAG1* expression. However, *YPK1* deletion in *ncr1*Δ cells did not further increase *LAG1* expression. These results suggest that Ypk1 regulates *LAG1* expression in the parental strain but the induction of *LAG1* in the yeast model of NP-C1 is Ypk1-independent.

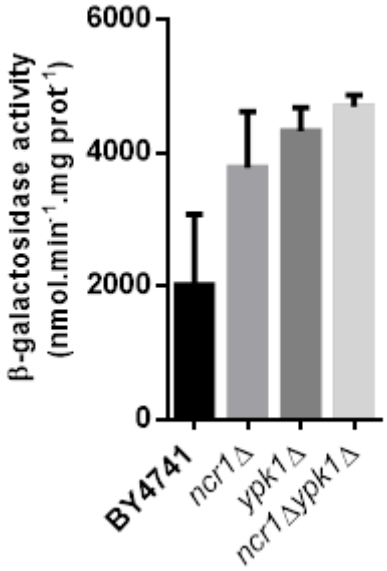


Figure 12 – **Upregulation of *LAG1* expression in *ncr1*Δ cells is Ypk1-independent.** Yeast cells expressing the lacZ gene under control of the *LAG1* promoter were grown overnight to log phase (OD600= 0.6-0.8) in SC-glucose lacking uracil. β-galactosidase activity was determined with 5 µg of total protein at 37°C for 25 min. Values are represented as a mean ± SD of three independent experiments.

# 3.2. Discussion

The current definition of NP-C1 is deep-seated in the fact that cholesterol and sphingolipid species have various alterations in abundance and subcellular location in human cells[1-3]. We became interested in how the alterations in sphingolipid abundance regulate cell survival in the context of the disease. For that purpose, we used a yeast model lacking *NCR1*, the yeast orthologue of the mammalian NPC1, as this model also presented sphingolipid accumulation and alterations in sphingolipid regulating proteins[128, 129].

Our results confirm that eisosomes are promoting the mitochondrial defects and shortened life span of *ncr1*Δ. Since eisosomes anchor Pkh1 to the PM, we propose that eisosomes act through Pkh1-Ypk1 signalling to promote the mitochondrial defects and shortened life span of *ncr1*Δ[56, 104, 128, 142]. Furthermore, since the upregulation of Pkh1-Ypk1 signalling was abolished by *PIL1* deletion, we also propose that there must be an upstream stimulus that is promoting Pkh1 activation by eisosomes. What could the upstream stimulus be? Given that TORC2 is also active in our model and that TORC2, Slm1/2 and Pkh1 are anchored to the membrane by PtdIns4,5P2, one pertinent subject that remains to be explored is the role of the PtdIns4,5P2 metabolism or subcellular location in Pkh1/TORC2 signalling and the effects of PtdIns4,5P2 synthesis inhibitors in in *ncr1*Δ.

The activation of Ypk1 in *ncr1*Δ cells led us to examine the phosphorylation of downstream targets of Ypk1 associated with sphingolipid metabolism. No alterations in Lag1 phosphorylation were observed between BY4741 or *ncr1*Δ cells. However, the method must be improved in order to clarify if Lag1 phosphorylation is altered or not in the mutant strain.

With the activation of Pkh1-Ypk1 and the generalized accumulation of sphingosine and ceramide species in *ncr1*Δ we questioned if this problem was derived only from alterations sphingolipid synthesis regulation. We found that the Lag1, a subunit of ceramide synthase is localized in small ER punctate structures in *ncr1*Δ cells. The results propose that other sphingolipid-related alterations that are not associated to cell signalling are affecting *ncr1*Δ sphingolipid accumulation. Moreover, the results propose that the accumulation of sphingolipids could also be originated from alterations in ceramide synthase expression, activity or location. Yet, the contribution of Lag1 subcellular structures to sphingolipid accumulation is unknown and is a relevant point for future research also because Lag1 seem to be a cross-talking point between calcium and TORC2/Pkh1-Ypk1 signalling. Notably, Lag1 is also phosphorylated by CK2, a

kinase that has been associated with DHS and PHS homeostasis and formation of Lag1 punctate structures in the ER that correlate with DHS and PHS accumulation in cells[110]. Thus, CK2 activity in *ncr1*Δ could be pertinent future study to understand the function of Lag1 subcellular structures. Regarding *LAG1* expression, we found that deletion of *YPK1* in the parental strain increased *LAG1* expression 2-fold but no major changes were observed when *YPK1* was deleted in *ncr1*Δ. Our findings conclude that regulation of *LAG1* expression by Ypk1 is indirect. With this, we propose that the increased in *LAG1* expression when YPK1 is deleted could be a cellular response to the shortage of sphingolipid species in the parental strain. Why deletion of *YPK1* in *ncr1*Δ has no effect in *LAG1* expression is unknown and a pertinent future inquiry to understand the regulation of Lag1 expression and its possible role in the sphingolipid abundance.

# 3.3. Calcineurin activation and its implication on the mitochondrial defects of *ncr1*Δ cells

## 3.3.1. Calcineurin-Crz1 pathway is mildly activated in *ncr1*Δ cells.

Human NP-C1 cells present a calcium homeostasis defect arising from an accumulation of sphingosine[38]. Strikingly, the accumulation of endogenous sphingosine-1-phosphate species promote an increase of cytosolic calcium that activates calcineurin[82]. We have previously observed that *ncr1*Δ cells are more sensitive to calcium in a calcineurin-dependent manner, as deletion of *CNB1* recovered cell growth under high calcium concentrations (unpublished data). Given the abnormal abundance of sphingosine species in *ncr1*Δ cells, we raised the hypothesis that calcineurin may be more activated in these cells contributing to cell death phenotypes of *ncr1*Δ cells. To evaluate this, we assessed the activity of calcineurin by transforming WT and *ncr1*Δ cells with a plasmid expressing β-galactosidase under the control of the Calcineurin Regulated Regulatory Element (CDRE) (FBp709 plasmid, kindly provided by Regina Menezes). This promoter is recognized by Crz1, a transcription factor directly activated by calcineurin. That way, β-galactosidase activity read-out is proportional to the activation of the calcineurin-Crz1 signalling pathway. The *cnb1*Δ and *cnb1*Δ*ncr1*Δ cells transformed with the same plasmid were used as negative control. In addition, cells were treated with 20 mM calcium for 15,30 and 60 min to stimulate calcineurin activity. A 2-fold increase in basal calcineurin activity was detected under no calcium stimulus (t = 0) between the parental and the *ncr1*Δ strain (Figure 13A). Both strains activated Crz1 in response to a calcium stimulus but for longer periods of treatment the differences in

activity between parental and *ncr1*Δ cells disappeared. Also, *cnb1*Δ and *cnb1*Δ*ncr1*Δ showed no β-galactosidase activity, consistent with the inactivation of calcineurin[145]. These results suggest a mild increase of calcineurin activity in *ncr1*Δ cells under normal growth conditions that is still sensitive to a calcium insult. The analysis of basal Cnb1 levels by western blot showed no differences between the parental strain and *ncr1*Δ cells (Figure 13B), indicating that the increase of calcineurin activity results from enzyme activation and is not due to an increase of Cnb1 levels.

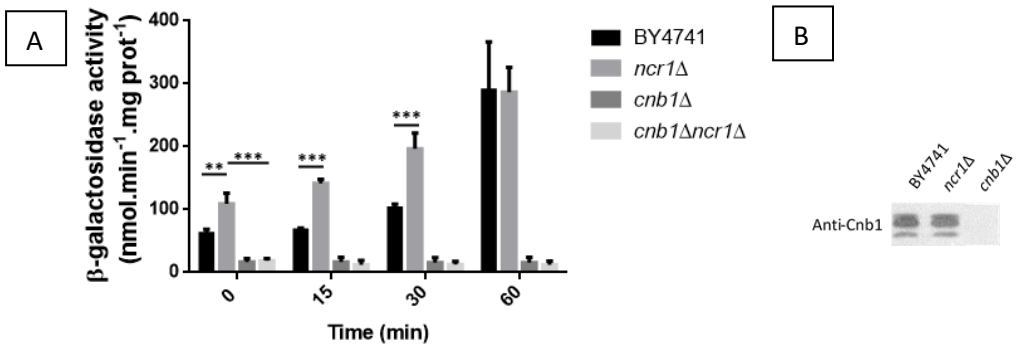


Figure 13 – **The calcineurin-Crz1 pathway is activated in *ncr1*Δ cells. A** – Yeast cells were grown to log-phase ($OD_{600}$= 0.6-0.8) in SC-Complete medium lacking uracil. Cells were subjected to a 20mM calcium stimulus for 0,15,30 or 60 mins before cell harvesting. β-galactosidase activity was determined with 50 µg of total protein, at 30° for 30 min. Values are represented as a mean ± SD of four independent experiments. ** - $p < 0.01$, *** - $p < 0.001$; Two-way ANOVA and Dunn's multiple comparison test. **B** – Yeast cells were grown overnight to log-phase in SC-Complete medium lacking uracil. Western blot analysis was done using an anti-cnb1 antibody.

## 3.3.2. Calcineurin does not regulate the dephosphorylation of Lag1

A previous report showed that Lag1/Lac1 phosphorylation was abolished by a 200 mM calcium stimulus and that deletion of both isoforms of the catalytic subunit of calcineurin - Cna1 and Cna2 - blocked the calcium-sensitive process[85]. To assess if the activation of calcineurin observed in *ncr1*Δ cells could regulate Lag1 activity, we analysed the phosphorylation state of Lag1 in WT and *ncr1*Δ cells. As a negative control, cells were also treated with FK506, a chemical inhibitor of calcineurin[146]. Yet, no difference between Lag1-9myc bands before and after treatment with FK506 were observed in WT and *ncr1*Δ cells (Figure 14). Still, these are preliminary data and further testing with other experimental conditions is required to improve the abundance of phosphorylated species.

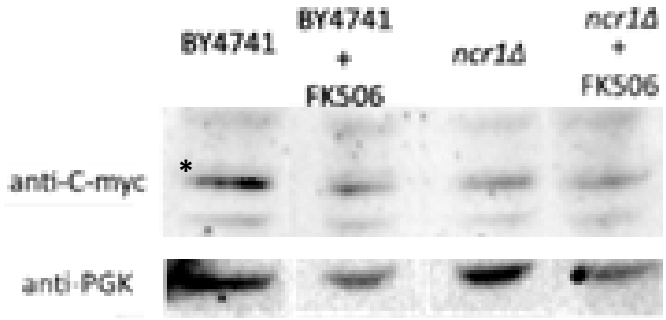


Figure 14 – **Inhibiton of calcineurin has no impact in the phosphorylation of Lag1 .** Yeast cells grown in SC-glucose media were treated with 50 µg/mL FK506 for 60 minutes. A total protein extract was used for western blot analysis using an anti-c-myc antibody. Pgk1 was used as a loading control. * - expected band for Lag1-9myc protein (59 kDa)

### 3.3.3. Abolishing calcineurin activity does not recover mitochondrial fitness in *ncr1*Δ.

We have previously shown that activation of Pkh1-Ypk1 pathway is detrimental to the mitochondrial respiratory function of *ncr1*Δ cells. Given that calcineurin is more activated in these cells, we questioned if this event could also impact mitochondrial function by affecting complex sphingolipid synthesis given the role of glycosphingolipids in the pathophysiological expression of NP-C1[35, 36]. As such, we measured the oxygen consumption rate and the growth on non-fermentable media of *ncr1*Δ cells and the *CNB1*-null counterpart, which has no calcineurin activity. The results show that deletion of *CNB1* did not revert the mitochondrial respiratory defects exhibited by the *ncr1*Δ cells (Figure 15). Thus, we conclude that calcineurin activity is not directly regulating the mitochondrial fitness of *ncr1*Δ cells.

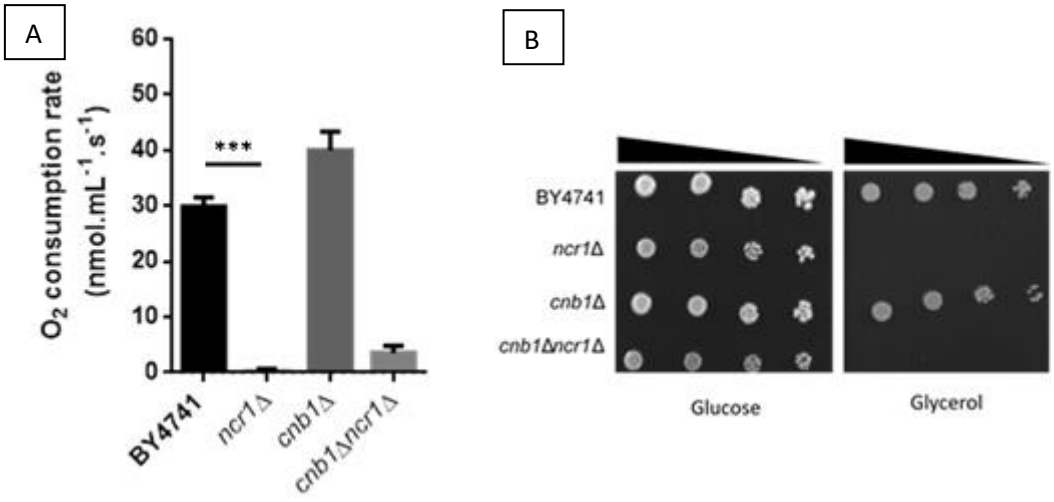


Figure 15 – **Suppression of calcineurin activity does not revert the mitochondrial respiratory defects of *ncr1*Δ cells. A** - Yeast cells were grown in SC-glucose medium to Post-Diauxic Shift. Oxygen consumption measurements were done with $3x10^8$ cells resuspended in PBS buffer in a sealed chamber oxygen electrode at room temperature. Values are represented as a mean ± SD of three different experiments. ***- $p < 0.001$; One-way ANOVA, Bonferroni test. **B** – Yeast cells were grown to log-phase in SC-glucose medium and 5-fold serial dilutions were plated in both Sc glucose and Sc glycerol plates. One of four representative experiments is shown.

### 3.3.4. Variations in the nuclear location of Crz1 were not discernible by standard fluorescence microscopy.

To explore the activation of Crz1 by calcineurin (see results above) in the yeast model of NP-C1, we further decided to assess its subcellular localization. We predicted that Crz1 accumulation in the nucleus, consistent with its activation, may be increased in *ncr1*Δ cells. Thus, the parental strain, *ncr1*Δ and their *CNB1*-null cells expressing a genomically tagged Crz1-GFP were stained with DAPI (for nucleus identification) and visualized with fluorescence microscopy. The expected co-localization of GFP signal with the nucleus was not significantly increased in *ncr1*Δ relative to WT (Figure 16) but the nuclear localization of Crz1 was reduced by *CNB1* deletion in both strains. Still, these are preliminary data and must be confirmed since the migration of Crz1 to the nucleus is highly sensitive to several stresses (reviewed in [118]). Further experiments are required to guarantee that sample manipulation and preparation has no effect on the migration of the transcription factor.

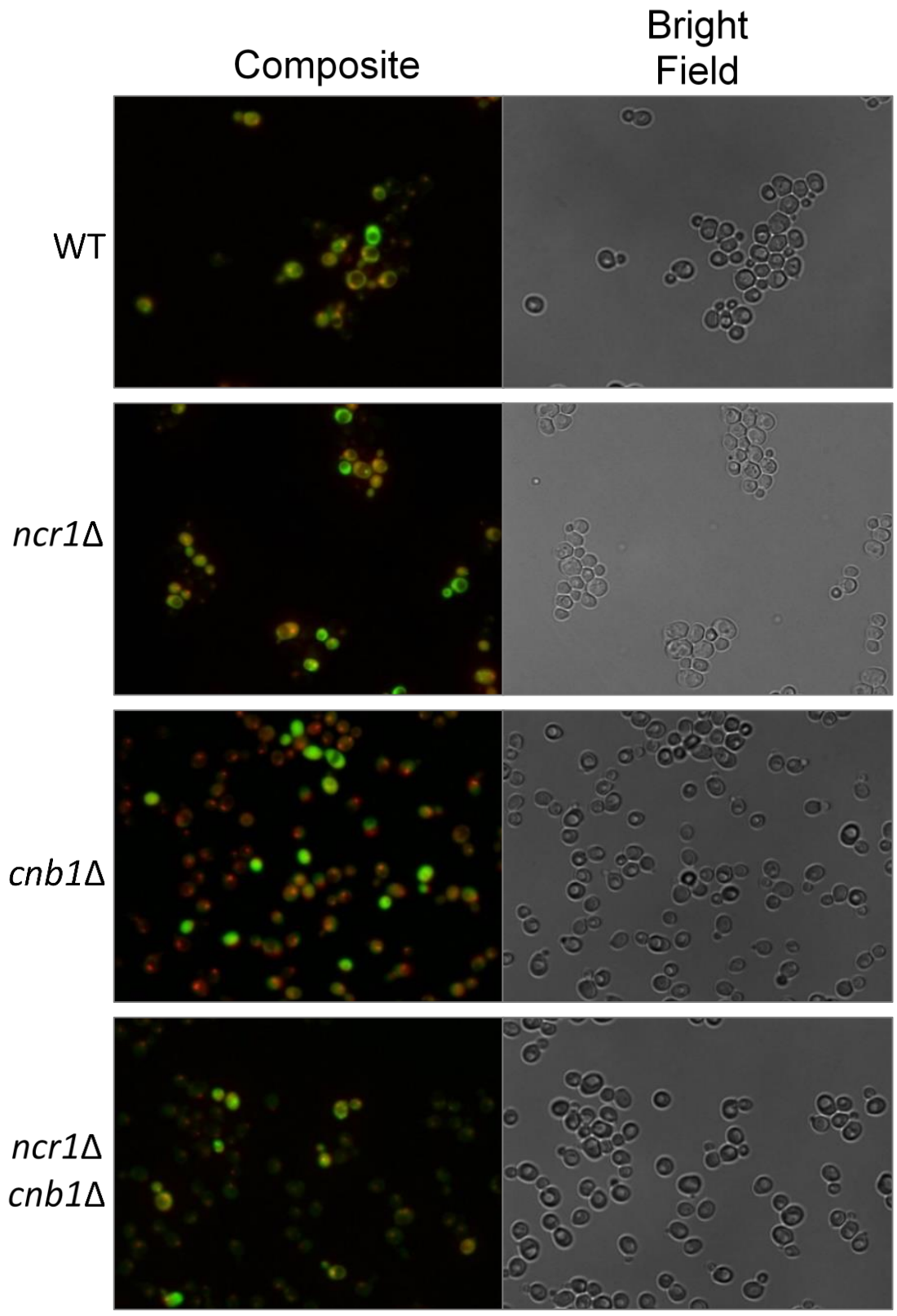


Figure 16 – **Effect of *NCR1* deletion on the nuclear location of Crz1.** *YYA3*, *ncr1*Δ and their cnb1-null counterparts expressing a genomic Crz1-GFP were grown to log phase in Sc-glucose media and stained with DAPI prior to fluorescence microscopy. DAPI colour was changed to red with imageJ to allow for easier identification of co-localization. Image editing was performed with ImageJ (1.52a).

# 3.4. Discussion

Calcineurin is a serine/threonine protein phosphatase conserved between yeast and mammals whose activation requires binding to a calcium-Calmodulin complex[145]. Lloyd-Evans et al showed that the cytosolic calcium levels of NP-C1 cells are altered due to sphingosine accumulation in lysosomes[38]. This was the first report linking sphingolipid abundance and calcium signalling in this disease. Others have reported a link between lysosomal calcium signalling and the activation of the human calcineurin[43]. This led us to pursue if calcium homeostasis was affected in *ncr1*Δ cells, which presents sphingolipid abundance alterations.

Using an indirect assay, we measured the calcineurin activation in *ncr1*Δ cells in normal growth conditions and under calcium treatment. The measurement of the β-galactosidase activity, which was under the control of a Crz1-regulated regulatory element, showed a 2-fold increase in calcineurin-Crz1 activation in *ncr1*Δ cells relative to the parental strain. Moreover, diminishing or upregulating calcineurin activity by *CNB1* deletion or calcium treatment respectively resulted in an equivalent alteration of β-galactosidase activity, consistent with other reports (Figure 13A)[145]. This lets us propose that calcineurin is more active in *ncr1*Δ cells. Furthermore, since we observed additional upregulation of calcineurin activity in *ncr1*Δ cells with a calcium stimulus, we propose that the phosphatase activity is only mildly increased under basal conditions. The levels of Cnb1, the regulatory subunit of calcineurin, were similar in *ncr1*Δ and parental cells. suggesting that the mild increase in calcineurin activity may be due to enzyme activation and not to protein overexpression.

Nevertheless, we cannot directly conclude that calcineurin is more active since Crz1 is regulated by phosphorylation events that may result from calcineurin activation or a kinase downregulation. For instance, Hrr25 is an ortholog to the mammalian Casein Kinase 1 that downregulates Crz1 activity and its nuclear migration[121]. Moreover, in yeast cells lacking Hrr25, calcineurin can still regulate Crz1 localization, indicating the involvement of other unknown kinases[121]. For this reason, further research is required to assess if Hrr25 or other kinases targeting Crz1 are altered in *ncr1*Δ cells and how it affects Crz1 location and activity. Moreover, it would be fundamental to measure the intracellular levels of calcium in *ncr1*Δ cells to directly relate with the activation of calcineurin.

Interestingly, calcineurin can also promote Lag1 dephosphorylation[85]. Indeed, it seems that Lag1 is a hub for various signalling pathways and understanding its regulation would allow us to better understand sphingolipid biosynthesis. To examine

Lag1 as a point of cross-talk with calcium signalling and its role in sphingolipid abundance in *ncr1*Δ, we evaluated if calcineurin could be affecting the activity of Lag1 kinases. Our preliminary results identified no differences in the phosphorylation of Lag1 between the WT and *ncr1*Δ (Figure 14). Still, this must be further confirmed. We reasoned that the protein extraction protocol should be improved before proceeding with any analysis in SDS-PAGE gels.

The downregulation of Lag1 activity and the increased *CSG1* and *CSG2* expression mediated by the calcineurin-Crz1 pathway indicates that calcineurin promotes the turnover of existing ceramides into complex sphingolipids[31, 85, 100, 101, 121] suggesting that calcineurin could be a regulator of complex sphingolipid synthesis. Moreover, our previous results with the deletion of *PIL1*, *PKH1* and *YPK1* in the recovery of the mitochondrial dysfunctions of *ncr1*Δ cells hint for a possible role of sphingolipids in the mitochondrial-related phenotypes. Yet, results revealed no recovery of oxygen consumption rates or growth on non-fermentable media by deleting *CNB1* in *ncr1*Δ cells (Figure 15A-B). We conclude that the mitochondrial defects exhibited by the yeast model are independent of calcineurin activity. Given the role of calcineurin in the regulation of complex sphingolipid synthesis, proposing that complex sphingolipids do not affect these mitochondrial phenotypes is possible.

The subcellular localization of Crz1 is controlled by calcineurin activity and its translocation to the nucleus occurs in short 2-minute bursts whose frequency is modulated by the intensity of the calcium stimulus[122, 145, 147, 148]. We looked for subcellular alterations in Crz1 location given that calcineurin-Crz1 pathway was upregulated in *ncr1*Δ cells. Yet, by standard fluorescence microscopy, no discernible difference was observed in the localization of a GFP tagged Crz1 between WT and *ncr1*Δ cells. We reasoned that the increase in Crz1 migration into the nucleus is not sufficiently detectable because of the mild activation of calcineurin paired with the sporadic and short retention of Crz1 in the nucleus that was documented previously[122]. Also, calcineurin is responsive to a wide array of stimuli, including pH, blue light and temperature and additional controls are required to confirm that Crz1 nuclear levels are not modified during the assay (reviewed in [118]).

For this reason, an improvement of the sample manipulation for observation under the fluorescence microscope must be considered in future studies to assure that this step does not compromise the transcription factor activation. Also, an appropriate detection of Crz1 location under mild calcium stimulus would require time-lapse

microscopy techniques and sufficient resolution to evaluate differences in Crz1 frequency migration into the nucleus.

# Chapter 4

# CONCLUSIONS AND FUTURE PRESPECTIVES

# Conclusions and future perspectives

The exuberance of sphingolipids in NP-C1 partakes in cellular alterations detrimental to normal cell function that are pivotal for the pathophysiological expression of the disease not only humans but also in simple eukaryotes like *S. cerevisiae*. Because sphingolipid synthesis and regulation are more characterized in yeast, a yeast model for NP-C1 potentiates sphingolipid research not currently possible in humans.

Identifying that activation of Pkh1-Ypk1 signalling and consequent mitochondrial defects are mediated by eisosomes in *ncr1*Δ cells brings us closer to the identification of the source of the dysregulated signalling that targets sphingolipid synthesis induction. A shared trait between Ypk1 regulators (TORC2, Pkh1, Slm1/2) is the necessity of PtdIns4,5P2 species to promote anchoring to the plasma membrane. This raised the question of what is the role of PtdIns4,5P2 metabolism or their subcellular location in *ncr1*Δ. If these lipids have some role in the disease, this may be an open way to identify novel drugs that inhibit PtdIns4,5P2 synthesis or antagonize PtdIns4,5P2-PH domain interactions to treat NP-C1. Indeed, future investigation of PtdIns4,5P2 dynamics and its role in the disease using yeast as a model might serve to improve the current therapeutics available for NP-C1 in humans.

Observing that Lag1 subcellular location in the ER forms punctate structures showed that alterations in the sphingolipid synthesis exhibited by *ncr1*Δ might not result only from TORC2/Pkh1-Ypk1 signalling. This is aided by the observation that *LAG1* is more expressed in *ncr1*Δ by an Ypk1-indirect mechanism. Lag1 is a target of various kinases and phosphatases and the fact that it localizes in ER punctate structures in *ncr1*Δ suggest that others signalling pathways are regulating sphingolipid abundance and metabolism.

Proposing that calcineurin-Crz1 signalling is active in *ncr1*Δ is strongly suggested by our results but still warrant for more analysis of other direct targets of calcineurin because Crz1 is regulated by various kinases and their contribution to Crz1 nuclear migration is unknown. The fact that Crz1 is promoting a transcriptional response in *ncr1*Δ is the only conclusion taken from our results and urge the need to study cytosolic calcium to conclude about calcineurin activation.

# Chapter 5

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