

Regulation of the yeast lysosome-like vacuole and its role in lifespan

Telma Filipa da Silva Martins

D
2024



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D.ICBAS 2024

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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Regulation of the yeast lysosome-like vacuole and its role in lifespan

Tese de Candidatura ao grau de Doutor em Biologia Molecular e Celular;

Programa doutoral da Universidade do Porto (Instituto de Ciências Biomédicas Abel Salazar e Faculdade de Ciências)

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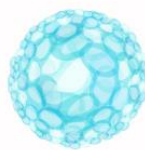
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Financial support

This work was funded by national funds through FCT - Fundação para a Ciência e a Tecnologia, I.P., under the project UIDB/04293/2020. The author was supported by a PhD fellowship from FCT (SFRH/BD/136996/2018 and COVID/BD/153217/2023).



DOCTORAL PROGRAM:
Molecular and Cell Biology
Programa Doutoral em Biologia Molecular e Celular

List of publications

Esta tese de doutoramento inclui dados científicos já publicados em revistas internacionais indexadas. A autora declara que esteve ativamente envolvida na conceção e execução das experiências que estiveram na origem dos dados, bem como na sua análise, interpretação e discussão, e na escrita dos manuscritos.

Publicações incluídas no corpo principal desta tese:

Telma Silva Martins, Rafaela S. Costa, Rita Vilaça, Carolina Lemos, Vitor Teixeira, Clara Pereira, Vítor Costa. Iron Limitation Restores Autophagy and Increases Lifespan in the Yeast Model of Niemann-Pick Type C1. *Int J Mol Sci* 2023, 24, 6221. <https://doi.org/10.3390/ijms24076221>

Telma Silva Martins, Miguel Correia, Denise Pinheiro, Carolina Lemos, Marta Vaz Mendes, Clara Pereira, Vítor Costa. Sit4 Genetically Interacts with Vps27 to Regulate Mitochondrial Function and Lifespan in *Saccharomyces cerevisiae*. *Cells* 2024, 13, 655. <https://doi.org/10.3390/cells13080655>

This doctoral thesis includes scientific data already published in international peer-reviewed journals. The author declares that she was actively involved in conceiving and executing the experiments that generated the data, as well as in their analysis, interpretation, and discussion, and in writing the manuscripts.

Publications included in the main body of this thesis:

Telma Silva Martins, Rafaela S. Costa, Rita Vilaça, Carolina Lemos, Vitor Teixeira, Clara Pereira, Vítor Costa. Iron Limitation Restores Autophagy and Increases Lifespan in the Yeast Model of Niemann-Pick Type C1. *Int J Mol Sci* 2023, 24, 6221. <https://doi.org/10.3390/ijms24076221>

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Agradecimentos

No final desta jornada, não posso deixar de agradecer a todas as pessoas que contribuíram para que eu hoje chegasse até aqui. Antes de mais, quero agradecer ao meu orientador Vítor Costa e à minha co-orientadora Clara Pereira, por todo o apoio, compreensão e amizade ao longo destes anos. Obrigada por terem acreditado em mim e por tudo o que me ensinaram. Quero também agradecer a todos os que passaram pelo laboratório 207 por sempre contribuírem para um bom ambiente. Um especial agradecimento ao Vitor Teixeira, pela paciência e amizade, por todas as discussões de trabalho que foram fundamentais para o meu percurso e por estares sempre disposto a responder às minhas dúvidas existenciais. Quero também agradecer especialmente ao Miguel e à Rafaela, que contribuíram diretamente para este trabalho. Foram uns excelentes alunos e colegas, aprendi imenso convosco! Muito obrigada pela vossa amizade! Um beijinho muito especial à Denise e à Mariana. Denise, obrigada pela tua contribuição nesta última fase e por estares sempre disposta a ajudar. Mariana, obrigada por me encheres as caixas com pontas, principalmente naquelas semanas caóticas de revisões do artigo! Quero também deixar um especial agradecimento à Cláudia, que sempre se disponibilizou para me ajudar e fez com que eu não me sentisse só nesta jornada!

A todos os membros do grupo Phenotypic Evolution, que sempre contribuíram para o bom ambiente do laboratório. À Cristina Vieira e ao Jorge Vieira, que estiveram sempre disponíveis para me ajudar e me fornecerem a “água de PCR maravilha” que me permitiu alcançar um PCR perfeito quando eu já estava a desesperar. Ao André, Pedro Duque, Pedro Ferreira e Ricardo pelo vosso companheirismo, boa disposição e ajuda. André, obrigada pelas tuas piadas e por estares sempre preocupado comigo. Cuida bem de ti! Ao Bruno, por estar sempre disposto a ajudar, foi bom partilhar estes últimos tempos contigo.

Quero também agradecer às pessoas que trabalham nos serviços do i3s e que estiveram sempre prontas a ajudar-me. Um especial agradecimento ao Hugo Osório, à Fátima Fonseca, ao Frederico Silva, à Tânia Meireles e à Paula Magalhães.

Por fim, quero agradecer aos pilares da minha vida, pois sem eles não sei se conseguiria chegar ao fim desta jornada. Aos meus pais, que sempre fizeram tudo por mim e, se hoje atingi este objetivo, é graças a eles. Obrigada pela vossa ajuda! Ao companheiro da minha vida, por estar sempre ao meu lado, por me dar força sempre que precisei e pela paciência nos dias menos bons. Se cheguei aqui, também foi por ti, Mário. Por último, e

mais importante do que tudo, quero agradecer ao meu amor mais “pequenino”, ao meu Tomás. Foste, sem dúvida, a pessoa que mais energia me deu para terminar este capítulo da minha vida. Mesmo sem saber e mesmo com as horas de sono que me tiraste nestes últimos anos, foste a maior força motriz desta etapa da minha vida. Espero que um dia te orgulhes disso.

OBRIGADA

Abstract

Lysosomes and vacuoles (in yeast) are organelles critical for cell fitness and longevity. They serve as storage compartments, have catabolic functions that prevent the accumulation of damaged molecules and organelles, and play key roles in cell adaptation to stress conditions and in the regulation of signaling pathways. Conversely, these organelles are also subject to modulation by conserved nutrient signaling pathways to ensure proper cell development and normal aging. In fact, downregulation of nutrient signaling pathways leads to lifespan extension by improving lysosomal and vacuolar function, highlighting their importance in cellular health and longevity. Declining lysosomal function is linked to aging, age-related diseases, and lysosomal storage diseases such as the Niemann-Pick type C (NPC) disease. NPC is caused by mutations in NPC1 (in 95% of cases) or NPC2 proteins, which are involved in the export of lipids from lysosomes. NPC1 or NPC2 loss of function causes aberrant accumulation of cholesterol and sphingolipids in the late endosomal/lysosomal network, impaired autophagy, mitochondrial dysfunction, and ion dyshomeostasis, leading to progressive neurodegeneration.

Despite the clear association between lysosomal function, aging, and disease, the regulation of lysosomal proteins in these contexts remains poorly understood. The budding yeast *Saccharomyces cerevisiae* is a well-established model organism for studying the molecular mechanisms underlying aging and the NPC disease. In this thesis, to identify proteins and pathways critical for vacuolar function and lifespan, we performed a proteomic analysis of vacuolar membranes from *ncr1* Δ cells, a yeast model of NPC1 that exhibits premature aging, and *sit4* Δ cells, a model of extended longevity. In cells lacking Ncr1, we found alterations in proteins associated with vesicle trafficking, autophagy and ion homeostasis. An in-silico analysis for potential regulators of genes encoding vacuolar proteins altered in *ncr1* Δ cells showed an enrichment in transcription factors regulating sulfur metabolism. The production of H₂S, a pro-longevity metabolite, decreased in *ncr1* Δ cells, but increasing H₂S levels did not extend *ncr1* Δ lifespan. Consistent with changes in proteins associated with intracellular trafficking, the *ncr1* Δ cells displayed increased induction of the cytoplasm to vacuole targeting (Cvt) pathway and compromised autophagy, which was not associated with the activation of TORC1 or inhibition of Snf1. Interestingly, we found that iron accumulation, associated with the activation of the Aft1 transcription factor, impairs autophagy and increases oxidative stress sensitivity in *ncr1* Δ cells. Iron chelation alleviated these phenotypes, extending *ncr1* Δ chronological lifespan. This study demonstrated that iron toxicity mediates the phenotypes observed in the yeast model of NPC1 and may be a relevant factor contributing to human NPC disease.

Sit4 is a PP2A-like Ser/Thr protein phosphatase, involved in the regulation of several cellular processes, including oxidative stress resistance, mitochondrial derepression, vacuolar acidification and longevity. In this study, the proteomic analysis of vacuolar membranes from *sit4Δ* cells revealed alterations in proteins associated with ion homeostasis, polyphosphate metabolism, vacuole fusion, vacuole and Golgi organization, autophagy, and late endosome to vacuole transport. In agreement, we found that Sit4 deficiency affects several trafficking pathways. Mitophagy and the Cvt pathway were induced, whereas autophagy, microautophagy and the multivesicular body pathway were decreased in *sit4Δ* cells. Moreover, we found that Vps27, a component of the endosomal sorting complex required for transport-0, was enriched in *sit4Δ* vacuolar membranes and was vital for *sit4Δ* lifespan extension. Vps27 did not extend the lifespan of *sit4Δ* cells by promoting vacuolar sorting pathways. However, *VPS27* deletion negatively impacted the vacuolar protein sorting pathway, potentially contributing to the premature aging of *sit4Δvps27Δ* cells. Vps27 was also essential for maintaining iron homeostasis in *sit4Δ* cells, but the increase of iron levels did not appear to be linked to the decrease of *sit4Δvps27Δ* lifespan. Interestingly, Vps27 was critical for the regulation of the oxidative phosphorylation proteins Atp2 and Cox2 and, consequently, for the mitochondrial respiration in *sit4Δ* cells. This study demonstrated, for the first time, a crosstalk between Vps27 and Sit4 in mitochondrial derepression, thereby contributing to our understanding of the molecular mechanisms underlying lifespan extension.

Resumo

Os lisossomas e os vacúolos em levedura são organelos críticos para a homeostasia e longevidade celular. Estes funcionam como compartimentos de armazenamento, têm funções catabólicas que evitam a acumulação de moléculas e organelos danificados, e desempenham funções importantes na adaptação das células às condições de stresse e na regulação das vias de sinalização. Por outro lado, estes organelos também estão sujeitos à modulação por vias conservadas de sinalização por nutrientes para garantir o desenvolvimento celular adequado e o envelhecimento normal. Na verdade, a inibição de vias de sinalização por nutrientes aumenta o tempo de vida através da melhoria da função lisossomal e vacuolar, demonstrando a sua importância para a função celular e a longevidade. A diminuição da função lisossomal está relacionada com o envelhecimento, com doenças associadas ao envelhecimento e com doenças lisossomais de sobrecarga, como a doença de Niemann-Pick tipo C (NPC). A doença NPC é causada por mutações nas proteínas NPC1 (em 95% dos casos) ou NPC2, estando ambas envolvidas no transporte de lípidos dos lisossomas. A perda de função das proteínas NPC1 ou NPC2 causa uma acumulação anormal de colesterol e esfingolípidos nos endossomas tardios e lisossomas, afeta a autofagia, a função mitocondrial e a homeostasia iónica, levando à neurodegeneração progressiva.

Apesar da clara associação entre a função lisossomal, envelhecimento e doenças, a regulação das proteínas lisossomais nestes contextos permanece pouco estudada. A levedura *Saccharomyces cerevisiae* é um organismo modelo bem estabelecido para estudar os mecanismos moleculares subjacentes ao envelhecimento e à doença NPC. Nesta tese, para identificar proteínas e vias críticas para a função vacuolar e longevidade, realizamos uma análise proteómica das membranas vacuolares das células *ncr1Δ*, o modelo de levedura da doença NPC1 que apresenta envelhecimento prematuro, e das células *sit4Δ*, um modelo de longevidade prolongada. Nas células sem Ncr1, encontramos alterações em proteínas associadas ao tráfego de vesículas, autofagia e homeostasia iónica. Uma análise in-silico para potenciais reguladores de genes que codificam proteínas vacuolares alteradas nas células *ncr1Δ* mostrou um enriquecimento em fatores de transcrição que regulam o metabolismo do enxofre. A produção de H₂S, um metabolito promotor da longevidade, diminuiu nas células *ncr1Δ*, mas o aumento dos níveis de H₂S não levou a um aumento do tempo de vida das células *ncr1Δ*. De acordo com as alterações nas proteínas associadas ao tráfego intracelular, as células *ncr1Δ* mostraram um aumento da indução da via de transporte de proteínas do citoplasma para o vacúolo e um comprometimento da autofagia, que não foi associado à ativação do complexo TORC1 ou à inibição da cinase Snf1. Curiosamente, descobrimos que a acumulação de ferro,

associada à ativação do fator de transcrição Aft1, afeta negativamente a autofagia e aumenta a sensibilidade ao stresse oxidativo nas células *ncr1Δ*. A quelatação de ferro aliviou estes fenótipos, aumentando o tempo de vida cronológico do mutante *ncr1Δ*. Este estudo demonstrou que a toxicidade do ferro medeia os fenótipos observados no modelo de levedura da doença NPC1 e pode ser um fator relevante que contribui para a doença de NPC nos humanos.

Sit4 é uma Ser/Thr proteína fosfatase do tipo PP2A, envolvida na regulação de vários processos celulares, incluindo resistência ao stresse oxidativo, desrepressão mitocondrial, acidificação vacuolar e longevidade. Neste estudo, a análise proteômica das membranas vacuolares das células *sit4Δ* revelou alterações em proteínas associadas à homeostasia iónica, metabolismo do polifosfato, fusão do vacúolo, organização do vacúolo e do Golgi, autofagia e transporte dos endossomas tardios para o vacúolo. De acordo com estes resultados, descobrimos que a ausência da fosfatase Sit4 afeta várias vias de tráfego. A indução da mitofagia e da via de transporte do citoplasma para o vacúolo aumentou, enquanto a indução da autofagia, microautofagia e a via de transporte através de corpos multivesiculares diminuíram nas células *sit4Δ*. Além disso, descobrimos que a proteína Vps27, um componente do complexo ESCRT-0 (do inglês endosomal sorting complex required for transport-0), estava enriquecida nas membranas vacuolares das células *sit4Δ* e foi vital para o aumento do tempo de vida destas células. A proteína Vps27 não prolongou o tempo de vida das células *sit4Δ* promovendo vias de transporte vacuolar. No entanto, a deleção do gene *VPS27* afetou negativamente a via VPS/CPY (vacuolar protein sorting), podendo esta alteração contribuir para o envelhecimento prematuro das células *sit4Δvps27Δ*. A proteína Vps27 também foi essencial para manter a homeostasia do ferro nas células *sit4Δ*, mas o aumento dos níveis de ferro não parece estar relacionado com a diminuição do tempo de vida das células *sit4Δvps27Δ*.

Curiosamente, a proteína Vps27 foi crítica para a regulação das proteínas envolvidas na fosforilação oxidativa Atp2 e Cox2 e, consequentemente, para a respiração mitocondrial nas células *sit4Δ*. Este estudo demonstrou, pela primeira vez, uma ligação entre as proteínas Vps27 e Sit4 na desrepressão mitocondrial, contribuindo assim para a nossa compreensão dos mecanismos moleculares subjacentes à regulação da longevidade.

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Abbreviations

Acc1 - acetyl-CoA carboxylase
ADP - adenosine diphosphate
AMP – adenosine monophosphate
AMPK - AMP-activated protein kinase
Ams1 - α -mannosidase
Ape1 - aminopeptidase I
Ape4 - aspartyl aminopeptidase
AP-3/ALP pathway - alkaline phosphatase pathway
ATP - adenosine triphosphate
BPS - bathophenanthrolinedisulfonic acid
cAMP - cyclic AMP
CDCFDA - 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate N-succinimidyl ester
CFUs - colony-forming units
CLS - chronological lifespan
CPY - carboxypeptidase Y
CR - caloric restriction
Cvt - cytoplasm to vacuole targeting
DFP - deferiprone
DHR123 - dihydrorhodamine 123
DHS - dihydrosphingosine
DMSO - dimethylsulfoxide
DR - dietary restriction
EDTA - ethylenediamine tetraacetic acid
ER - endoplasmic reticulum
ESCRT - endosomal sorting complex required for transport
FDR - false Discovery Rate
GABA - gamma-aminobutyric acid
GAPs - GTPase activating proteins
GDP - guanosine diphosphate
GEFs - guanine nucleotide exchange factors
GO - gene Ontology
GTP - guanosine triphosphate
HPLC-MS/MS - High-performance liquid chromatography coupled to tandem mass spectrometry

ISC - iron sulfur clusters
LE/Lys - late endosomes/lysosomes
Log phase – exponential phase
LSDs - lysosomal storage disorders
mApe1 - mature Ape1
mCPY - mature CPY
MM - minimal medium
mtDNA - mitochondrial DNA
mTORC1 - mammalian TORC1
MVB - multivesicular body
nanoLC-MS/MS – nanoscale liquid chromatography coupled to tandem mass spectrometry
NPC - Niemann-Pick type C
NPC1 - Niemann–Pick type C1
NPC2 - Niemann–Pick type C2
NTCB - 2-nitro-5-thiocyanatobenzoic acid
OCR - oxygen consumption rate
OD - optical density
PAGE - polyacrylamide gel electrophoresis
PAS - pre-autophagosomal structure
PBS - phosphate-buffered saline
PDS – post-diauxic shift
PHS - phytosphingosine
PhytoCer - phytoceramide
PI3P - phosphatidylinositol-3-phosphate
PKA - cAMP-dependent protein kinase A
polyP - polyphosphate
PP6 - protein phosphatase 6
prApe1 - precursor form of Ape1
prCPY - precursor form of CPY
qPCR - quantitative PCR
Rap – rapamycin
ROS - reactive oxygen species
Rpm – revolutions per minute
Rps6 - ribosomal protein S6
Sak1 - Snf1 Activating Kinase 1
SAPs - Sit4 associated proteins

SC - synthetic complete
SDS - sodium dodecyl sulphate
SD - standard deviation
SEM - standard error of the mean
SMPD1 - sphingomyelin phosphodiesterase 1
SNARE - soluble NSF attachment receptor
SPSS - Statistical Package for the Social Sciences
SP3 - solid-phase-enhanced sample-preparation
TCA - trichloroacetic acid
TEAB - triethylammonium bicarbonate
TGN - trans-Golgi network
TOR – target of rapamycin
TORC1 - TOR Complex 1
TFs – transcription factors
VPS - vacuolar protein sorting
YEASTRACT - Yeast Search for Transcriptional Regulators And Consensus
Tracking
YPD - yeast extract peptone dextrose
YPG - yeast extract peptone glycerol
vCLAMP - vacuole and mitochondria patch
VTC - vacuolar transporter chaperone
V-ATPase - vacuolar H⁺-ATPase

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CHAPTER I

General introduction

1.1 Yeast as a eukaryotic model organism

The yeast *Saccharomyces cerevisiae* was the first eukaryotic organism to have its genome fully sequenced in 1996 [1] and stands out as one of the most extensively studied model organisms [2]. This single-cell eukaryotic organism is easy and relatively inexpensive to cultivate in the laboratory, as it does not require complex growth medium or conditions. Its rapid growth rate and suitability to genetic manipulation allow for straightforward genetic modifications through plasmid expression or the integration of DNA fragments at precise genomic loci via homologous recombination, making it especially convenient for research. Furthermore, there are several essential tools available for studying the link between specific genes and their cellular functions in yeast. These tools include collections of yeast deletion mutants [3,4], GFP-tagged yeast strains [5,6], temperature-sensitive mutants [7], and an overexpression library of *S. cerevisiae* genes [8]. Additionally, numerous databases support yeast research, with one notable example being the *Saccharomyces* Genome Database (SGD, <http://www.yeastgenome.org>) [9,10].

S. cerevisiae has been used as a model to study a plethora of biological processes, including cell cycle, signal transduction, metabolism, apoptosis, vesicular transport, protein folding and quality control, and stress-response [11]. Key regulators of autophagy, vesicle trafficking and the cell cycle have been firstly identified in yeast cells [2]. Yeast and human proteins show an average amino acid identity of about 32%, and there are human orthologs for 47% of the genes that are indispensable for yeast survival [12]. Furthermore, 22% of genes implicated in human diseases have orthologs in yeast, and the introduction of many of these genes into yeast deletion strains can effectively restore their lost functions [9,11,13]. Consequently, yeast has been useful to study fundamental aspects of human diseases, including cancer, neurodegenerative diseases, and mitochondrial disorders.

Yeast cells have also been used to study the biological function of disease-associated human proteins that do not have functional counterparts in yeast, by heterologous expression of human proteins in yeast [9,14]. These studies are designated as humanized yeast systems. This approach has been used to study the molecular mechanisms underlying those diseases and to identify potential regulators that may attenuate the phenotypes of pathologies, such as Parkinson's disease and Huntington's disease [9,14]. For instance, humanized yeast has enabled the identification of N-aryl benzimidazole as a protective agent against α -synuclein toxicity in the yeast model of Parkinson's disease [15]. This approach also revealed the molecular mechanisms that mitigate the toxic effects of α -synuclein. Additionally, the enzyme kynurenine 3-monooxygenase was identified as a new therapeutic target for Huntington's disease [16]. Humanized yeast systems have made significant contributions to our understanding of

cancer research as well [9,14]. For instance, heterologous expression of p53, BRCA1, and BRCA2 mammalian tumor suppressors in yeast have been contributing to dissecting the molecular mechanisms underlying their functions [17]. A mutagenesis screen in yeast has identified mutations in p53 that result in growth suppression or lethality, thereby potentially guiding the development of novel cancer therapies [18].

The yeast *S. cerevisiae* is also a well-established model in aging research since the hallmarks of aging are well conserved in this cell model [19]. Several key pathways and regulators of aging processes were first characterized in yeast, along with the discovery of anti-aging strategies. Yeast has been used to study two aspects of lifespan: replicative lifespan (RLS), which refers to the number of buds generated by each mother cell before reaching senescence, and chronological lifespan (CLS), which refers to the survival of non-dividing cells overtime [20]. RLS and CLS are recognized as aging models for mitotic and post-mitotic cells, respectively, offering valuable insights into the aging process.

Despite the advantages discussed above, the yeast model also presents limitations that should also be considered. Being an unicellular organism, yeast cells are much less complex than humans and some pathways and mechanisms important for human cells (e.g., intercellular communication) and tissues do not exist in yeast [11]. Regardless the high degree of conservation, we can observe phenotypes that are uniquely associated with yeast when we are studying orthologous proteins [11].

1.2 Aging in yeast

Aging is characterized by the progressive accumulation of cellular damages that leads to loss of functions, tissue failure, and increased susceptibility to the development of diseases and the probability of death. It is associated with numerous changes that are considered hallmarks of aging, such as genomic instability, telomere shortening, epigenetic alterations, loss of protein homeostasis, impaired autophagy, deregulation of nutrient sensing, mitochondrial malfunction, cellular senescence, stem cell depletion, altered intercellular communication, long-term inflammation and dysbiosis [21]. Aging is a complex process, and the interconnection of these hallmarks contributes to the accumulation of cellular damage and loss of homeostasis.

The efficiency of DNA repair mechanisms decreases with age, resulting in increased accumulation of genomic damages induced by either external or internal factors contributing to aging [21]. On the other hand, improved DNA repair mechanisms are associated with increased longevity. For instance, increased activity of SIRT6 protein was associated with increased longevity by improving DNA double-strand break repair [22]. As organisms age, telomeres naturally shorten, a feature of aging observed across various species [21].

Accordingly, activating telomerase, a ribonucleoprotein responsible for maintaining telomere length, delays aging in mouse models [23,24]. Several epigenetic changes associated with aging have also been identified, including DNA methylation variations, unconventional posttranslational modifications of histones, irregular chromatin remodeling, and impaired function of non-coding RNAs [21].

Disturbances in protein homeostasis are associated with aging and age-related diseases, whereas enhancing proteostasis can decelerate the aging process [21,25]. Proteostasis relies on mechanisms that stabilize and ensure proper folding of proteins, as well as on mechanisms that facilitate protein degradation. Treatment with exogenous chaperones and increased proteasome activity are linked to improved cognitive function and extended lifespan in mice [26,27]. Boosting protein degradation through lysosomes, specifically via chaperone-mediated autophagy, alleviates pathology in two distinct Alzheimer's disease mouse models [28]. A decline in autophagy also occurs with aging in many organisms, contributing not only to the loss of proteostasis but also to the accumulation of dysfunctional organelles, such as mitochondria [21,29,30]. Additionally, blocking autophagy accelerates aging in model organisms, while promoting it extends both lifespan and health span. Mitochondrial function declines with age due to decreased turnover via autophagy, loss of proteostasis causing the destabilization of respiratory chain complexes, and accumulation of mtDNA mutations [21,31]. These changes affect cellular bioenergetics and increase oxidative stress [21,31].

Several of these hallmarks of aging are conserved in yeast and will be discussed here. Genomic instability and epigenetic modifications have been shown to exert substantial effects on yeast lifespan [19,32,33]. Extrachromosomal rDNA circles (ERCs) accumulate in yeast old mother cells and its accumulation is associated with decreased RLS [34], while decreasing the formation of ERCs extends lifespan [35]. The histone deacetylase Sir2 plays a key role in the regulation of ERCs and histones. The levels of histones [36] and Sir2 [37] decrease during aging whereas the increase of histone levels [36] or the overexpression of Sir2 [37,38] increases yeast RLS. The deletion of *ASF1* that encodes for a central chaperone of histones H3 and H4 also decreases CLS [36]. The regulation of RLS by Sir2 is associated with various cellular processes, including the suppression of ERCs formation [38], maintenance of rDNA stability, asymmetric retention of damaged proteins in mother cells [39], epigenetic modifications of histones [37], and regulation of cytoskeleton function and polarity [40]. In contrast, Sir2 has been associated with chronological aging [19,41]. The deletion of *SIR2* can either have no impact or lead to a modest extension of CLS, varying with the strain background and nutrient conditions [42,43].

During yeast aging, a decline in protein homeostasis is also observed, characterized by increased protein oxidative damage and aggregation [19,32]. This decline is partly due

to impaired proteasome activity with aging. Boosting proteasomal degradation has been demonstrated to enhance both yeast RLS and CLS [44,45]. Similarly, autophagy decreases during aging contributing to the accumulation of aberrant proteins and organelles [19,41,46]. Additionally, the activation of autophagy has been shown to be crucial for several interventions that extend both CLS and RLS [46]. Downregulation of nutrient signalling kinases TORC1, Sch9, and PKA promote autophagy and have been shown to increase both RLS and CLS [41,46]. Cells lacking the nutrient-sensing kinase Snf1, a promoter of autophagy, exhibit a decrease in CLS [47,48] albeit an increase in RLS (Figure 1) [49].

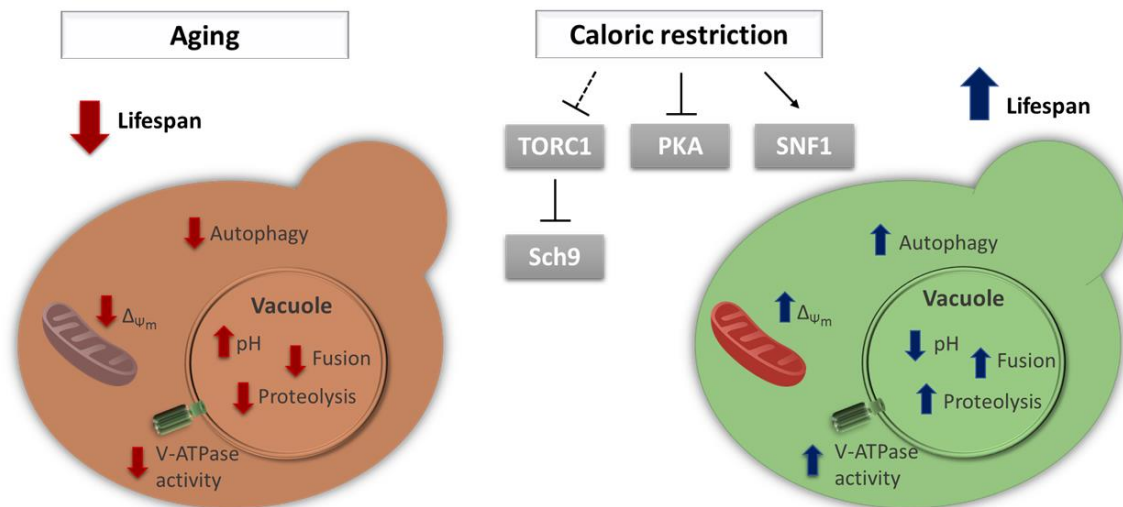


Figure 1 - Vacuolar function and lifespan in yeast. During yeast aging, a decline in autophagy and vacuolar function, including decreased proteolysis, acidification and fusion, is observed and contribute to mitochondrial dysfunction. Downregulation of TORC1, Sch9, and PKA and activation of Snf1 protein kinase (e.g., by caloric restriction) promote autophagy and have been shown to increase yeast lifespan.

Mitochondrial function also declines with aging in yeast, as shown by increased mitochondrial fragmentation, reduced membrane potential, and elevated reactive oxygen species (ROS) production [20,32]. Nonetheless, inducing mitochondrial respiration and ROS production during early growth phases, either by reducing TORC1 signalling [50,51] or subjecting cells to caloric restriction (CR) [52,53], activates a mitochondrial adaptive stress response that extends yeast CLS.

In a wide range of model organisms, including yeast, worms, flies, rodents and primates, the genetic or pharmacological modulation of nutrient signalling pathways extends lifespan. Notably, the anti-aging potential of compounds that impact signalling pathways, such as rapamycin, resveratrol, and spermidine, was first identified in yeast [54]. Dietary restriction (DR), which refers to the decrease of one or more macronutrients without

malnutrition, with or without reducing calories, is the most effective non-genetic intervention that modulates these pathways and promotes longevity [55–57]. DR is performed by decreasing the levels of glucose in the diet/growth media (a frequently used condition) or by restricting a single amino acid. These conditions extend lifespan in part by inducing oxidative stress responses and by improving mitochondrial and lysosomal fitness [50,56–61].

1.3 Lysosomes and vacuoles in aging

Lysosomes and lysosome-like vacuoles (in yeast) are organelles surrounded by a single-layer lipid membrane rich in carrier proteins. They contain a wide variety of soluble hydrolases in the lumen, including proteases, nucleases, lipases, sulfatases, phosphatases, and glycosidases. Lysosomes and vacuoles function as major catabolic centers due to their high concentration of hydrolases and their characteristically acidic pH, which is maintained by the activity of the vacuolar H⁺-ATPase (V-ATPase) [62,63]. The dynamic feature of lysosomes and vacuoles allows cells to adapt to physiological and environmental cues, by changing their size and number via cycles of fission and fusion [64,65]. These organelles are critical for cellular and organismal longevity due to its roles in cellular processes such as autophagy, nutrient signalling, amino acid and ion homeostasis, and interaction with other organelles, including mitochondria [62,63,65–68].

Autophagy is an evolutionarily conserved process critical for cell survival as it removes damaged material or organelles produced under stress conditions, during aging, or as a result of diseases. Moreover, it is critical for the beneficial effects of DR and other lifespan extension conditions [21,29,30]. Metabolites generated by lysosomal/vacuolar digestion can then be exported through specific proteins or stored for later use [62,63,65]. The recycling of macromolecules via autophagy enables cells to survive under nutrient scarcity. Notably, an age-dependent decline in lysosome proteolytic function and autophagy has been demonstrated in several organisms, compromising cellular homeostasis [66,69]. Moreover, studies in different model organisms showed that defects in autophagy results in a decrease of lifespan and an increase of metabolic and age-related diseases, including cancer and neurodegenerative diseases, which are characterized by the accumulation of damaged molecules and organelles [29,30,66,69]. Conversely, strategies that enhance autophagy (e.g., by overexpression of Atg5 or by supplementation with spermidine) have shown promising results in attenuating disease progression and extending lifespan in several organisms [46,69–77]. Notably, the longevity of centenarians has been associated with enhanced autophagy-lysosomal function and proteasome activity [78,79]. A similar correlation was found in long-living queen bees [80].

In yeast, vacuolar dysfunction is an early event during replicative aging and precedes the decline of mitochondrial function [81]. The link between vacuoles and lifespan is further supported by genome wide screens of the yeast deletion strain collection, revealing that proteins involved in vacuolar functions have prominent roles in chronological longevity [82,83]. In addition, vacuolar proteins are differentially expressed or phosphorylated under conditions that modulate nutrient signalling pathways relevant to aging (e.g., inhibition of the TOR Complex 1 (TORC1) or DR) [84–89]. In the scope of this thesis, the role of vacuoles in yeast aging is discussed below.

1.4 Trafficking pathways and aging

1.4.1 Biosynthetic pathways

Vesicular trafficking pathways are essential for vacuole degradative functions as well as for vacuole biogenesis and function. The biosynthetic pathways, including the cytoplasm to vacuole targeting (Cvt), the alkaline phosphatase (ALP/AP-3) and the vacuolar protein sorting (VPS/CPY) pathways, target resident proteins to vacuoles, while endocytosis and autophagy are responsible for delivering proteins for degradation (Figure 2) [90].

In the CPY pathway, different adaptors concentrate the cargo proteins at the trans-Golgi network (TGN) and recruit the clathrin coat, facilitating the formation of vesicles. These vesicles then fuse with the endosomal membrane, where vacuolar soluble proteins are delivered to the lumen of the endosomes and vacuolar membrane proteins remain at the endosomal membrane. Membrane proteins targeted for the vacuolar lumen, such as carboxypeptidase S (Cps1), must be selectively sorted into vesicles that bud into the lumen of the late endosome or the multivesicular body (MVB). This inward budding process is mediated by the ubiquitination of membrane proteins by Rsp5. Protein ubiquitination marks them for the MVB sorting machinery, consisting of four different protein complexes known as ESCRT (endosomal sorting complex required for transport): ESCRT-0 (Vps27 and Hse1), ESCRT-I (Mvb12, Vps28, Vps23, and Vps37), ESCRT-II (Vps22, Vps36, and Vps25), and ESCRT-III (Vps20, Snf7, Vps24, Vps2) [90–93]. Firstly, the ESCRT-0 complex is recruited to the late endosomal membrane through interaction between the FYVE zinc finger domain of Vps27 with the endosomal phospholipid phosphatidylinositol-3-phosphate (PI3P). Due to several ubiquitin interaction motifs, the ESCRT-0 complex clusters ubiquitinated proteins and subsequently Vps27 binds to Vps23 and recruits the ESCRT-I complex. The ESCRT-I complex also binds to ubiquitinated cargo and its subunit Vps28 binds to Vps36 and recruits the ESCRT-II complex. Additionally, the ESCRT-II complex interacts with PI3P, cargo, and ESCRT-III through binding between Vps25 and Vps20 subunits. ESCRT-III sequesters cargo and mediates inward budding of the vesicle. To

finalize, the Vps4 complex is recruited by ESCRT-III and catalyses both the disassembly of ESCRT-III and the scission of the vesicle, releasing it into the MVB lumen. In the final step of this pathway, cargo proteins are delivered to vacuoles through fusion between late endosomes/MVB and vacuoles. The carboxypeptidase Y (CPY) is the most thoroughly studied soluble protein that is directed to the vacuole via this pathway [90]. The soluble pro-enzyme of CPY binds to its transmembrane receptor Vps10 at the Golgi and both are sorted to endosomes. Then CPY is released into the endosomal lumen and Vps10 is recycled back to late Golgi, a mechanism that also depends on ESCRT complexes [94,95]. Effectors of the CPY pathway were identified based on genetic screens that led to the isolation of mutant cells mis-sorting CPY [96]. The efficient delivery of proteins to the vacuole is crucial for maintaining organelle function. Thus, mutations that disrupt this pathway can negatively impact lifespan [82].

Proteins utilizing the ALP/AP-3 pathway undergo sorting within a distinct class of vesicles at the TGN, facilitated by the adaptor complex AP-3, consisting of Apl6, Apl5, Apm3, and Aps3 subunits. These proteins are directed to vacuoles without passing through endosomes [90]. Defects in this pathway are expected to contribute to vacuolar dysfunction and aging. For instance, Vtc5 is mislocalized in cells lacking components of the AP-3 complex, leading to defects in polyphosphate (polyP) accumulation [97]. Vtc5 acts as a regulatory subunit of the vacuolar transporter chaperone (VTC) complex, serving as the sole known polyP synthetase in yeast [97]. Additionally, the VTC complex is involved in maintaining the stability of vacuolar V-ATPase, facilitating vacuole fusion, and regulating microautophagy [98–100]. PolyP has been associated with the regulation of pH and ion balance, phosphate metabolism, stress response, cell cycle progression, genomic stability, and lifespan [101–104]. Defects in the ALP/AP-3 pathway also impair sorting of amino acid carriers involved in the export of basic amino acids from vacuoles and lysosomes both in yeast and mammals [105].

The Cvt pathway, identified exclusively in yeast, specifically and constitutively transports vacuolar hydrolases, including α -mannosidase (Ams1), aspartyl aminopeptidase (Ape4) and aminopeptidase I (Ape1), to vacuoles [106]. Cvt is classified as a form of selective autophagy and shares molecular elements with the autophagic process. However, it differs in its classification as it is considered a biosynthetic pathway rather than a degradative one. Protein cargos are synthesized in the cytoplasm, where they are enclosed in Cvt vesicles, which are smaller than autophagic autophagosomes. This process requires a receptor protein (Atg19), which binds Cvt substrates and recruits Atg8 and Atg11, forming the Cvt complex. The subsequent recruitment of the core autophagy machinery by the Cvt complex triggers the formation of the Cvt vesicle, which then undergoes fusion with the

vacuole. This fusion event results in the release of the Ape1 complex, where the precursor form of Ape1 (prApe1) is processed into its mature form (mApe1) [106,107].

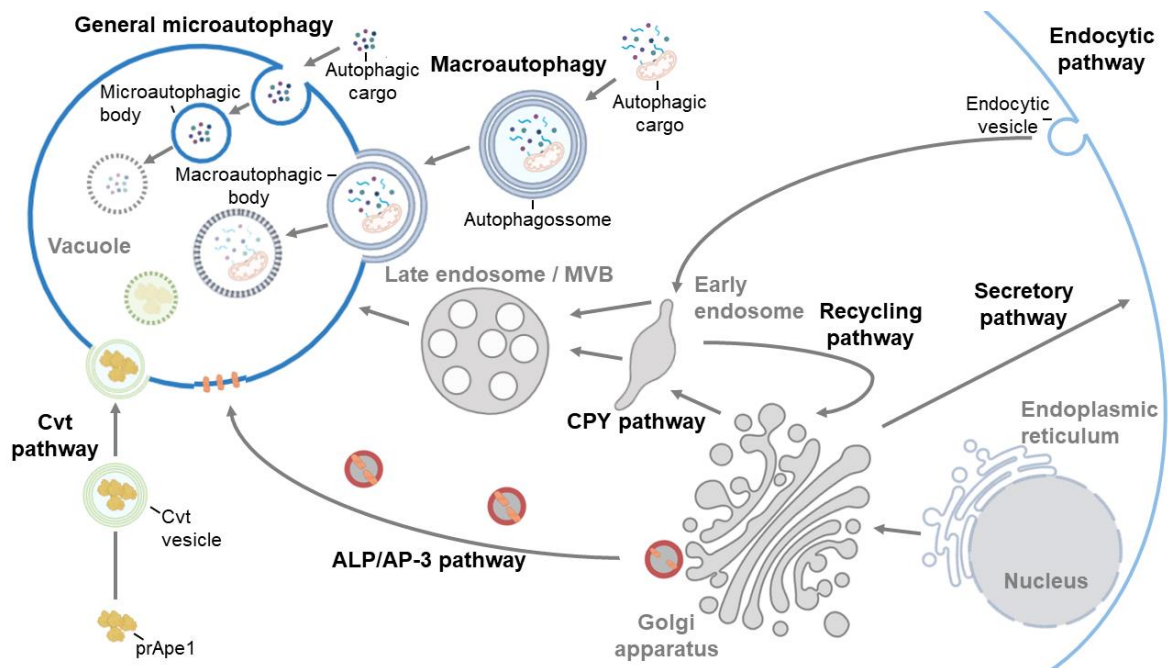


Figure 2 – Trafficking pathways in the yeast *S. cerevisiae*. Vacuolar proteins are synthesized within the endoplasmic reticulum before being conveyed to the Golgi apparatus where they can follow one of two pathways to reach the vacuole: the alkaline phosphatase (ALP/AP-3) pathway or the vacuolar protein sorting (VPS/CPY) pathway. In the ALP/AP-3 pathway, proteins are transported directly from the Golgi to the vacuole, whereas the CPY pathway involves the transport of proteins from the Golgi to the vacuole via endosomes. Additionally, certain vacuolar hydrolases, such as aminopeptidase I (Ape1), utilize a different route, known as the Cytoplasm to Vacuole Targeting (Cvt) Pathway, which transports the proteins from the cytoplasm directly to the vacuole. The Endocytic Pathway is responsible for internalizing plasma membrane proteins and extracellular components into early endosomes. Subsequently, the internalized materials have two potential paths: they can either be targeted for degradation within the vacuole via late endosomes or multivesicular bodies (MVBs), or they can undergo recycling back to the plasma membrane. Autophagy is a major pathway for delivering substrates to the vacuole for degradation. In macroautophagy, a double-membrane structure surrounds the cytoplasmic cargo, forming an autophagosome. The autophagosome then fuses with the vacuolar membrane, allowing the degradation of its inner membrane and enclosed cargo. In microautophagy, the vacuolar membrane directly engulfs the cytoplasmic cargo via invagination, sorting it into the vacuolar lumen for degradation.

1.4.2 The endocytic pathway

The endocytic pathway allows for the internalization of extracellular and plasma membrane protein cargos, thereby regulating cell function. This process encompasses several distinct steps - initiation, invagination, scission, and vesicle release - each requiring distinct protein complexes [90,108]. After internalization, endocytic vesicles fuse with the early endosome where protein cargos undergo sorting. Proteins can proceed to late endosomes/MVB for degradation in the vacuole or can be directed back to the TGN for

recycling. Those routed through the recycling pathway enter the secretory pathway to be redirected to the plasma membrane [109,110].

Protein ubiquitination functions as a crucial recognition signal to divert cargo via the ESCRTs into intraluminal vesicles of the MVB [90,91]. The intricate process involves sequential interactions among ESCRT complexes, allowing cargo sequestration, MVB vesicle formation and finally membrane budding/scission, resulting in PI3P vesicle release into the MVB lumen.

The degradation of plasma membrane proteins through the MVB pathway plays a role in cellular fitness during aging, especially under conditions of nutrient scarcity [111,112]. When nutrients are depleted, the ESCRT-dependent degradation of plasma membrane proteins through the MVB pathway becomes crucial to sustain an essential reservoir of amino acids necessary for protein synthesis. The synchronized function of the MVB pathway and autophagy is vital for cell survival under nutrient restriction [111] as well as for lifespan extension through phosphate restriction [112].

1.4.3 Autophagy

Autophagy is an evolutionarily conserved recycling pathway critical for cell survival [29,46,113,114]. It ensures the quality control of proteins and organelles by eliminating damaged material (e.g., proteins, lipids and organelles), preventing its accumulation during aging. Moreover, autophagy enables cells to resist nutrient scarcity by recycling macromolecules to generate new nutrients (amino acids, simple sugars, fatty acids, and nucleotides) and energy. Consistently, the modulation of nutrient signalling pathways extends the lifespan in part through the induction of autophagy (discussed in section 1.6) [46,115].

Depending on the mechanism by which intracellular cargo is transported to vacuoles, we can distinguish two main types of autophagy in yeast: macroautophagy and microautophagy [116]. In macroautophagy, cytoplasmic material and/or organelles are sequestered in a double-membrane structure called autophagosomes [117]. The process of macroautophagy, hereafter named autophagy, can be subdivided into different steps, including induction and nucleation of the phagophore, expansion and maturation of the phagophore to form the autophagosome, autophagosome tethering and fusion with the vacuole, and, finally, cargo release and degradation [118,119]. Several autophagy-related (*ATG*) genes were first identified in yeast and the coordinated action of Atg proteins is crucial for the effective fulfilment of autophagy [120]. The initial step of autophagy involves the formation of Atg1 multiprotein complex composed by Atg1, Atg13 and the Atg17–Atg31–Atg29 ternary subcomplex, which subsequently recruits multiple autophagy proteins to the phagophore assembly site (PAS) [118]. Vesicles containing Atg9 derived from the Golgi

interact with Atg17 and Atg13 and are recruited to the PAS to generate the phagophore. Other membrane sources for phagophore formation have been described, including COPII-coated vesicles [121]. The vacuolar membrane protein Vac8, the Atg1 complex and Atg9 interact with the PI3K complex I, composed by the PI3K Vps34, Vps15, Vps30/Atg6, Atg14 and Atg38, recruiting it to the PAS [118,122,123]. The PI3K complex produces phosphoinositol 3-phosphate (PtdIns3P) that recruits the Atg2–Atg18 complex, which is involved in lipid transfer and phagophore expansion [118,124,125]. Atg21 also binds to PtdIns3P and Atg16, recruiting the Atg12–Atg5–Atg16 complex, and Atg12 promotes the conjugation of Atg8 with phosphatidylethanolamine (PE) [118,126]. Atg8 lipidation is associated with cargo recognition, autophagosome formation, trafficking and fusion with the vacuole. Bulk autophagy is generally a nonspecific process, and its activation is associated with nutrient depletion during starvation. However, there are instances in which organelles are degraded by selective autophagy pathways, such as mitophagy (mitochondria) and pexophagy (peroxisomes), to maintaining the number and integrity of cellular organelles [116]. This selectivity is ensured by the association of autophagic receptors with scaffold proteins, which serve as a link connecting the phagophore and the cargo.

In microautophagy, the cytoplasmic cargo is engulfed by the vacuolar membrane and directly sorted into the vacuolar lumen for degradation [116,127,128]. Several targets of microautophagy degradation have been identified, including the cytoplasm, ER, portions of the nucleus, mitochondria, lipid droplets, peroxisomes, and vacuole membrane proteins. Similar to autophagy, microautophagy plays a crucial role in adapting to metabolic changes, remodeling organelles and ensuring quality control within the cell. The mechanisms of distinct microautophagy processes greatly differ and can depend on the core autophagy machinery, ESCRT components and SNARE proteins [127,128]. Recently, microautophagy was classified in two types: the fission-type that is mediated by ESCRT proteins and the fusion-type that relies on the core autophagy machinery [128]. In the fission-type, similarly to the sorting of ubiquitinated cargo to MVB endosomal membranes, ESCRT-0 recognizes and sorts ubiquitinated cargo, such as vacuolar membrane and associated proteins, into intraluminal vesicles destined for direct degradation within the vacuole. ESCRT machinery also drives selective microautophagy of the endoplasmic reticulum (ER) and proteasome [129,130]. In the fusion-type, membrane invaginations or extensions are formed to englobe the cargo and then sealed with a phagophore-like structure by fusion, a process that might rely on SNARE proteins. This type of microautophagy encompasses micropexophagy, micronucleophagy and apparently micromitophagy [128]. Microautophagy can target several cellular components, implying this process in a variety of cellular functions and suggesting a significant role in promoting cell survival. For instance, lysosome membrane repair through ESCRT-driven microautophagy is essential to prevent aging in

Caenorhabditis elegans, as failure to induce it increases the rate of senescent cells and decreases lifespan [131]. The RAB7-dependent delivery of endosomes to lysosomes via microautophagy plays a crucial role in early embryonic mouse development, with loss of RAB7 leading to embryonic lethality [132]. Defects in microautophagy have been linked to aging-related disorders, including various neurodegenerative diseases and cancer [133].

1.5 Role of vacuolar storage in aging

In addition to serve as the terminal degradation compartment in autophagy, yeast vacuoles have essential roles in cellular homeostasis by storing ions and amino acids (the most abundant vacuolar metabolites) as well as sugars, lipids and nucleotides [65]. Vacuolar nutrient storage is critical for cell adaptation to stress conditions (e.g., fluctuations in nutrients availability) and for cellular lifespan. The transport of nutrients is ensured by the presence of multiple proteins at the vacuolar membrane and by the proton gradient established by the V-ATPase, which uses ATP to drive protons into the lumen of the vacuole [65]. The vacuole is also the main storage site of polyP, which can be mobilized when needed, e.g., for the synthesis of nucleotides, phospholipids, nucleic acids or ATP [134–136]. The accumulation in vacuoles of glycogen (delivered by autophagy) is also crucial for cell longevity as it prevents glycogen degradation in the cytosol, mediated by glycogen phosphorylase, and allows its use in late stationary phase [137,138]. Consistent with the key role of vacuoles in nutrient storage and with the complexity of the metabolites accumulated within this organelle, vacuoles are considered as central regulators of nutrient sensing and signaling [63,139].

1.5.1 Vacuolar amino acid homeostasis and aging

The availability of amino acids significantly influences the lifespan of yeast. Amino acids are essential for the synthesis of proteins and other molecules (e.g., nucleotides, lipids, Heme) and can be used for energy production. It is increasingly recognized that lifespan can be modulated by changing the levels of amino acids, either by increasing or decreasing them. For example, high levels of essential amino acids, such as leucine, increases the resistance to oxidative and heat stress conditions and extends CLS in both autophagy-deficient and autophagy-proficient cells [140,141]. This effect is not observed with supplementation of other essential amino acids (histidine and lysine), indicating that leucine regulates lifespan by other mechanisms rather than only providing an additional source of nitrogen [141]. Nonessential amino acids also increase yeast CLS in minimal media but some of these amino acids (e.g., isoleucine, valine and threonine) have a more noticeable effect [141]. However, methionine restriction also extends lifespan, to levels

similar to that observed in calorie restriction (CR) conditions, by increasing autophagy and promoting vacuolar acidification [142]. This is evolutionarily conserved, as the reduction of methionine and CR similarly increase longevity in rats, mice and fruit flies [143–145].

Amino acid homeostasis, including their transport between vacuoles and the cytosol and other organelles, is also critical for lifespan [62]. Interestingly, amino acids are distinctly compartmentalized depending on their characteristics. In yeast, basic and neutral amino acids (lysine, histidine, and arginine) are preferentially stored within the vacuolar lumen whereas acidic amino acids (glutamate and aspartate) are excluded from the organelle to the cytosol [66,146–148]. Several pieces of evidence suggest that sequestering specific amino acids within vacuoles may shield cells from their potential toxicity. This hypothesis is supported by observations that mutations impacting vacuolar storage of particular amino acids result in impaired growth when cells are exposed to high concentrations of those amino acids [149–151]. The increase in cytoplasmic amino acid pools favours their import at high rates to mitochondria, surpassing the capacity of mitochondrial catabolic pathways. For instance, high levels of basic amino acids are associated with mitochondria-dependent cell death [152]. The imbalance in amino acid storage within vacuoles has been linked to a decrease in lifespan, in part due to its toxic effects on mitochondrial function, but changes in amino acids levels may also impair mitochondria and lifespan by influencing nutrient sensing pathways which are recognized to regulate lifespan (see 1.6).

The transport of amino acids across the vacuolar membrane is mediated by three transporter families: amino acid vacuolar transporters (AVT; Avt1-7), vacuolar basic amino acid transporters (VBA; Vba1-3) and lysosomal cystine transporters (LCT; Ypq1-3) [153]. Vacuolar uptake of basic amino acids is mediated by VBA and LCT transporters [154,155]. While the significance of vacuolar storage for basic amino acids in cell survival is recognized, the potential correlation between these transporters and the aging process remains elusive.

The AVT family of vacuolar transporters is related to the vesicular GABA-glycine transporters and homologous transporters have been found in many different organisms [156,157]. Avt1 is involved in the vacuolar uptake of neutral amino acids and histidine [157,158]. The deletion of *AVT1* disrupts amino acid homeostasis, increases age-induced mitochondrial dysfunction and decreases RLS, while Avt1 overexpression prevents alterations in mitochondrial function and extends the yeast lifespan [81]. A recent study also showed that the age-associated decline in vacuole acidification prevents storage of the neutral amino acid cysteine, leading to cysteine-mediated iron limitation and mitochondrial dysfunction [159]. Iron is required for iron sulfur clusters (ISC), which are essential cofactors for proteins in several cellular processes, including mitochondrial respiration [160]. During aging, elevated non-vacuolar cysteine promotes ROS, which limit intracellular iron

bioavailability and mitochondrial respiration [159]. These results indicate that neutral amino acids hold particular significance in the vacuolar regulation of mitochondrial function and lifespan. Moreover, these results suggest that vacuolar alkalization in aged cells may lead to mitochondrial dysfunction due to impaired vacuolar amino acid storage rather than reduced capacity of vacuolar protein degradation [81,161].

Avt3, Avt4 and Avt7, similarly to Atg22 (a member of VBA family), are involved in the transport of neutral amino acids out of vacuoles and are required for cellular viability under starvation conditions [157,162,163]. Likewise, loss of SLC38A9, which mediates lysosomal efflux of many essential non-polar amino acids, impairs the capacity of mammalian cells to survive amino acid starvation [164]. The efflux of basic and acidic amino acids (glutamate and aspartate) is mediated by Avt4 and Avt6, respectively [165,166]. The contribution of these transporters for lifespan is unknown. However, the export of vacuolar amino acids generated by autophagy is essential for cell adaptation to the environmental conditions and longevity, as autophagy deficient cells rapidly loses viability when deprived of amino acids [167].

1.5.2 Vacuolar ion storage on aging

In addition to amino acids, the vacuole serves as the primary storage site for ions such as calcium and iron. Disruption of ion homeostasis have been directly linked to aging and age-associated diseases [66,168,169]. Calcium is an important second messenger that plays a pivotal role in the regulation of numerous biological processes, including cell proliferation, muscle contraction, programmed cell death, neurotransmission, vesicular transport, vacuole fusion, secretion, and transcription [170]. In yeast, vacuoles are crucial for the maintenance of calcium homeostasis. Indeed, more than 90% of total cellular calcium is stored in vacuoles [171] through the $\text{Ca}^{2+}/\text{H}^{+}$ exchanger Vcx1 [172] and the P-type Ca^{2+} -ATPase Pmc1 [173]. The export of vacuolar calcium to the cytosol is mediated by Yvc1, e.g. in response to extracellular hypertonic shock [65,174]. When cytosolic calcium increases, calmodulin binds calcium and activates the protein phosphatase calcineurin, which in turn activates the Crz1 transcription factor, leading to the upregulation of genes associated with vesicle transport, cell wall synthesis and ion transport [175]. This signaling pathway is essential for cellular adaptation to high ion stress conditions [176] and its proper regulation is required for a normal lifespan, as both the absence and hyperactivation of calcineurin signaling decreases yeast lifespan [177]. Moreover, the impairment of vacuolar function results in a rapid increase in cytoplasmic calcium levels [178] and to a decrease in yeast RLS [177]. The absence of a functional V-ATPase also increases the sensitivity to calcium, as Vcx1 relies on the V-ATPase-established H^{+} gradient to store calcium into vacuoles [179]. However, vacuolar calcium overload is also detrimental. For example, cells

lacking Pmr1 and Cod1, involved in calcium sequestration in the Golgi and ER, exhibit calcium influx and vacuolar calcium uptake, and display fragmented vacuoles [180]. Consistently, mutants featuring impaired fusion machinery exhibit vacuolar fragmentation and decreased RLS, whereas vacuolar fusion has been associated with increased longevity [64]. This suggests a potential link between calcium, vacuolar organization, and lifespan. In mammalian cells, calcium release from lysosomes and subsequent calcineurin activation have been associated with the induction of autophagy [181]. In contrast, calcineurin activation was found to downregulate autophagy in yeast [182].

Changes in iron homeostasis also impact aging and age-related diseases [168]. Iron is necessary as prosthetic group for metalloproteins participating in several biological processes. To fulfil their iron requirements, yeast cells activate the Aft1-dependent iron regulon under low iron conditions, increasing the expression of genes involved in iron uptake and intracellular iron mobilization [183]. However, iron is a redox active metal and its excess increases oxidative stress and cell damage. Vacuoles play a critical role in preventing iron toxicity by storing the excess of iron through the iron importer Ccc1, as cells lacking Ccc1 have increased sensitivity to high levels of iron [184]. Defects in the V-ATPase activity and the consequent decrease in vacuolar acidification perturbs the vacuolar storage of iron and activates an iron deficiency response, even when iron levels are abundant, resulting in iron overload [185,186]. Despite the activation of the iron regulon, loss of V-ATPase activity affects iron availability and decreases the levels of ISC, leading to depletion of iron-containing respiratory chain complexes and impairment of mitochondrial respiration. This phenotype is suppressed by increasing the levels of iron through iron uptake induction or iron supplementation [159,187]. This suggests that the decline in vacuolar acidification affects aging by disrupting iron homeostasis. On the other hand, under conditions of low iron availability, vacuolar export of iron is facilitated by a high-affinity transport system comprised of Fet5 [188] and Fth1 [189], along with the low-affinity transporter Smf3 [190]. The mobilization of vacuolar iron is crucial for mitochondrial metabolism, a key determinant of yeast lifespan [191], as preventing vacuolar iron export affects the capacity of cells to transit from a fermentative to a respiratory metabolism at the post-diauxic shift (PDS) phase [189,192].

Nutrient signaling pathways governing yeast lifespan have also been linked to the regulation of iron homeostasis [183]. For instance, the Snf1/AMPK protein kinase induces the iron regulon at the PDS phase and regulates iron toxicity resistance by increasing the expression of *CCC1*, promoting iron storage within vacuoles [193,194]. In addition, iron deprivation, achieved by treating cells with bathophenanthrolinedisulfonic acid (BPS), reduces TORC1 activity, leading to the dephosphorylation of Atg13 and induction of

autophagy that extends yeast lifespan [195]. These findings suggest a two-way regulation of iron and signaling pathways impacting aging.

1.6 Nutrient signaling in vacuole-mediated yeast longevity

In yeast, nutrient and energy signaling through TORC1, Ras-PKA, and Snf1/AMPK have pivotal roles in the regulation of vacuoles and lifespan (Figure 3) [46,196,197]. The TORC1 protein kinase is a conserved master regulator of cell growth that is activated at the vacuole/lysosome membrane in response to growth factors and nutrients, in particular amino acids [198]. In response to amino acids availability, mammalian mTORC1 shuttles between the cytosol and the lysosomal surface [199], whereas the yeast TORC1 relocates from vacuole associated puncta to the vacuolar membrane [200–202]. Studies using yeast uncovered several proteins that sense amino acids and relay the signal to TORC1 mostly via the RAG family of small GTPases Gtr1/Gtr2 (reviewed in [198,203]). GTPase activity is regulated by conserved GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) in response to amino acids availability. In yeast, the activity of LeuRS and Vam6 on Gtr1 and Lst4-Lst7 on Gtr2 promote the assembly of the Rag heterodimer in its active conformation (Gtr1_{GTP} Gtr2_{GDP}), while SEACAT counteracts the inhibitory impact of the Gtr1 GAP, SEACIT [198,203].

The PtdIns3P binding protein Pib2 is also a key regulator of TORC1 [204,205]. The deletion of both *PIB2* and *GTR1/2* is synthetic lethal due to the complete loss of TORC1 activity [204]. It has been suggested that Pib2 and Gtr1/2 act in parallel to activate distinct pools of TORC1 in response to amino acids [206], or that they act cooperatively and are both required to fully activate TORC1 [207]. TORC1 is fully active under nutrient-rich conditions but is completely inhibited when cells are transferred to growth media containing a poor-quality nitrogen source. During cell adaptation to poor-quality nitrogen conditions, Pib2 is reactivated and TORC1 shifts to an intermediate activity state, phosphorylating a subset of its targets. Pib2-TORC1 activation restarts cell growth by phosphorylating Sch9, while not suppressing the branch that increases amino acid transport and synthesis essential for mass accumulation. Upon activation, TORC1 promotes anabolic processes, such as the synthesis of proteins, lipids, and nucleotides, and represses catabolic processes, like autophagy [208]. Under nutrient restriction, TORC1 is negatively regulated, resulting in downregulation of protein synthesis and in the activation of autophagy and stress responses. These changes promote the clearance of damaged protein and organelles as well as the induction of antioxidant defenses and other protective mechanisms that mitigate cellular damage. Consistently, the inhibition of TORC1 signaling by decreasing the levels of amino acids, rapamycin treatment or genetic manipulation

increase yeast lifespan [83]. Similar observations have been described in other model organisms [71,75–77].

In yeast, TORC1 inhibits autophagy by phosphorylating Atg1 and Atg13, which inhibits their association with the PAS during autophagy initiation [209]. TORC1 also controls autophagy through phosphorylation of the protein kinase Rim15, leading to its inhibition. Moreover, TORC1 acts indirectly through activation of the protein kinase Sch9 (S6K homologue) that in turn phosphorylates and inhibits Rim15. This blocks autophagy since Rim15 is a positive regulator of ATG gene expression, by activating the transcription factor Ume6 [210] and inhibiting the negative regulator of the transcription Rph1 [211]. TORC1 also inhibits microautophagy through phosphorylation of Vps27, a component of ESCRT-0, without disrupting MVB sorting [212]. TORC1 additionally suppresses the Rim15-dependent activation of transcription factors like Msn2/Msn4 and Gis1. Consequently, this inhibition hampers the induction of genes containing stress responsive and PDS elements in their promoters, which are linked to stress resistance and mitochondrial respiration. Thus, TORC1 inactivation leads to the induction of autophagy, stress responses and mitochondrial respiration (Figure 3), which increases redox signaling during exponential growth and further induces cell protection systems [50,58,213].

Besides amino acids, the TORC1-Sch9 pathway responds to glucose availability and its downregulation is also associated with the benefits of CR [213]. Although little is known about the mechanisms underlying the activation of TORC1-Sch9 by glucose, it was found that glucose metabolism promotes cytosolic acidification, which is sensed by the V-ATPase subunit Vph1. V-ATPase in turn activates TORC1 by interacting with Gtr1/2 [214,215]. Moreover, Sch9 physically interacts with V-ATPase and modulates its assembly/disassembly in response to glucose in order to control vacuolar pH and aging [216].

Lifespan extension triggered by CR is also mediated by decreased Ras-PKA signaling in yeast [213]. The Ras-PKA pathway is activated by glucose (and other fermentable sugars) and promotes fermentative metabolism, anabolic processes and cell growth while decreasing respiratory metabolism, catabolic processes and stress responses [217]. During fermentative growth, PKA directly phosphorylates a wide variety of enzymes and gene expression regulators. Similarly to TORC1-Sch9, PKA phosphorylates and inhibits Rim15, preventing the activation of Msn2/4 transcription factors, thereby suppressing the induction of autophagy and the expression of stress-response genes [46,218] (Figure 3). PKA also exerts direct control over Msn2/4 by regulating their nuclear localization [219] and inhibits autophagy in a TORC1-independent manner by directly phosphorylating Atg1 and Atg13 [220–222]. PKA inactivation is crucial for proper diauxic shift, when glucose is depleted, namely for the induction of respiratory metabolism and

stress resistance during the PDS phase and for cell survival at stationary phase [223]. Under CR conditions, yeast lifespan increases due to PKA inactivation and Rim15-dependent activation of Msn2/Msn4 and Gis1, associated with the upregulation of stress resistance proteins such as the mitochondrial Sod2 [213,224,225]. Notably, downregulation of PKA also increases lifespan through induction of autophagy [223,226,227]. PKA is also involved in glucose-dependent V-ATPase regulation [161,226]. Although the exact mechanism by which PKA regulates V-ATPase was not yet uncovered, mutant cells with constitutively active PKA exhibit impaired disassembly of the V-ATPase when glucose levels are depleted [226].

The AMPK energy-sensing pathway is also involved in mediating the lifespan extension under CR [228,229]. The yeast AMPK homolog is the SNF1 complex, a heterotrimer comprising Snf1 (catalytic α subunit), Snf4 (activating γ subunit) and one of three alternative β subunits (Gal83/Sip1/Sip2) that tether Snf1 and Snf4 together [230]. Snf1 is inhibited by both TORC1-Sch9 and Ras-PKA. Thus, Snf1 is activated under CR in part due to downregulation of those pathways [231,232]. Snf1 is activated by phosphorylation mediated by Sak1 (Snf1 Activating Kinase 1) in response to stresses encountered during aging, such as glucose depletion and oxidative stress, and is involved in the regulation of metabolism, cell growth, and autophagy, among other cellular processes [139,233]. Snf1 activates downstream effectors that are critical for lifespan extension, including the Cat8 and Adr1 transcription factors [228], which control the expression of genes linked to the utilization of ethanol and acetate through the glyoxylate and gluconeogenesis routes [234–236]. These changes favor the production of trehalose, a stress protective disaccharide that has antioxidant properties and inhibits the aggregation of denatured protein [237]. Moreover, Snf1 promotes mitochondrial biogenesis and respiration through inhibition of the transcriptional repressor Mig1 [238–241]. Snf1 induces autophagy by inhibiting TORC1 through phosphorylation of one of its subunits, Kog1, and by directly phosphorylating Atg1 [242,243]. Snf1 also regulates microautophagy upon glucose depletion by facilitating the relocation of Vps27 from endosomes to the vacuolar membranes [129]. Overall, by promoting mitochondrial biogenesis and autophagy, Snf1 (and Rim15-Gis1) stimulates the maintenance of a healthy mitochondrial network and, therefore, increases its oxidative capacity, decreases the accumulation of molecular damages, and improves overall cellular homeostasis. Downregulation of Sit4, a PP2A-like Ser/Thr protein phosphatase, homologue of the mammalian protein phosphatase 6 (PP6) and a negative regulator of Snf1, also increases yeast lifespan in a Snf1-dependent manner, by promoting mitochondrial derepression and oxidative stress resistance [244–246] (Figure 3). The benefits of *SIT4* deletion on lifespan are also linked to the regulation of vacuolar integrity and ATPase activity [247].

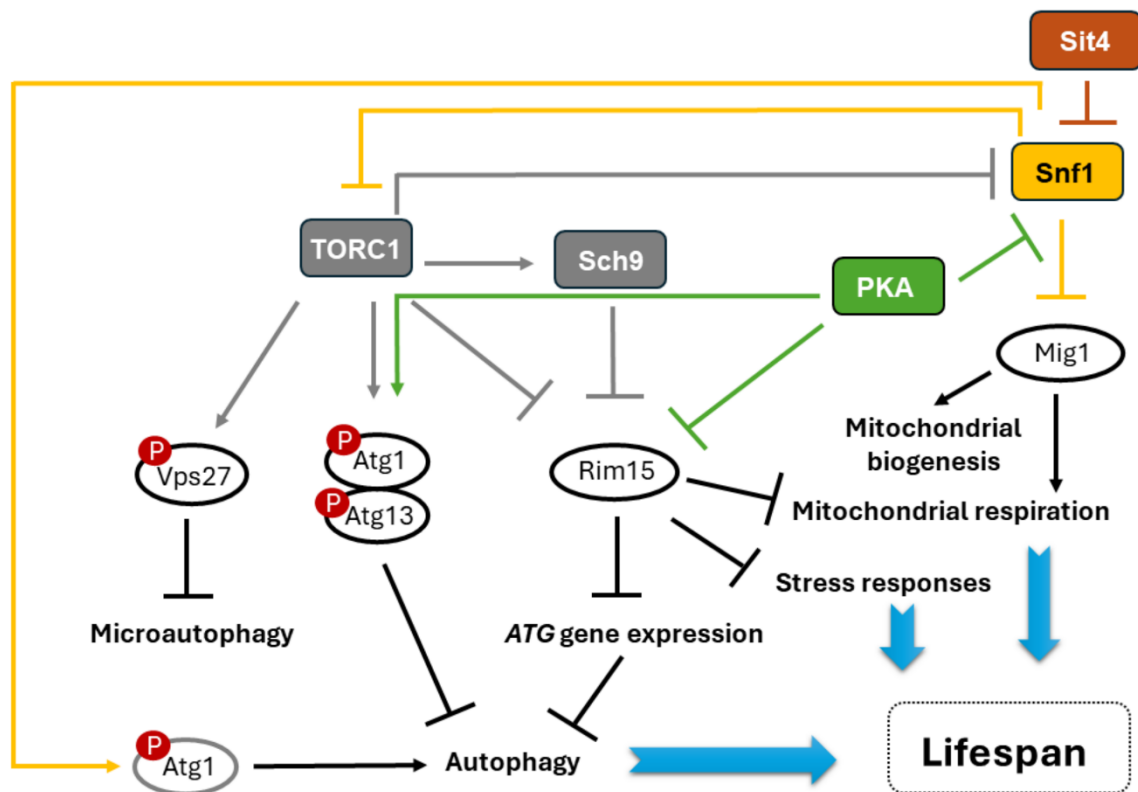


Figure 3 - Lifespan regulation by nutrient signaling pathways in yeast. TORC1 and PKA downregulate autophagy by directly phosphorylating Atg1 and Atg13, as well as by inhibiting the transcription factor Rim15 and the Snf1 protein kinase. TORC1-mediated activation of Sch9 also contributes to the inhibition of Rim15 and autophagy. The inhibition of Rim15 further suppresses the expression of genes related to stress responses and mitochondrial respiration. Additionally, TORC1 negatively regulates microautophagy through the phosphorylation of Vps27. Snf1 promotes autophagy by phosphorylating Atg1 and inhibiting TORC1. It also enhances mitochondrial respiration and biogenesis by inhibiting the transcription factor Mig1. Downregulation of TORC1, Sch9, PKA, and Sit4, along with activation of Snf1, via genetic modifications or caloric restriction, are associated with increased lifespan in yeast by promoting autophagy, stress responses, and mitochondrial respiration.

Protein phosphorylation is the primary post-translational modification in eukaryotes, playing a pivotal role in regulating diverse cellular processes and activating signaling transduction pathways [248]. As discussed above, modulation of nutrient signaling kinases by CR or rapamycin treatment increases lifespan in part by regulating vacuolar function. As expected, phosphoproteomic studies showed that the phosphorylation of vacuolar proteins is altered in yeast cells treated with rapamycin [85,87,88,249] or subjected to CR [84].

Proteins regulated by (de)phosphorylation in response to rapamycin are associated with amino acid and ion transport, vacuole organization, autophagy and autophagosome assembly and organization [85,87,88,249]. Avt1 and Avt4 were identified to be regulated by (de)phosphorylation in response to rapamycin in four different studies. Avt1 and Avt4 are involved in the transport of amino acids between vacuoles and the cytosol and exhibit

identical substrate specificity, yet they transport neutral and basic amino acids in opposite directions [157]. Notably, the function of these proteins has been related with the regulation of mitochondrial function. The absence of Avt1 accelerates age-associated mitochondrial dysfunction [81,250], and Avt4 is present at membrane contact sites between vacuoles and mitochondria, named vCLAMP (vacuole and mitochondria patch) [81,250]. Additionally to the regulation of these transporters by phosphorylation, microarray data show that genes encoding the amino acid transporters Atg22, Avt1, Avt4 and Avt7 are upregulated in response to rapamycin treatment [89], suggesting a role of amino acid homeostasis in lifespan extension.

In response to CR, 32 proteins related to vacuolar functions were found to be regulated by (de)phosphorylation [84]. Notably, several of these proteins overlap with those identified in response to rapamycin, including Avt1, Pep12, Svl3, Vma2, Vtc2, and Vtc3 [85,87,88,249]. They are involved in vacuolar transport, autophagy, organelle fusion, vacuolar organization, and polyP metabolism. Pep12 is a multi-functional syntaxin that controls all recognized membrane fusion events at the prevacuolar compartment, and its absence was associated with reduced CLS [251]. Svl3 function seems to play a role on vacuolar function as its absence results in defective localization of the vacuolar lumen vital dye CDCFDA [252]. A large-scale study showed that *SVL3* deletion decreases CLS [253]. Vma2 is an integral part of the V1 domain of V-ATPase and, together with Vma1, constitutes the catalytic binding site responsible for ATP hydrolysis into ADP and phosphate [254]. It is essential to the establishment of the membrane potential across the vacuolar membrane and to the acidification of the vacuolar lumen, but is also associated with polyP metabolism [255,256]. Cells lacking Vma2 display increased susceptibility to elevated concentrations of extracellular ions, reduced stress resistance, accelerated onset of mitochondrial dysfunction during aging, and diminished CLS and RLS [81,186,251].

Vtc2 and Vtc3 are accessory subunits of the VTC complex, together with Vtc1, Vtc5 and the catalytic subunit Vtc4 [257,258]. The VTC complex containing Vtc3 is mainly found on vacuoles whereas the Vtc2-containing complex is more abundant in the cell periphery (probably in the peripheral ER and nuclear envelope), but it localizes to the vacuole under phosphate starvation. It has been suggested that Vtc2 and Vtc3 play a regulatory role as they have two hydrophilic N-terminal domains facing the cytosol that contain numerous phosphorylation sites. The VTC complex has been associated with V-ATPase stability and trafficking, vacuolar fusion, microautophagy, and synthesis and translocation of polyP across vacuolar membrane [98–100]. Vacuolar polyP stands out as the primary phosphate reservoir within cells [135,136]. Phosphate, a fundamental element crucial to life, is required for the synthesis of nucleic acids and phospholipids. Additionally, it is indispensable for energy metabolism, protein modification, and signal transduction processes [259].

Moreover, storage of polyP functions as a cytosolic phosphate buffer during short term depletion of extracellular phosphate [260] and aids in the absorption and retention of positively charged ions and amino acids [65,261].

These proteomic studies have identified vacuolar proteins that may govern longevity. However, the link between lifespan extension and the phosphoregulation of specific vacuolar proteins remains elusive.

1.7 Lysosomal storage diseases

Lysosomal dysfunction is a hallmark of aging and age-associated diseases such as cancer and neurodegeneration [62,63,66,67,262]. It is also characteristic of lysosomal storage diseases (LSDs), a group of heterogeneous metabolic disorders arising from inherited mutations in proteins crucial for maintaining optimal lysosomal function [263–265]. LSDs are characterized by progressive accumulation of biomolecules (lipids, proteins, oligosaccharides, among others) in lysosomes. The accumulation primarily occurs in visceral tissues and the central nervous system, resulting in both physical and neurological defects. LSDs can be caused by defects in vesicular trafficking and sorting of proteins, although most of them results from defects in sulfatases and glycosidases involved in the degradation of glycoconjugates [265]. Around 70 LSDs have been described so far [263–265]. Gaucher disease is the most frequent LSD with a prevalence of about 1/40,000. This disease is caused by mutations in *GBA* leading to a deficiency of glucocerebrosidase and lysosomal accumulation of glucosylceramide [264]. Mutations in the gene *GLA* causing deficiencies in alpha-galactosidase leads to the accumulation of globotriaosylceramide characteristic of the Fabry disease [264]. Niemann-Pick type A/B disease is associated with defects in acid sphingomyelinase leading to sphingomyelin accumulation [264]. Niemann-Pick type C is caused by mutations in *NPC1* or *NPC2* involved in cholesterol and lipid trafficking [264]. Other LSDs associated with defects in sphingolipid metabolism include the GM1 gangliosidosis, metachromatic leukodystrophy, and the Tay-Sachs, Sandhoff, and Krabbe diseases [264]. Cystinosis can result from mutations in *CTNS*, which encodes for a lysosomal cystine exporter, leading to the buildup of insoluble cysteine within lysosomes [265]. Batten disease, a form of neuronal ceroid lipofuscinosis, is marked by the buildup of autofluorescent material containing lipopigments. The primary cause of this condition is mutations in *CLN3*, a lysosomal transmembrane protein crucial for vesicular trafficking [265].

Understanding the function of disease-associated proteins is crucial for the development of effective therapies aimed at restoring their functionality or attenuating their detrimental effects. Model organisms such as yeast play a crucial role in unravelling the

intricate molecular mechanisms underlying these pathologies [265]. The yeast *S. cerevisiae* expresses twenty-three yeast orthologs of human genes encoding lysosomal proteins linked to LSDs [266]. Studies using yeast have been made important contributions in the characterization of LSDs, including Batten disease, cystinosis, and Niemann Pick Type-C disease [265].

1.7.1 Niemann Pick Type C

Niemann-Pick disease is a group of autosomal recessive LSDs characterized by lipid accumulation in late endosomes and lysosomes (LE/Lys). These incurable neurodegenerative visceral disorders exhibit a broad spectrum of symptoms and varying degrees of severity. This disease can be categorized into types A, B, or C [267]. Niemann-Pick type A and B result from mutations in *SMPD1*, causing a deficiency in acid sphingomyelinase activity, responsible for the decomposition of sphingomyelin into ceramide and phosphocholine [268,269]. This deficiency leads to the buildup of sphingomyelin, cholesterol, and various other lipids within lysosomes. Mutations causing type A disease result in a significantly deficient or dysfunctional sphingomyelinase, whereas in type B disease, mutations lead to an enzyme that is defective but still retains partial activity [269]. Patients with Niemann-Pick type A disease experience rapid neurodegeneration, profound hypotonia, developmental defects, and typically do not survive past their third year of life [268,269]. Niemann-Pick type B patients do not exhibit alterations in the central nervous system, but they may present a profound swelling and enlargement of the liver and spleen accompanied by defects in liver function. The pulmonary function is also commonly affected. Among type B patients, there is significant variability in both the age at which symptoms begin and the speed at which the disease progresses. Individuals with intermediate phenotypes between these two types of Niemann-Pick disease have also been identified [268–270].

Niemann-Pick type C is caused by point mutations leading to the loss of function in *NPC1* (in 95% of cases) or *NPC2*, being subcategorized into Niemann-Pick type C1 (*NPC1*) or Niemann-Pick type C2 (*NPC2*) diseases, respectively [269]. Both *NPC1* and *NPC2* are involved in lipids and cholesterol efflux from lysosomes. Thus, the clinical manifestations of *NPC1* and *NPC2* diseases are similar. NPC is a neurovisceral progressive disorder that exhibits a broad spectrum of ages at symptom onset, closely linked with the severity and progression of the condition. Patients can be categorized based on the age of onset: pre/perinatal (<2 months), early-infantile (between 2 months and 2 years old), late-infantile (2-6 years), juvenile (6-15 years), and adolescent/adult (>15 years) [271]. Visceral alterations normally precede the neurologic manifestations, being the liver, spleen, and the lungs the most frequent organs affected. The most prevalent neurologic alterations are

progressive ataxia, dystonia, dysarthria, dysphagia, and dementia. Other common features are seizures, cataplexy, and vertical supranuclear gaze palsy. Additionally, patients with adult-onset disease may exhibit psychiatric manifestations like psychosis and depression [269,271].

NPC1 is a multi-pass transmembrane protein localized in LE/Lys, featuring 3 large and 4 small luminal loops, 6 small cytoplasmic loops, and a cytoplasmic tail [272]. With approximately 300 mutations identified across various domains, alterations in the NPC1 protein have been linked to NPC1 disease due to compromised activity [269]. The most prevalent mutation, found in approximately 20% of patients of Western European ancestry, is a missense mutation at position 1061 in the cysteine-rich domain, associated with severe juvenile onset [273]. This mutation hinders the proper folding of NPC1, leading to its degradation. Other disease-causing mutations in NPC1 lead to delayed transport through the secretory pathway or impaired cholesterol binding [274,275].

NPC2 is a small globular glycoprotein located in the lumen of LE/Lys, responsible for binding unesterified cholesterol and transferring it to NPC1 for subsequent insertion into the lysosomal membrane [275–277]. NPC1 then facilitates the export and distribution of cholesterol to various cellular locations, including the ER, trans-Golgi network, recycling endosomes, plasma membrane, and mitochondria (Figure 4) [278,279]. The activation of NPC1 is mediated by the GTPase Rab7, which, in turn, is activated by the trimeric GEF complex MON1-CCZ1-C18orf8 [280]. At membrane contact sites, NPC1 interacts with lipid-binding proteins such as GRAM1b and ORP5 at the ER and plasma membrane, respectively, promoting cholesterol transfer [281,282]. Defects in NPC1-mediated cholesterol export from lysosomes compromise the delivery of cholesterol to the ER and the cell membrane, resulting in abnormal accumulation within LE/Lys [283,284]. Impaired intracellular trafficking of cholesterol is associated with changes in histone methylation, hypomyelination and defects in oligodendrocyte lineage maturation [285].

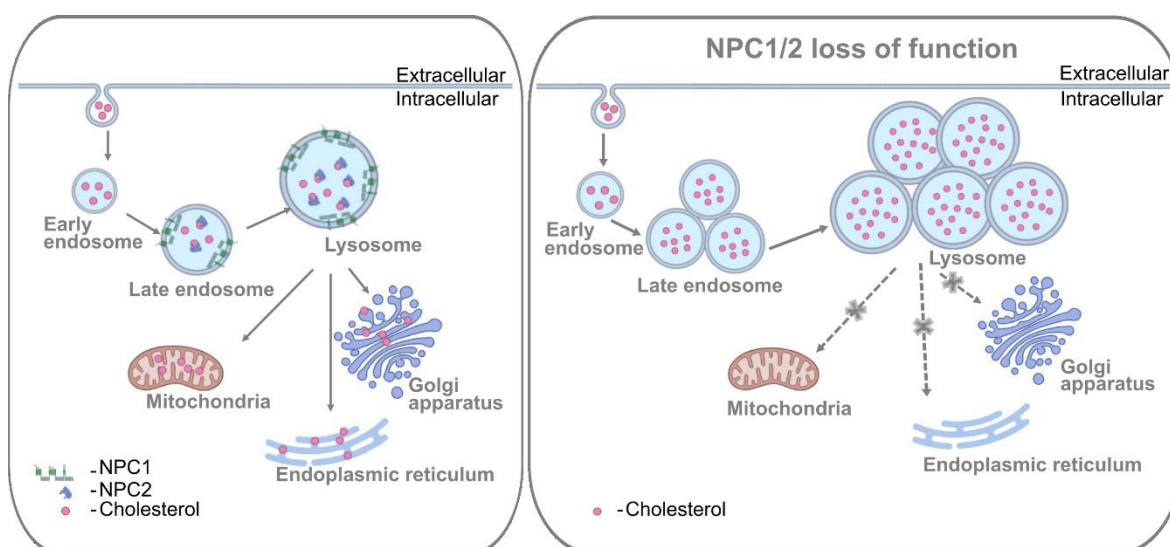


Figure 4 - NPC1 and NPC2 mediate the intracellular transport of cholesterol. NPC2 binds cholesterol in the lumen of late endosomes/lysosomes (LE/Lys) and transfers it to the multi-transmembrane protein NPC1. NPC1 then facilitates the export and transfer of cholesterol to other cellular locations, including the Golgi apparatus, endoplasmic reticulum, and mitochondria. A loss of function in either NPC1 or NPC2 results in the aberrant accumulation of cholesterol within the LE/Lys network and a depletion of cholesterol in other intracellular compartments.

In addition to cholesterol, NPC disease is characterized by lysosomal accumulation of several sphingolipid species, including sphingosine, sphingomyelin and glycosphingolipids [286]. Defects in the endocytic pathway at the LE/Lys level, which are typical in NPC cells, contribute to the pathologic accumulation of sphingolipids [286,287]. The initial lipid that accumulates upon NPC inactivation is sphingosine. Its accumulation in the LE/Lys system has been shown to lead to calcium depletion in these organelles, subsequently resulting in the accumulation of cholesterol, sphingomyelin, and glycosphingolipids [287]. Chelation of LE/Lys calcium leads to a series of cellular abnormalities similar to those observed in NPC, such as impaired endocytic transport and subsequent accumulation of cholesterol, glycosphingolipids and sphingomyelin [287]. Conversely, elevating cytosolic calcium levels by treating cells with thapsigargin induces fusion between late endosomes and lysosomes and corrects endocytic uptake of horseradish peroxidase [287]. Moreover, increasing TRPML1-mediated lysosomal calcium release corrects late endosome and lysosome-to-Golgi transport and reduces cholesterol accumulation [288]. Sphingomyelin has been shown to contribute to cholesterol accumulation in NPC cells by inhibiting vesicular trafficking of cholesterol between the late endosome and the Golgi [289].

Several pieces of evidence suggest a reciprocal relationship between impaired intracellular cholesterol transport and dysfunction in autophagy. While inhibition of cholesterol transport leads to a decrease in the autophagic flux due to the inhibition of

autophagosome fusion with lysosomes, impairment of the autophagic flux may contribute to cholesterol accumulation [290–292]. NPC1 deficiency is marked by the accumulation of autophagosomes, as evidenced by an elevated number of LC3/Atg8-positive vesicles and heightened levels of the lipidated form of LC3 (LC3-II), attributed to a blockage in autophagic flux [290–292]. The underlying mechanisms involve impairments in autophagosome–late endosome fusion resulting in defective amphisome formation, primarily attributed to the compromised recruitment of SNARE machinery components to late endosomes [290]. In addition, cargo degradation in lysosomes is impaired due to inhibition of lysosomal cathepsin activity by lipids stored in NPC1-deficient cells [292]. Elevated levels of cholesterol in the lysosomal membrane contribute to the accumulation of axonal autophagosomes in NPC mice, compromising autophagy and leading to axonal dystrophy [293]. This occurs due to the sequestration of motor-adaptor proteins involved in lysosome transport, which inhibits lysosome transport into axons and affects axonal homeostasis. Lowering cholesterol levels in the lysosomal membrane enhances lysosome transport, resulting in reduced axonal autophagic stress and neuronal death in NPC mice. Promoting autophagy has been shown to enhance the viability of NPC1-deficient cells, albeit without influencing cholesterol levels [290,294]. However, another study suggest that induction of autophagy may have a deleterious effect in NPC disease pathogenesis by contributing to cholesterol accumulation [292].

Disruption of autophagy in NPC disease has been reported to be caused by cholesterol-induced aberrant activation of mTORC1 [295–297]. Accordingly, reducing cholesterol with 2-hydroxypropyl- β -cyclodextrin (HP- β -CD) restores autophagy in NPC1 cells by activating AMPK and inhibiting mTORC1. However, some studies have indicated no alterations [298] or inhibition [299] of mTORC1 in NPC disease. Defects in autophagy contribute to faulty clearance of mitochondria and the fragmentation of mitochondria in human-derived neuronal cells lacking NPC1 [300]. Mitochondrial dysfunction is a characteristic feature of various neurodegenerative diseases, including NPC [301,302]. Elevated cholesterol levels within mitochondrial membranes of NPC1 cells have been associated with mitochondrial dysfunction, as shown by the reduced mitochondrial membrane potential, ATP synthase activity and oxygen consumption, and increased secretion of lactate [303–307]. Notably, accumulation of cholesterol contributes to mitochondrial dysfunction in NPC1 cells by enlarging the points of contact between lysosomes and mitochondria. This expansion enhances the transfer of cholesterol to mitochondria through mechanisms involving STARD1 and STARD3 proteins. Consequently, mitochondrial function and redox homeostasis are disrupted [282,308]. Consistently, downregulation of STARD3 in NPC1 deficient cells increases mitochondrial respiration and decreases lactate secretion [305]. Defects in mitophagy, decreased

mitochondrial motility, loss of mitochondrial DNA, changes in mitochondrial dynamics and decreased mitochondrial biogenesis have also been associated with NPC1 disease [309–312]. Calcium dyshomeostasis also contributes to mitochondrial dysfunction in NPC1. Loss of NPC1 leads to altered IP3R1 distribution and activity, increasing Ca^{2+} release from the ER and causing mitochondrial calcium cytotoxicity [313]. Another report demonstrates that alterations in membrane contact sites and increased interaction between $\text{Ca}_v1.2\text{--K}_v2.1$ channels lead to abnormal increases in mitochondrial Ca^{2+} levels, resulting in elevated ROS production, decreased mitochondrial membrane polarization and neurotoxicity in NPC1 neurons [314].

Changes in iron homeostasis have also been reported in both NPC1 models and NPC1 patients, including alterations in the brain, suggesting a potential role in the disease's pathophysiology [315–321]. Iron levels have been observed to either decrease or increase depending on the tissue. A recent report demonstrates that defects in autophagy contribute to the disruption of iron homeostasis in NPC1-deficient HEI-OC1 auditory cells [322]. Abnormal autophagy promotes the release of iron from ferritin through ferritinophagy, increasing lipid peroxidation and ferroptosis. In a recent study using label-free proteomics, researchers found that ferroptosis was the most significantly altered pathway in two NPC1 cell models. Mitophagy also emerged as one of the most altered pathways, alongside significant changes in biological processes such as autophagy and cellular iron ion homeostasis [323].

1.7.2 Therapeutic approaches for Niemann Pick Type C

Currently, there is no cure for NPC disease and treatment strategies primarily aim at reducing the effects of its symptoms. The use of medicines to diminish cholesterol levels has been tested, but no beneficial effects on neurological symptoms were observed [324–326]. For instance, studies in an NPC1 mouse model showed that using nifedipine and probucol as cholesterol-lowering treatments significantly reduced liver cholesterol accumulation. However, these drugs did not affect the neurological progression of the disease, likely because they do not cross the blood-brain barrier [324]. Up to date, only one disease-modifying drug, Miglustat (Zavesca®, Actelion Pharmaceuticals Ltd.), is approved for the treatment of NPC by several regulatory authorities, except by the US Food and Drug Administration (FDA) [325,327]. Miglustat can cross the blood-brain barrier and inhibit glucosylceramide synthase, thereby blocking the initial step in glycosphingolipid biosynthesis and reducing their accumulation. Since its initial approval in 2009, treatment with miglustat has been associated with the stabilization or even improvement of neurological symptoms. The benefits of this treatment are more pronounced in adult-onset patients, especially when treatment is initiated early after diagnosis. Administration of HP-

β -CD has also shown to be an efficient therapy in NPC mouse models [328,329], cat models [330], and human patients [331–334]. Despite its inability to penetrate the blood-brain barrier, HP- β -CD is one of the most promising therapeutic agents for NPC currently in clinical trials [325]. HP- β -CD is a cyclic oligosaccharide with a hydrophobic core capable of binding cholesterol, thereby reducing its accumulation in endolysosomes. Simultaneous administration of HP- β -CD through intraperitoneal, subcutaneous, and intrathecal routes to young NPC animal models has demonstrated benefits against disease progression [325]. These benefits include improved liver function, reduced lipid accumulation, and neurological improvement. However, significant side effects were observed in these animal models [335]. Other cyclodextrins, such as hydroxypropyl- γ -cyclodextrin, show therapeutic potential, but further research is required [325,336–338]. There is still a need for effective drug therapies for NPC disease, ideally with a convenient and safe administration route.

Inhibitors of histone deacetylases (HDACs) have been shown to reduce endolysosomal cholesterol accumulation by increasing the expression of NPC1 [339]. The FDA-approved HDAC inhibitors vorinostat and Panobinostat increase the expression of mutant NPC1 and reduce cholesterol accumulation in several NPC1 mutant fibroblast cell lines [340]. Another FDA-approved HDAC inhibitor, valproic acid (VPA), also significantly reduces cholesterol buildup and alleviates impairments in self-renewal and in the process of neuronal differentiation in induced neural stem cells obtained from fibroblasts of patients with NPC [341]. Recently, it was demonstrated that VPA corrects the folding and trafficking defect of I1061T-NPC1, the most common NPC1 mutation, restoring cholesterol balance [342]. VPA easily crosses the blood-brain barrier and has been prescribed for decades to patients with epilepsy and bipolar disorder as well as to manage neurological symptoms in advanced NPC1 [342]. The authors suggest that combining VPA with HP- β -CD could enhance the clinical effectiveness of HP- β -CD-based therapies.

Arimoclomol, a small molecule capable of penetrating the blood-brain barrier, induces the expression of heat shock proteins (HSPs), including HSP70, which plays a crucial role in the proper processing and folding of the NPC1 protein [325,343]. A recent phase 2/3 clinical trial evaluating arimoclomol for NPC treatment reported positive results, indicating a reduction in disease progression [344].

1.7.3 The yeast model of NPC1

The yeast *S. cerevisiae* has been used as a model to study molecular mechanisms underlying NPC disease. Yeast cells express an orthologue of human NPC1 named Nrc1 (Niemann-Pick type C-related protein 1), with *NCR1* and NPC1 sharing 32% sequence identity [345,346]. Both NPC1 and Nrc1 proteins localize to the membranes of the lysosomal (vacuolar)/endosomal system and have similar functions [345,346]. Moreover, expression

of Ncr1 in NPC1-deficient mammalian cells has been shown to abolish disease phenotypes [345,346]. Likewise, the Npc2 protein in yeast is orthologous to human NPC2, and these proteins share 22% primary sequence identity and possess a conserved structure and function [346]. As in mammalian cells, Ncr1 and Npc2 work together to deliver sterols to the vacuolar membrane for subcellular redistribution [346]. Npc2 binds sterol molecules in the vacuolar lumen and transfers them to Ncr1, where sterols can be integrated into the vacuolar membrane or distributed by nonvesicular transport throughout the cell, either directly through membrane contact sites or via cytosolic shuttle proteins [347]. Notably, cells lacking Ncr1 recapitulate several phenotypes observed in NPC1 mammalian cells. These include the disruption of ergosterol homeostasis (the functional equivalent of cholesterol in mammalian cells) and alterations in sphingolipid metabolism, leading to increased levels of the bioactive sphingolipids phytosphingosine, dihydrosphingosine, and phytoceramide [348,349]. Defects in the processing of lipid droplets within yeast vacuole were also reported. Ncr1 and Npc2 are essential for the formation of membrane domains enriched in sterols, which are necessary for microautophagy of lipid droplets. In *ncr1Δ* and *npc2Δ* cells, the engulfment of lipid droplets into the vacuoles is impaired [350]. Egebjerg and colleagues suggested that these cells efficiently uptake lipid droplets into the vacuole but their degradation is compromised, resulting in the accumulation of lipid vesicles [351]. Furthermore, cells lacking Ncr1 exhibit elevated levels of oxidative stress indicators and defects in mitochondrial function, leading to premature aging [348,349]. Mitochondrial defects include mitochondrial network fragmentation, mitochondrial membrane depolarization, decreased oxygen consumption and an inability to grow under exclusively respiratory conditions [348,349]. These phenotypes were associated with the hyperactivation of Pkh1 and its downstream target Sch9 by sphingosine signalling (Figure 5). The deletion of *PKH1* or *SCH9* suppresses oxidative stress sensitivity, mitochondrial defects, and premature lifespan in cells lacking Ncr1 [348]. The accumulation of phytoceramide also leads to the activation of Sit4 phosphatase, which contributes to *ncr1Δ* impaired mitochondrial function, oxidative stress sensitivity and premature lifespan (Figure 5). The deletion of *SIT4* suppresses these phenotypes, and this protective effect is partly attributed to the downregulation of the Pkh1-Sch9 pathway, as evidenced by decreased activation of Sch9 in *ncr1Δsit4Δ* cells [349].

Recently, we reported that autophagic flux is disrupted in yeast cells lacking Ncr1 due to impaired iron homeostasis (Figure (5) [352]. Lowering intracellular iron levels by treating cells with the iron chelator BPS reduces oxidative stress, restores autophagy, and extends lifespan in *ncr1Δ* cells. Additionally, we demonstrated that TORC1 signalling is decreased in the yeast model of NPC1, suggesting that TORC1 activation is not the cause of autophagy inhibition [352].

Unbiased screens in yeast also revealed alterations in pathways already linked to NPC disease phenotypes, including mitochondrial function, metal ion homeostasis, calcium signaling, and lipid trafficking [310]. Additionally, these screens uncovered changes in genes associated with cytoskeleton organization and nutrient sensing, and showed that Ncr1 physically interacts with Pmc1, a Ca^{2+} ATPase, Fth1, an iron transporter, and Apc11, an anaphase-promoting protein. Notably, the cytoskeletal defects suggested by the yeast studies were confirmed in cells derived from NPC1 patients [310], underscoring the relevance of yeast as a model system for uncovering novel pathways implicated in NPC1 pathology.

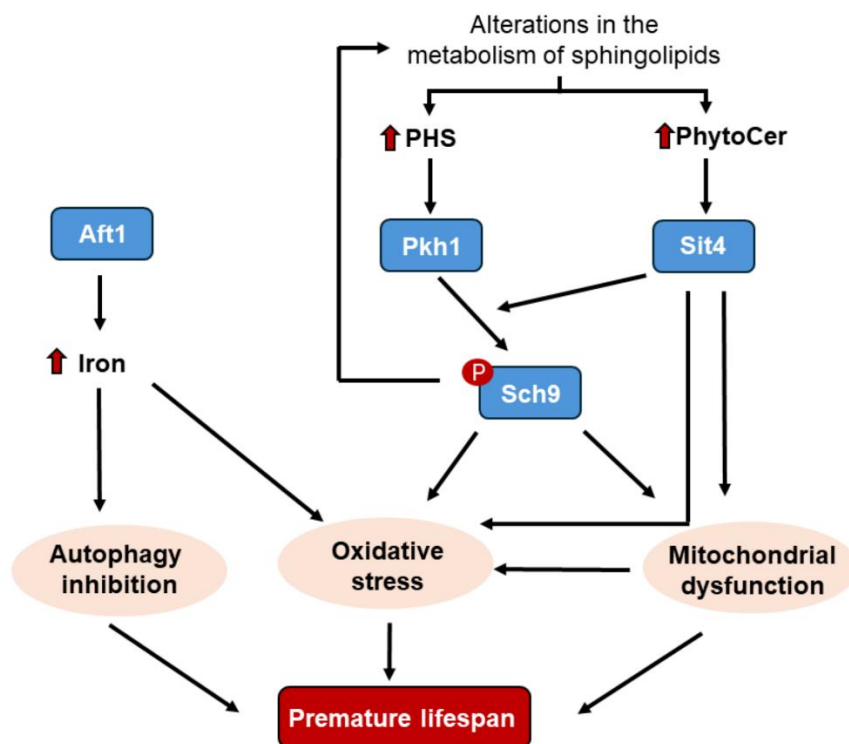


Figure 5 - Accumulation of iron and sphingolipids species mediate the premature lifespan of the yeast model of NPC1. Deletion of *NCR1* leads to increased levels of phytosphingosine (PHS), which enhances the activation of the Pkh1-Sch9 pathway. This activation results in increased oxidative stress, mitochondrial dysfunction, and premature aging. Additionally, the elevated accumulation of phytoceramide (PhytoCer) activates the Sit4 phosphatase. Sit4, either directly or through further activation of Sch9, contributes to mitochondrial dysfunction, increased oxidative stress, and premature aging in cells lacking Ncr1. Sch9 activation also contributes to the observed alterations in sphingolipid metabolism in *ncr1Δ* cells. Moreover, iron accumulation, triggered by the activation of the low-iron sensing transcription factor Aft1, impairs autophagy induction and increases oxidative stress, further contributing to the premature aging of *ncr1Δ* cells.

1.8 Research aims

Lysosomes and lysosome-like vacuoles (in yeast) are critical for cellular homeostasis and longevity. A decline in lysosomal function is associated with aging and age-related diseases, whereas improving lysosomal function is linked to increased lifespan and health span. Several interventions, such as the modulation of nutrient signaling pathways (e.g., TORC1, Sch9, PKA, Snf1/AMPK), increase lifespan by enhancing lysosomal/vacuolar function. This indicates that the levels and phosphorylation status of specific vacuolar proteins may change during aging, and these alterations are mitigated under conditions of extended lifespan. Understanding the regulation of lysosomes during aging and lifespan extension conditions may provide new insights into the biology of this organelle, increasing its potential as a therapeutic target to promote a healthier lifespan.

The main aim of this thesis was to identify proteins and pathways important for vacuolar function and critical for cellular homeostasis during aging. Specifically, it aimed to identify vacuolar proteins modulated under conditions of altered longevity using phosphoproteomic approaches and assess the impact of those alterations on aging. For this purpose, we characterized the yeast model of Niemann-Pick type C (*ncr1Δ*) that exhibits premature aging (Chapters II and III) and yeast cells lacking Sit4, a model of extended longevity (Chapter IV).

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CHAPTER II

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann-Pick Type C1

This chapter is based on the following published article:

Telma S. Martins, Rafaela S. Costa, Rita Vilaça, Carolina Lemos, Vitor Teixeira, Clara Pereira and Vítor Costa. Int J Mol Sci, 2023, 24, 6221. <https://doi.org/10.3390/ijms24076221>.

Abstract

Niemann–Pick type C1 (NPC1) is an endolysosomal transmembrane protein involved in the export of cholesterol and sphingolipids to other cellular compartments such as the endoplasmic reticulum and plasma membrane. NPC1 loss of function is the major cause of NPC disease, a rare lysosomal storage disorder characterized by an abnormal accumulation of lipids in the late endosomal/lysosomal network, mitochondrial dysfunction, and impaired autophagy. NPC phenotypes are conserved in yeast lacking Ncr1, an orthologue of human NPC1, leading to premature aging. Herein, we performed a phosphoproteomic analysis to investigate the effect of Ncr1 loss on cellular functions mediated by the yeast lysosome-like vacuoles. Our results revealed changes in vacuolar membrane proteins that are associated mostly with vesicle biology (fusion, transport, organization), autophagy, and ion homeostasis, including iron, manganese, and calcium. Consistently, the cytoplasm to vacuole targeting (Cvt) pathway was increased in *ncr1*Δ cells and autophagy was compromised despite TORC1 inhibition. Moreover, *ncr1*Δ cells exhibited iron overload mediated by the low-iron sensing transcription factor Aft1. Iron deprivation restored the autophagic flux of *ncr1*Δ cells and increased its chronological lifespan and oxidative stress resistance. These results implicate iron overload on autophagy impairment, oxidative stress sensitivity, and cell death in the yeast model of NPC1.

Keywords: Ncr1; TORC1; Aft1; vacuoles; vesicle trafficking; iron; autophagy; oxidative stress; chronological aging

2.1 Introduction

Lysosomes and yeast lysosome-like vacuoles are acidic organelles that play critical roles in the degradation of material delivered by autophagy or by the endocytic pathway, compartmentation of metabolites, including ions and amino acids, and nutrient signaling. Plus, they interact with other organelles, including mitochondria and endoplasmic reticulum (ER), to promote cellular homeostasis [1–3]. The functional decline of lysosomes has been implicated in aging and numerous diseases. For example, lysosomal acidification and autophagy decrease with age, leading to defects in nutrient storage and in the degradation of damaged proteins and organelles, cellular dyshomeostasis, and ultimately to cell death [4]. Lysosomal impairment and defects in the lysosomal-mitochondrial axis are also hallmarks of lysosomal storage disorders (LSDs), including Niemann–Pick type C (NPC) disease [5].

NPC disease is a rare LSD caused by loss-of-function mutations in NPC1, in 95% of cases, or NPC2 [6,7]. It affects nearly 1/120,000 live births with clinical manifestations such as malfunction of the liver and lung and progressive neurodegeneration that cause ataxia and neurological dysfunction [8–10]. NPC1 and NPC2 are late endosomal/lysosomal proteins involved in the transport of endocytosed cholesterol. NPC2 is a soluble glycoprotein that has a high affinity to cholesterol and transfers it to NPC1, a transmembrane protein that promotes cholesterol export and transfer to the ER, plasma membrane, recycling endosomes, trans-Golgi network, and mitochondria [11–13]. In NPC disease, unesterified cholesterol accumulates in endolysosomal compartments, concomitantly with sphingolipids (e.g., sphingomyelin, glycosphingolipids, and sphingosine), which become depleted in other cellular compartments [14,15].

Disruption of cholesterol dynamics in NPC results in vesicular trafficking defects, a decrease in endosome and lysosome motility, and stalled autophagic flux associated with induction of autophagy and defects in the fusion of autophagosomes with late endosomes/lysosomes [9,16–26]. A proteomic analysis of isolated lysosomes showed that loss of NPC1 leads to the upregulation of several proteins associated with autophagy, namely autophagosome elongation and closure (GABARAPL2), endocytosis (RAB5A), lysosomal biogenesis (CLN3, IGF2R), and selective autophagy (SQSTM1/p62) [27]. Different cathepsins are also upregulated in NPC [28,29], but the activity of cathepsin D decreases due to defects in its maturation [30]. Several pieces of evidence indicate that lysosomal hydrolases are depleted and autophagy is inhibited in cell models of NPC1 disease due to an aberrant activation of the mammalian Target of Rapamycin Complex 1 (mTORC1) by cholesterol [31–33]. Conversely, cholesterol depletion with methyl- β -cyclodextrin restores the autophagic flux in NPC1 cells by inducing AMP-activated protein

kinase (AMPK)-dependent activation of ULK1/ATG1 and inhibition of mTORC1 [34]. Nevertheless, other studies reported that mTORC1 is inhibited [35] or not affected [16] in NPC disease.

Defects in autophagy and mitophagy, concomitant with a decrease in mitochondrial motility, lead to mitochondrial impairment in NPC disease [20]. Indeed, NPC1 fibroblasts exhibit depletion of mtDNA and changes in mitochondrial membrane potential and mitochondrial dynamics [36]. However, several other factors have been implicated in mitochondrial dysfunction. These include the downregulation of sphingosine-1-phosphate receptors and sphingosine-1-phosphate signaling that leads to KLF2- and ETV1-dependent transcriptional repression of mitochondrial biogenesis [37,38]. Notably, mitochondrial dysfunction of NPC1 cells is also caused by the accumulation of cholesterol due to the expansion of lysosome-mitochondria contact sites, which increases cholesterol transfer to mitochondria, disrupting its function and redox homeostasis [39,40]. Mitochondrial dysfunction in NPC has also been associated with calcium cytotoxicity resulting from the activation of the IP3R type 1 in the ER that increases calcium release [41]. Numerous studies have also shown that iron homeostasis is disrupted in the plasma and tissues of NPC patients and in animal and cell models of this disease [42–47]. Iron is a redox-active metal that catalyzes the Fenton reaction, in which hydrogen peroxide is converted into highly reactive hydroxyl radicals. Thus, iron accumulation has been linked to neurodegenerative disorders [48] and may contribute to oxidative distress and ferroptosis in NPC [49].

The yeast *Saccharomyces cerevisiae* has been used as a model for neurodegenerative diseases [50] and LSDs, including NPC [51]. Yeast cells express orthologues of NPC1 (named Ncr1) and NPC2, which are localized to lysosome-like vacuoles and involved in lipid trafficking [52,53]. Recent studies showed that sterols are delivered to the luminal membrane leaflet by the N-terminal domain of Ncr1 and transported by a proton-driven mechanism [54]. Ncr1 and Npc2 play a role in the formation of sterol-rich membrane domains that are required for microlipophagy [55]. Notably, yeast cells lacking Ncr1 recapitulate molecular features of NPC including trafficking defects [52], impaired transport of sterols to the vacuolar membrane under starvation conditions [54], accumulation of long-chain sphingoid bases and ceramide leading to a shortened lifespan [56,57], mitochondrial dysfunction [36,56,57], and changes in calcium and metal ion homeostasis and lipid trafficking [36], as well as alterations in sphingolipid signaling and nutrient signaling [36,56,57] and in cytoskeleton organization [36].

In this work, we performed a MS-based phosphoproteomic analysis of the vacuolar membranes from *ncr1Δ* mutant cells to characterize how loss of Ncr1 affects yeast lysosome-like vacuoles. Proteins associated with vesicle fusion, transport and organization

and with iron homeostasis were the most significantly altered. We found that the cytoplasm to vacuole targeting (Cvt) pathway was induced and autophagy was impaired in *ncr1* Δ cells. Notably, *ncr1* Δ cells exhibited iron overload, and iron deprivation restored the autophagic flux and improved longevity and oxidative stress resistance of *ncr1* Δ cells. Overall, these results implicate iron overload on autophagy impairment and cell death in the yeast model of NPC1.

2.2. Results and Discussion

2.2.1. Characterization of the vacuolar membrane proteome in the yeast model of NPC1

To characterize how Ncr1 affects cellular functions mediated by the yeast lysosome-like vacuoles, the effects of Ncr1 deficiency on the phosphoproteome of vacuolar membranes were investigated. For that, vacuolar membranes were isolated from wild type and *ncr1* Δ cells, and samples were analyzed by nanoLC-MS/MS (an overview of the experimental design is shown in Figure 1). Vacuolar membranes were analyzed instead of intact vacuoles to avoid the interference of non-vacuole constituents that are delivered from other compartments for degradation. The enrichment and purity of the vacuolar membranes were qualitatively analyzed by Western blot using antibodies against specific organelle markers (not shown).

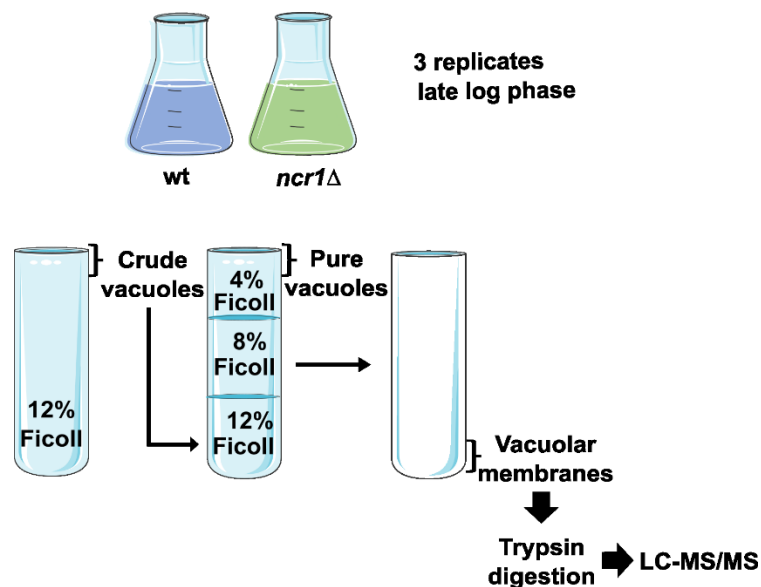


Figure 1. Overview of the experimental design. Wild type (wt) and *ncr1* Δ cells were grown in SC medium to late exponential phase [late log; ($OD_{600nm} \cong 2$)]. Vacuoles were isolated by density centrifugations using Ficoll gradients and vacuolar membranes recovered after osmotically lysis of vacuoles. Samples were then digested with trypsin, and protein identification and quantitation were performed by nanoLC-MS/MS.

We were able to detect 230 proteins annotated as vacuolar membrane proteins, representing around 80% of the total number of vacuolar membrane proteins in *S. cerevisiae* (protein levels and statistical analysis are presented in the Supplementary table S1). From these, we identified phosphorylated residues in 54 proteins (Supplementary table S2 contains information about the percentage of phosphorylation of each identified residue and the respective statistical analysis). Our results showed that the levels of 45 proteins changed significantly in *ncr1* Δ vacuolar membranes, and 18 proteins were differently phosphorylated, with 4 proteins (Yck3, Ybt1, Fet5 and Fth1) presenting alterations in both levels and phosphorylation (Figure 2A-C). In *ncr1* Δ vacuolar membranes, 21 proteins were less abundant and 24 proteins more abundant. Moreover, 14 amino acid residues were less phosphorylated and 8 more phosphorylated. Gene Ontology (GO) analysis on biological processes showed an enrichment in proteins related to vesicle and vacuole fusion and organization, ion homeostasis and transport (namely, iron, manganese, and calcium), polyphosphate metabolism, Golgi organization, autophagy, and protein targeting to vacuole (Figure 2D and Supplementary table S3). These results suggest that vesicle trafficking pathways and ion homeostasis are altered in yeast cells lacking Ncr1, being consistent with previously reported trafficking defects and calcium/iron dyshomeostasis [9,16–26,42–44,46,47,58] and alterations in the expression of lysosomal proteins related to autophagy in NPC [27]. A previous study with the yeast model also showed that Ncr1 interacts with vacuolar proteins, including the Pmc1 Ca²⁺ ATPase and the Fth1 iron transporter. Moreover, the yeast model of NPC exhibits mislocalization of proteins involved in vacuole protein sorting (Vps41, Did4, Pmr1 and Yip5) and *NCR1* is synthetically lethal with genes associated with protein sorting (*MVB12* and *APS1*) [36].

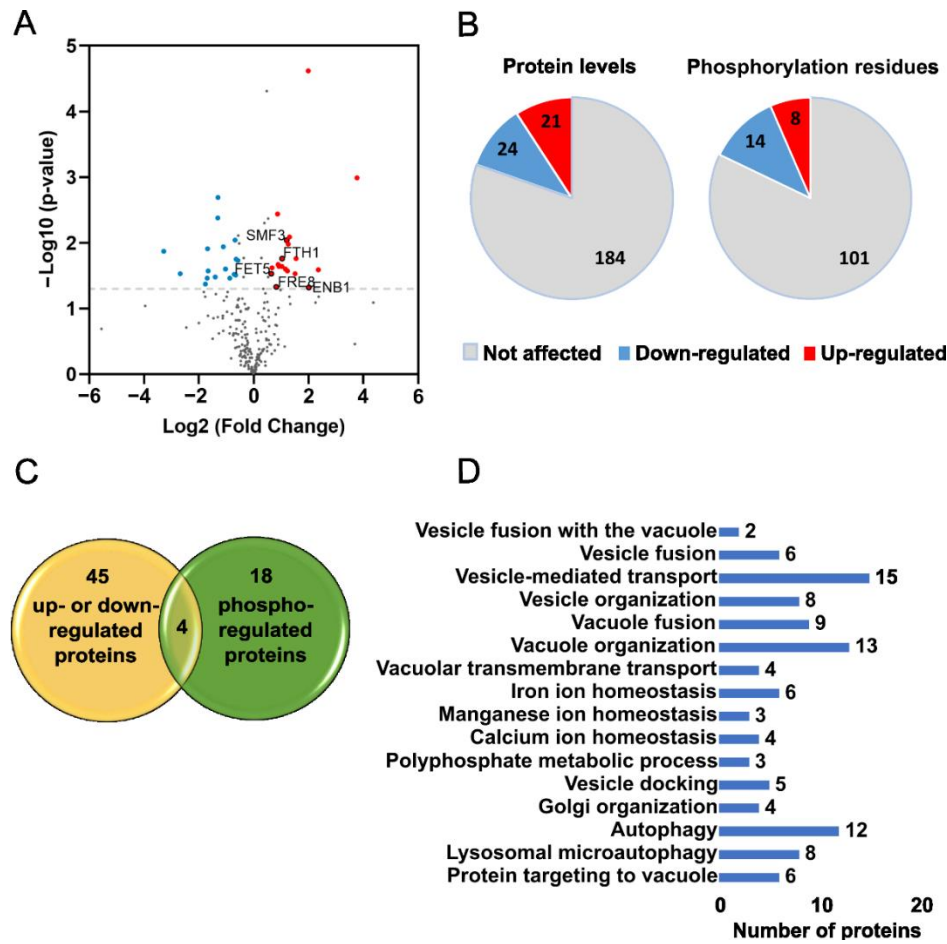


Figure 2. Overview of the phosphoproteomic analysis from *ncr1Δ* vacuolar membranes. (A) Volcano plot for differentially expressed proteins in *ncr1Δ* vs. wild type samples. Log-transformed *p*-values (Student's *t*-test) in the vertical axis are plotted against log-transformed fold change in the horizontal axis. The horizontal dashed line marks a *p*-value of 0.05. Red plots represent the up-regulated proteins with a 1.5-fold change threshold, and blue plots represent the down-regulated proteins (log2 fold change of ± 0.58). Red plots with black outlines represent the proteins associated with iron homeostasis. (B) Number of proteins and number of phosphorylation sites significantly up- or down-regulated in the *ncr1Δ* mutant. (C) Overlap of proteins with alterations in the levels and phosphorylation in *ncr1Δ* samples. (D) Gene Ontology (GO) term enrichment analysis on biological processes was performed for proteins with statistical alterations in *ncr1Δ* samples (levels and phosphorylation).

2.2.2. Cvt pathway is induced and autophagy compromised in the yeast model of NPC1

Trafficking pathways are critical for vacuole biogenesis and function. In eukaryotic cells, organellar proteins are synthesized in the ER and then transferred to the Golgi apparatus for sorting into their cellular compartments. In yeast, proteins are targeted to the vacuole either through endosomes via the vacuolar protein sorting (VPS/CPY) pathway or directly through the alkaline phosphatase (AP-3) pathway [59]. Our proteomic data revealed that *ncr1Δ* vacuolar membranes had lower levels of several proteins (Yck3, Vam3, Nyv1) transported to the vacuole through the AP-3 pathway, suggesting a defective route. To

assess the AP-3 pathway, cells were transformed with a plasmid expressing a GFP-tagged Nyv1-Snc1 fusion protein that localizes to both vacuolar and plasma membrane in cells defective in AP3 transport (e.g., *yck3* Δ cells) [60] (Figure 3A). Our results showed that GFP-Nyv1-Snc1 was restricted to the vacuolar membrane in wild type and *ncr1* Δ cells, suggesting that the AP-3 pathway is not compromised (Figure 3A). We also observed that Yck3-S48 phosphorylation decreased in the *ncr1* Δ mutant (Supplementary table S3). However, this alteration does not seem to affect the AP-3 pathway and, therefore, may not impact *ncr1* Δ phenotypes.

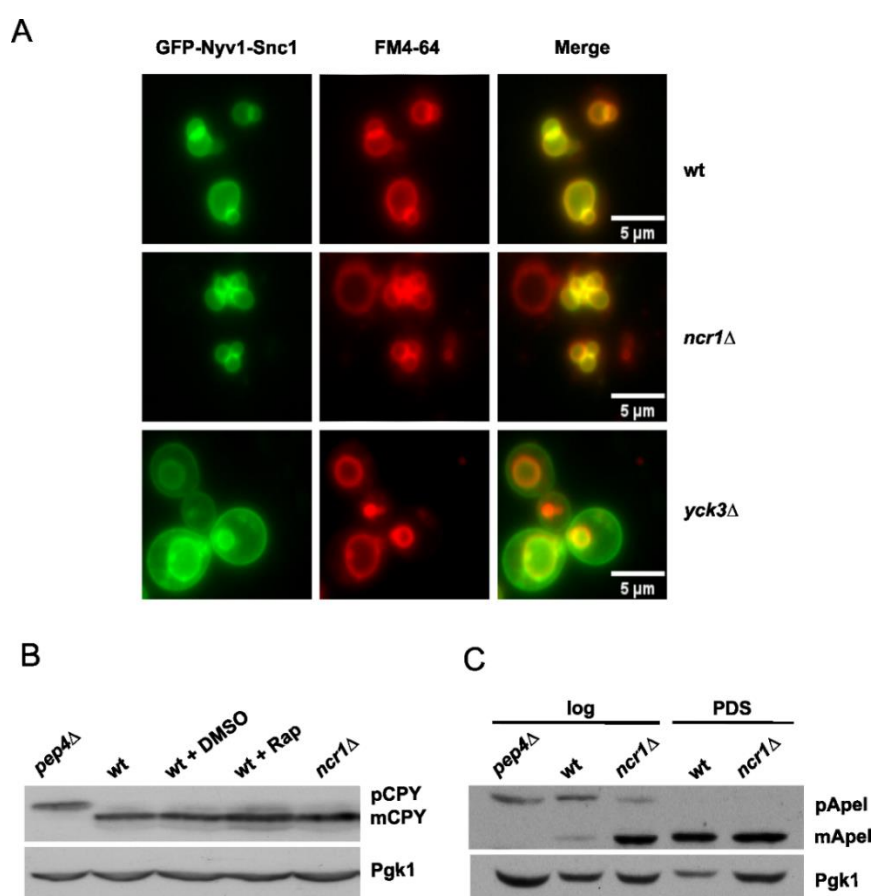


Figure 3. Loss of Ncr1 does not impair protein trafficking to the yeast vacuole. (A) ALP-AP-3 pathway: wild type (wt), *ncr1* Δ , and *yck3* Δ cells carrying a plasmid expressing a GFP-tagged Nyv1-Snc1 fusion protein grown to exponential phase were incubated with 0.024 mM FM4-64 (vacuolar membrane probe) for 1 h and observed by fluorescence microscopy. (B) CPY pathway: cells were grown to exponential phase and proteins were analyzed by immunoblotting, using anti-CPY antibody. Bands corresponding to the proenzyme (prCPY) or the mature enzyme (mCPY) are indicated. Pgk1 was used as loading control. Cells lacking Pep4, which is involved in the proteolytic activation of prCPY to mCPY, were used as a negative control. As a positive control, wild type cells were treated with rapamycin (200 ng mL⁻¹) for 4 h (wt + Rap). (C) Cvt pathway: cells were grown to exponential (log) and post-diauxic shift [(PDS), 48 h after log] phases, and the processing of pApel to mApel was analyzed by immunoblotting, using anti-Apel antibody. Pgk1 was used as loading control. Cells lacking Pep4, which is involved in the proteolytic activation of pApel to mApel, were used as control. Representative images of at least three independent experiments are shown.

Carboxypeptidase Y (CPY) is the best-studied soluble protein targeted to the vacuole through the CPY pathway and has been largely used as a read-out of this pathway [61]. CPY is transported to the vacuole where it is processed to its mature form by Pep4-dependent proteolytic activation [62]. To assess this pathway, CPY was analyzed by Western blotting. Cells lacking Pep4 and wild type cells treated with rapamycin were also analyzed to facilitate the identification of the precursor and mature forms of CPY. The results revealed that CPY was fully processed in both wild type and *ncr1* Δ cells, indicating that the CPY pathway is not defective in Ncr1-deficient cells (Figure 3B). These results are consistent with a previous study showing that Ncr1 deficiency does not affect endocytosis, CPY maturation, or AP-3 pathway [63].

The cytoplasm to vacuole targeting (Cvt) pathway is another biosynthetic route for vacuolar proteins in yeast [64]. This pathway uses most of the molecular machinery of autophagy to transport vacuolar hydrolases such as aminopeptidase I (Ape1) and α -mannosidase (Ams1). Biosynthetic cargo is sequestered by a cytosolic double-membrane vesicle that subsequently fuses with the vacuole, allowing the resident enzymes access to their final destination. Our proteomic analysis showed a 13.6-fold increase of Ams1 in the vacuolar membranes of the *ncr1* Δ mutant. This led us to postulate that the Cvt pathway may be induced in this mutant. To test this hypothesis, we analyzed the processing of Ape1 to its mature form by Western blotting, a well-established assay for Cvt. Since this pathway is induced during growth from exponential (fermentative) to post-diauxic shift (PDS; respiratory) phase, we assessed Cvt in both phases. The results demonstrated that the levels of the mature form of Ape1 increased in *ncr1* Δ cells grown to the exponential phase (Figure 3C), suggesting that the Cvt pathway flux is enhanced in cells lacking Ncr1. It should be noted that Ape1 was not detected in our study since it is not a vacuolar membrane protein.

In addition to the Cvt pathway, autophagy is induced during growth to the PDS/respiratory and stationary phases. These cellular adaptations are important for cell longevity and their impairment has been associated with numerous diseases, including neurodegenerative disorders and aging [4]. We assessed the autophagic flux by monitoring the processing of GFP-Atg8 [65]. Since the GFP moiety is resistant to proteolysis and remains stable upon vacuolar delivery of GFP-Atg8, the detection of free GFP signal by Western blotting can be used to evaluate the autophagy flux. Autophagy is a crucial process for cell survival that is negatively regulated by TORC1 [66]. Autophagy is induced as cells start to age and nutrients become scarce, removing damaged macromolecules that are recycled and allowing cells to survive under nutrient deprivation. In agreement, we observed an increase in autophagy in wild type cells grown to the PDS phase or treated with rapamycin (to inhibit TORC1). Contrary to the wild type, cells lacking Ncr1 were unable to induce autophagy at the PDS phase (Figure 4A), which may underlie the premature aging

observed in these cells [56]. Changes in autophagy have been largely described in NPC1-deficient mammalian cells. The number of autophagosomes increases but autophagic progression and proteolysis are stalled [16,22,25,67]. This has been mostly associated with defects in the cargo-targeting step and in the fusion step of autophagosomes and lysosomes to form autolysosomes. The role of TORC1 signaling in the autophagy defects of NPC1-deficient cells is controversial. Some studies report that loss of function of NPC1 results in TORC1 hyperactivation and that inhibition of TORC1 signaling is sufficient to restore lysosomal function and autophagy [31–33]. However, others reported no alterations or even a decrease in TORC1 activity upon NPC1 deficiency [22,35]. To evaluate TORC1 activity, we assessed TORC1-mediated phosphorylation of ribosomal protein S6 (Rps6 in yeast) at S232/233 (S235/236 in human S6), a specific readout for TORC1 activity [68]. TORC1 responds to growth conditions and nutrient availability and, as expected, its activity decreased in wild type cells grown to PDS phase, which agrees with the induction of autophagy. Our results also demonstrated that cells lacking Ncr1, compared to wild type cells, displayed significantly lower levels of phospho-Rps6 (Figure 4B,C). The phosphorylation of Sch9, another target of TORC1, also decreased in *ncr1Δ* cells (Supplementary Figure S1). Overall, these results indicate that TORC1 signaling is downregulated in the yeast model of NPC1, suggesting that the autophagic flux is impaired in a TORC1-independent manner.

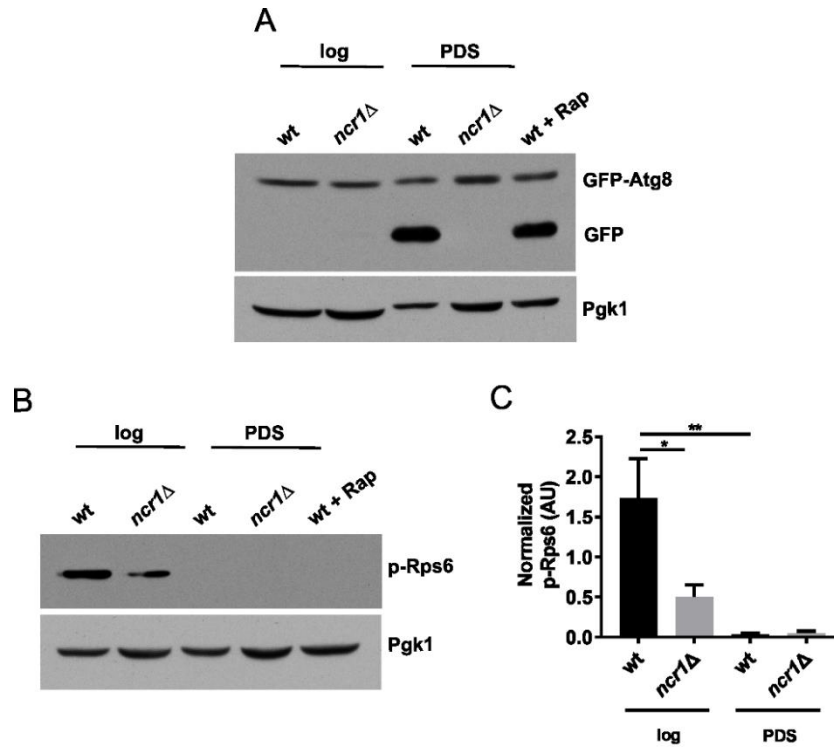


Figure 4. Cells lacking *Ncr1* exhibit autophagic defects despite reduced TORC1 activity. (A) Wild type (wt) and *ncr1*Δ cells carrying pRS416-*GFP-ATG8* were grown to the exponential (log) and post-diauxic shift [(PDS), 48 h after log] phases, and proteins analyzed by immunoblotting using anti-GFP antibody. As positive control, wild type cells grown to exponential phase were treated with rapamycin (200 ng mL⁻¹) for 4 h (wt + Rap). (B) Cells were grown to exponential (log) and post-diauxic shift [(PDS), 48 h after log] phases, and the levels of phospho-Rps6 (S232/233), used as read-out for TORC1 activity, were evaluated by Western blotting. Pgk1 was used as loading control. Representative images of at least three independent experiments are shown. (C) Quantification of phospho-Rps6 levels were calculated by the ratio between p-Rps6 and Pgk1 signals. Values are the mean ± SEM. * $p \leq 0.05$; **, $p \leq 0.01$ (one-way ANOVA).

2.2.3. *Ncr1* deficiency induces the iron regulon and Aft1-dependent iron overload

Iron is an important cofactor essential for cell viability, but iron in excess increases the formation of harmful hydroxyl radicals through the Fenton reaction, promoting oxidative stress [69]. Loss of iron homeostasis associated with NPC1 disease has been documented in several studies. Hung et al. reported higher levels of iron in the brain and decreased levels in the liver and spleen of the NPC1 mouse model. A tendency for iron levels to be higher in post-mortem human NPC1 cerebellar tissue and lower in plasma was also observed [42]. Recently, Chen et al. detected lower levels of ferritin in the cerebrospinal fluid of NPC1 patients, consistent with alterations in iron homeostasis in the central nervous system [46]. Indeed, the authors showed that *Npc1*^{-/-} mice and NPC1 patients have significantly reduced serum iron and several haematological changes characteristic of iron deficiency anaemia. A lower expression of L-ferritin and reduced iron levels in the liver of

Npc1^{-/-} mice was also observed together with higher levels of serum ferritin in NPC patients [46]. NPC1 fibroblasts from patients also exhibit enhanced expression of genes encoding proteins involved in iron homeostasis (ferritin and sideroflexin 1) [70]. However, several studies report a ferritin deficiency in various NPC tissues [44,46,71,72] and a decrease in FTH1 (H-ferritin) protein levels in NPC1-deficient HeLa cells [47]. It has been proposed that lower levels of ferritin lead to an excess of unbound iron, thus favoring lipid peroxidation caused by reactive oxygen species (ROS) [73]. Recently, it was demonstrated that changes in autophagy contribute to the loss of iron homeostasis upon NPC1 deficiency [47]. Abnormal autophagy in NPC1-deficient HEI-OC1 auditory cells induces ferritinophagy (autophagy-dependent iron release from ferritin), resulting in loss of iron homeostasis and increasing lipid peroxide that induces ferroptosis [47].

Interestingly, our proteomic analysis showed that around 24% of proteins with increased levels at vacuolar membranes of cells lacking *Ncr1* were associated with iron homeostasis (Fth1, Fet5, Smf3, Fre8 and Enb1). We also identified alterations in the phosphorylation status of three iron homeostasis-related proteins: Fth1, Fet5 and Ccc1 (Supplementary table S3). This led us to assess whether iron levels were altered in *ncr1Δ* cells. Although iron content was similar in wild type and *ncr1Δ* cells grown to the exponential phase, we observed that *ncr1Δ* cells accumulate iron at the PDS phase (Figure 5A). This led us to postulate that loss of iron homeostasis may be a contributing factor for oxidative stress sensitivity and premature aging of *ncr1Δ* cells [56]. Changes in iron levels cannot be attributed to growth defects of the *ncr1Δ* mutant as they were analyzed at time points where both strains clearly entered the PDS phase (see Supplementary Figure S2). Since proteins involved in vacuolar iron mobilization were upregulated and vacuolar iron storage is known to protect against iron toxicity [74], iron is probably accumulating in the cytoplasm and, as consequence, it may also accumulate in other organelles. Further research is needed to characterize how the dysregulation of vacuolar iron transporters, including changes in its phosphorylation, impacts *ncr1Δ* phenotypes.

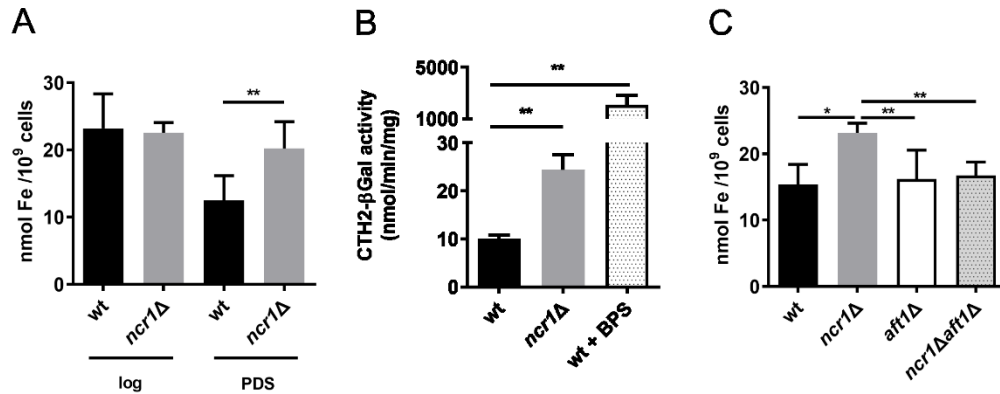


Figure 5 - Ncr1-deficient cells accumulate iron at post-diauxic shift (PDS) phase in an Aft1-dependent manner. (A) Total iron levels were quantified in wild type (wt) and *ncr1Δ* cells grown to exponential (log) and PDS (48 h after log) phases. (B) Wild type and *ncr1Δ* cells expressing the Aft1 binding sequence from *CTH2* promoter fused to a LacZ reporter were grown to the exponential phase, and β -galactosidase activity was determined. Wild type cells treated with 20 μ M of bathophenanthrolinedisulfonic acid (BPS) for 4 h (wt + BPS) were used as positive control. (C) Iron levels were quantified in wt, *ncr1Δ*, *aft1Δ*, and *ncr1Δaft1Δ* cells grown to the PDS (48 h after log) phase. Data are the mean $\pm$ SD of at least three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$ (Student's *t*-test).

Iron uptake and mobilization in the yeast *S. cerevisiae* is mainly regulated at the transcriptional level by the Aft1 transcription factor. When iron availability is low, Aft1 upregulates the iron regulon, a set of genes associated with iron import and mobilization of vacuolar iron stores. However, activation of Aft1 in iron replete conditions can result in iron overload [75]. To assess the involvement of Aft1 in the accumulation of iron in the *ncr1Δ* mutant, cells were transformed with a plasmid containing the Aft1 binding sequence from *CTH2* promoter fused to a LacZ reporter, and β -galactosidase activity was determined. As expected, *CTH2*-LacZ was induced in wild type cells treated with the iron chelator bathophenanthrolinedisulfonic acid (BPS) (Figure 5B). Under normal conditions, β -galactosidase activity was significantly higher in *ncr1Δ* cells than in parental cells. Moreover, iron overload was abolished in *ncr1Δaft1Δ* cells (Figure 5C), indicating that early activation of Aft1 (in exponential fermentative phase) mediates iron accumulation in a later stage (PDS phase) in *ncr1Δ* cells. The vacuolar defects described in this study for *ncr1Δ* cells might contribute to the iron overload in response to abnormal activation of Aft1 (lack of vacuolar iron buffering capacity). On the other hand, premature aging of *ncr1Δ* cells can lead to defective intracellular iron homeostasis and abnormal activation of the iron regulon (Aft1) [76]. *AFT1* deletion did not suppress the premature aging of cells lacking Ncr1 (Supplementary Figure S3). However, *aft1Δ* cells also exhibited a shortened lifespan. Since Aft1 also has iron-independent functions [77], *AFT1* deletion likely affects other processes important for cell longevity, which may explain why it did not increase the lifespan of *ncr1Δ* cells. Moreover, *aft1Δ* cells display mitochondrial defects similar to the *ncr1Δ* mutant, which

can also explain its shortened lifespan [76]. The mechanism leading to Aft1 activation in cells lacking Ncr1 remains to be disclosed. These cells exhibit glutathione depletion [56], which may contribute to Aft1 activation as dysregulation of glutathione levels triggers a starvation-like response [78]. Depletion of glutathione and mitochondrial dysfunction in *ncr1* Δ cells might impair iron-sulfur cluster assembly machinery and compromise the iron-dependent nuclear export of Aft1 [79]. Changes in lipids may also contribute to iron dyshomeostasis, as previous studies have unravelled a functional crosstalk between lipid metabolism and iron maintenance [74]. Ergosterol [80] and sphingolipids [81], both altered in *ncr1* Δ cells [56,57,82], modulate Aft1 activity upon iron depletion. This raises the hypothesis that accumulation of ergosterol and sphingolipids in *ncr1* Δ cells could contribute to Aft1 deregulation.

2.2.4. Iron chelation with BPS increases lifespan, oxidative stress resistance, and restores autophagic flux in the yeast model of NPC1

Recently, it was shown that iron limitation, by treating cells with BPS, prolongs yeast lifespan in an autophagy-dependent manner [83]. Since cells lacking Ncr1 display an impairment in autophagic flux and iron accumulation, this led us to test whether BPS would produce beneficial effects. For that, we analyzed the chronological lifespan. We considered the PDS phase as t0 in this assay, instead of the beginning of the stationary phase (3 days after PDS; standard conditions used in most studies), as *ncr1* Δ cells have a short lifespan [56,57]. Our results showed that BPS treatment caused a significant lifespan extension in *ncr1* Δ mutant cells (Figure 6A,B), indicating that iron accumulation is involved in the *ncr1* Δ mutant shortened lifespan. In the wild type strain, however, BPS did not extend longevity, and even decreased it (Figure 6A,B), contrarily to a previous report [83]. This may arise from differences in growth conditions since, in addition to BPS supplementation, Montella-Manuel et al. used an SD medium prepared with an iron-free nitrogen base [83]. Notably, incubation with BPS also suppressed the hydrogen peroxide sensitivity and high ROS levels of *ncr1* Δ cells (Figure 6C,D), corroborating the initial hypothesis that iron overload promotes intracellular oxidation and oxidative stress sensitivity in cells lacking Ncr1. Studies using a mouse model of NPC1 suggest that iron chelation with deferiprone (DFP) is ineffective in slowing the disease progression or in prolonging lifespan [43]. However, another study reported that DFP conjugated with β -cyclodextrin partially rescues the increase in lysosomal volume in NPC1-deficient cells [84]. Thus, more studies are required to evaluate the use of iron chelators as potential therapeutic strategies against NPC.

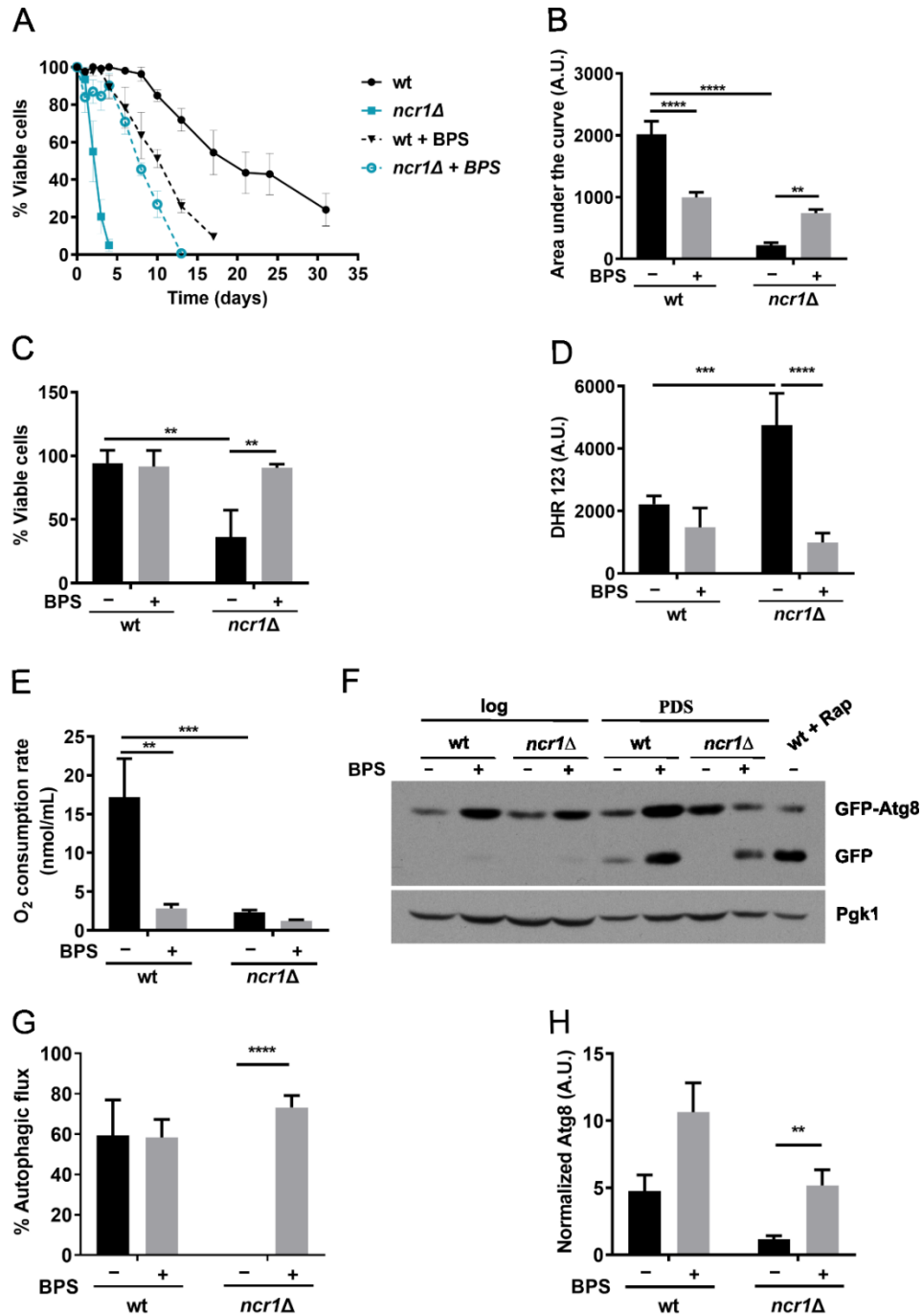


Figure 6. Chelating iron with BPS increases the chronological lifespan, oxidative stress resistance, and autophagic flux of *ncr1Δ* cells. (A) Wild type and *ncr1Δ* cells were grown to post-diauxic shift [(PDS), 24 h after log] phase and maintained overtime in SC medium (wt, *ncr1Δ*) or SC medium supplemented with 80 μ M of BPS (wt + BPS, *ncr1Δ* + BPS). Cellular viability was expressed as the percentage of the colony-forming units in relation to day 0. Data are the mean $\pm$ SEM of at least three independent experiments. (B) The area under each lifespan curve was computed using GraphPad in arbitrary units (A.U.). Data are the mean $\pm$ SEM. ** $p \leq 0.01$; ****, $p \leq 0.0001$ (one-way ANOVA). (C) Cells were grown to PDS (24 h after log) phase in medium supplemented or not with 80 μ M of BPS and exposed to 5 mM H₂O₂ for 1 h. Cell viability was measured as the percentage of the colony-forming unit (treated cells vs. non-stressed cells). Values are the mean $\pm$ SD. ** $p \leq 0.01$ (two-way ANOVA). (D) Cells were grown to PDS (48 h after log) phase in medium supplemented or not with 80 μ M of BPS and intracellular reactive oxygen species were analyzed by flow cytometry, using dihydrorhodamine 123 (DHR123) as probe. Data are the mean $\pm$ SD. *** $p \leq 0.001$; **** $p \leq 0.0001$.

0.0001 (two-way ANOVA). (E) Cells were grown to PDS (24 h after log) phase in medium supplemented or not with 80 μ M of BPS and oxygen consumption rates were measured. Values are the mean $\pm$ SD (two-way ANOVA), ** $p \leq 0.01$, *** $p \leq 0.001$. (F) Cells carrying pRS416-*GFP-ATG8* were grown to exponential (log) and PDS (48 h after log) phases in medium supplemented or not with 80 μ M of BPS. Proteins were analyzed by immunoblotting using anti-GFP antibody. As positive control, wild type cells grown to exponential were treated with rapamycin (200 ng mL⁻¹) for 4 h (wt + Rap). A representative blot of at least three independent experiments is shown. (G) Autophagic flux was calculated by the ratio between the free GFP signal and the sum of free GFP and GFP-Atg8 signals. Values are the mean $\pm$ SEM. **** $p \leq 0.0001$ (Multiple Student's *t*-tests). (H) Atg8 levels were calculated by the ratio between free GFP plus GFP-Atg8 and Pgk1 signals. Values are the mean $\pm$ SEM. ** $p \leq 0.01$ (Multiple Student's *t*-tests).

To further investigate if the beneficial effects imparted by iron chelation were associated with an improvement of other *ncr1* Δ phenotypes, we assessed mitochondrial respiration and autophagy in the mutant upon BPS treatment. Our results showed that BPS did not suppress the mitochondrial dysfunction exhibited by *ncr1* Δ cells, suggesting that it is not mainly caused by iron overload (Figure 6E). Interestingly, BPS significantly reduced the oxygen consumption rate in wild type cells, which is consistent with defective respiration of cells in iron-depleted medium or *aft1* Δ mutant cells [85]. As mitochondrial function is crucial for lifespan [86], this result may explain why iron limitation decreased chronological lifespan of wild type cells (Figure 6A,B). To monitor autophagy, we used the GFP-Atg8 assay (Figure 6F-H). We observed that Atg8 levels tend to increase in wild type cells treated with BPS, although the autophagic flux was not affected. Notably, BPS induced autophagy and restored the autophagic flux of cells lacking Ncr1, suggesting that iron chelation may improve *ncr1* Δ longevity by restoring the autophagic flux.

Several compounds are known to increase lifespan in part by decreasing free iron, protecting against oxidative damage [87]. Iron deficiency downregulates TORC1 activity [88], whose inhibition is largely associated with lifespan extension and healthspan in several organisms [89]. In yeast, iron limitation increases lifespan by inhibiting TORC1 and inducing autophagy [83]. Epigallocatechin gallate, an iron chelator, extends mice and rats' lifespans by increasing the activation of autophagy and decreasing oxidative stress [90,91]. As TORC1 activity was lower in *ncr1* Δ cells (Figure 4B), the increase in the autophagic flux of the mutant treated with BPS is, probably, TORC1 independent. These data point to the existence of another mechanism by which autophagy is regulated by iron. Our results also implicate that loss of iron homeostasis impacts the autophagic flux in the yeast model of NPC disease. The identification of the molecular players involved in this regulation will contribute to our understanding of the molecular basis of the NPC1 disease. Notably, high levels of iron increase sphingolipid synthesis and sphingolipid signaling through the Pkh1 protein kinase [92], and we have previously shown that phytosphingosine (PHS) but not dihydrosphingosine (DHS) levels increase in *ncr1* Δ cells, leading to Pkh1 activation and a shortened chronological lifespan [56]. Plus, the hydroxylation of DHS to PHS is mediated

by the iron-dependent protein Sur2 [93,94], and iron chelation decreases the accumulation of PHS, but not of DHS [95]. More studies are required to define if iron depletion increases the longevity of the yeast model of NPC1 through modulation of Pkh1 signaling. In summary, our results suggest that vesicle trafficking pathways and iron homeostasis are deregulated in cells lacking Ncr1 and implicate iron overload on the high oxidative stress sensitivity, impaired autophagic flux, and premature aging exhibited by the yeast model of NPC1 (Figure 7). This study supports a link between iron toxicity and NPC phenotypes, but more studies are required to clarify if iron chelation may be explored as a therapeutic strategy aimed at counteracting the development of this disease.

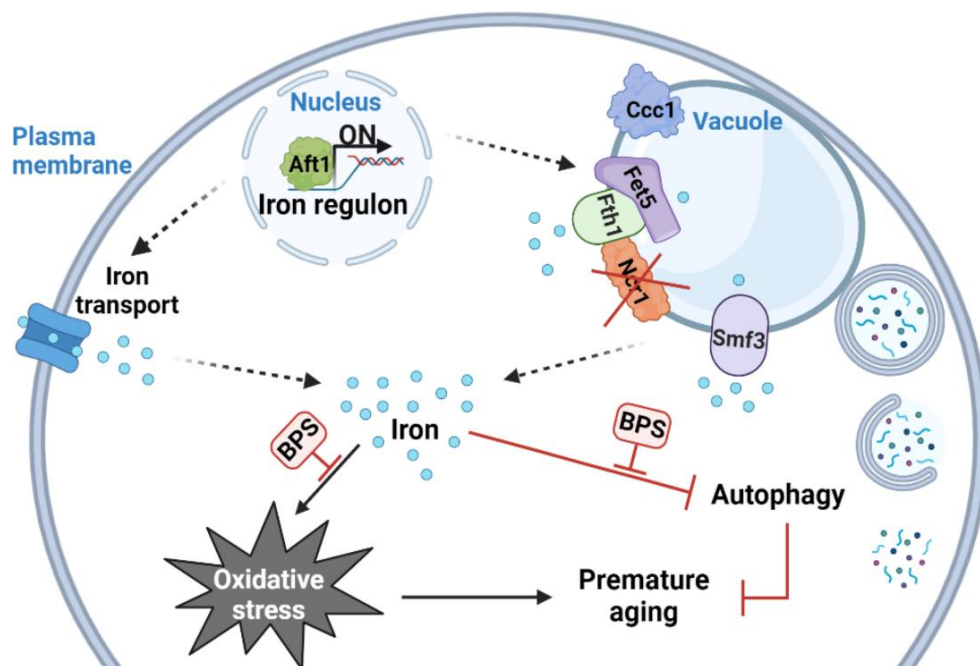


Figure 7. Working model. Absence of Ncr1 leads to Aft1 activation, increasing the transcription of the iron regulon and causing iron dyshomeostasis. Our results show that the levels of vacuolar iron exporters (Fet5, Fth1, and Smf3) increase in vacuolar membranes of *ncr1Δ* cells. The expression of plasma membrane iron transporters may also be induced (due to Aft1 activation), but this remains to be demonstrated. Iron accumulation promotes oxidative stress and blocks autophagy. Iron limitation, by treating cells with BPS, decreases oxidative stress, restores the autophagic flux, and, consequently, improves the lifespan in the yeast model of NPC1. Dashed arrows represent cellular functions and mechanisms demonstrated in other studies.

2.3. Materials and methods

2.3.1 Yeast strains and growth conditions

S. cerevisiae strains used in this study are listed in Table 1. Yeast cells were grown aerobically at 26 °C in an orbital shaker (at 140 rpm), with a ratio of flask volume:medium volume of 5:1. The growth medium used was synthetic complete (SC) medium, containing drop-out, 2% (w/v) glucose (ThermoFisher Scientific, Waltham, MA, USA), and 0.67% (w/v) yeast nitrogen base without amino acids (BD BioSciences, San Jose, CA, USA), supplemented with appropriate amino acids or nucleotides [0.008% (w/v) histidine (Sigma Aldrich, St. Louis, MO, USA), 0.038% (w/v) methionine (Sigma Aldrich, St. Louis, MO, USA), 0.04% (w/v) leucine (Sigma Aldrich, St. Louis, MO, USA) and 0.008% (w/v) uracil (Sigma Aldrich, St. Louis, MO, USA)]. Deletion of *AFT1* in *ncr1Δ* cells was performed using a deletion fragment containing *HIS3* and the flanking regions of *AFT1*. Deletion of *YCK3* in wild type cells was performed using a deletion fragment containing *KanMX4* and the flanking regions of *YCK3*. Yeast cells were transformed using the lithium acetate/single-stranded carrier DNA/PEG method as described [96]. Cells were selected by growing in medium lacking histidine or containing geneticin (300 µg mL⁻¹) and gene deletion was confirmed by standard PCR procedures.

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

Strain	Genotype	Source
BY4741 ^{a,b,c,d}	Mata, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i>	EUROSCARF
<i>ncr1Δ</i> ^{a,b,c,d}	BY4741 <i>ncr1::KanMX4</i>	[56]
<i>yck3Δ</i> ^a	BY4741 <i>yck3::KanMX4</i>	This study
<i>pep4Δ</i>	BY4741 <i>pep4::KanMX4</i>	EUROSCARF
<i>aft1Δ</i>	BY4741 <i>aft1::HIS3</i>	[75]
<i>ncr1Δaft1Δ</i>	BY4741 <i>ncr1::KanMX4 aft1::HIS3</i>	This study

Cells harboring ^apGNS416-*GFP-NYV1-SNC1*, ^bpRS416-*GFP-ATG8*, ^cpCTH2-*LacZ*, and ^dpRS416-*SCH9-5HA*, are indicated.

2.3.2. Vacuolar membranes isolation

2.3.2.1. Spheroplasts preparation and lysis

Membrane vacuoles were isolated as previously described [97]. Cells were grown in 1 L of SC medium until the late exponential phase ($OD_{600nm} \cong 2$) and harvested by centrifugation at 5,000 rpm for 8 min (rotor JLA-8.1000; High speed Centrifuge Beckman Avanti J-26 XP). Cells were washed with 50 mL of double-distilled water and centrifuged again. The wet weight of cells from a 1 L culture was 2–3 g. Cells were resuspended in 2.5

mL of 1.1 M sorbitol (Merck, Darmstadt, Germany) plus 4.5 mg zymolyase (AMSBIO, Cambridge, USA) and 2.5 μ L of β -mercaptoethanol (Merck, Darmstadt, Germany) per 1 g of cells and incubated at 37 °C with shaking at 80 rpm. Light microscopy was used to evaluate the extent of spheroplasts formation. After digestion of the cell wall, the spheroplasts were cooled on ice. Each 2.5 mL of spheroplast suspension was pipetted on top of a layer of 2.5 mL of ice-cold solution containing 7.5% (w/v) Ficoll (Corning, NY USA) and 1.1 M sorbitol in a centrifuge tube. The spheroplasts were washed through this Ficoll-sorbitol layer by centrifugation at 5,000 rpm for 20 min at 4 °C. The spheroplasts were lysed by a 6-fold dilution of the spheroplast pellet with the ice-cold lysis buffer containing 10 mM Tris-MES, pH 6.9, 12% (w/v) Ficoll, 0.1 mM $MgCl_2$. After this step, all solutions were supplemented with a cocktail of protease inhibitors (Sigma Aldrich, St. Louis, MO, USA) and phosphatase inhibitors [1 mM sodium orthovanadate (Sigma Aldrich, St. Louis, MO, USA), 50 mM sodium fluoride (Sigma Aldrich, St. Louis, MO, USA), 5 mM sodium pyrophosphate (Fluka, Buchs, Switzerland)]. The suspension was homogenized on ice in a Dounce homogenizer (40 mL) by 15 strokes with a large clearance pestle “A” (Kontes Glassware, Vineland, NJ, USA). The cells and solutions were kept at 4°C throughout the procedure.

2.3.2.2. Isolation of intact vacuoles

Samples of the spheroplast lysate were transferred to a centrifuge tube and overlaid with lysis buffer until 38 mL. Centrifugation was performed in a swing-out bucket rotor (Beckman, rotor type SW32Ti) at 20,000 rpm for 30 min at 4 °C. The fraction floating on top of the tube, containing the crude vacuoles, was collected and resuspended in lysis buffer by homogenization with a loosely fitting Dounce homogenizer (5–6 strokes, pestle A). The homogenized crude vacuoles were overlaid in a centrifugation tube with a layer of 14 mL of 10 mM Tris-MES pH 6.9, 8% (w/v) Ficoll and 0.5 mM $MgCl_2$ plus a second layer of 14 mL of the same buffer containing 4% (w/v) Ficoll. After centrifugation at 20,000 rpm for 45 min, intact vacuoles were floating on top of the 4% (w/v) Ficoll solution as a white wafer. Purified vacuoles were collected with a spoon-shaped spatula prewetted in 4% (w/v) Ficoll buffer.

2.3.2.3. Isolation of vacuolar membranes

Vacuoles were lysed osmotically in the same volume of buffer consisting of 20 mM triethylammonium bicarbonate (TEAB) pH 8.0, 10 mM $MgCl_2$ and 50 mM KCl. The lysates were then diluted with 2 volumes of buffer containing 10 mM TEAB pH 8.0, 5 mM $MgCl_2$ and 25 mM KCl. Immediately (without incubation), vacuolar membranes were recovered by centrifugation at 80,000 rpm for 1 h (rotor TLA100.3, Beckman, Brea, CA, USA). To remove peripheral proteins, the pellet was resuspended in 100 mM sodium carbonate pH 11.8, and subsequently incubated in the same buffer plus 2 mM EDTA for 15 min on ice. The

membranes were recovered by centrifugation for 3 h at 80,000 rpm (fixed angle rotor 70 Ti, Beckman, Brea, CA, USA). The protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA), after solubilization in 2% (w/v) SDS.

2.3.3. Proteomics

2.3.3.1. Sample preparation and data acquisition

Proteins were solubilized with 100 mM Tris pH 8.5, 1% (w/v) sodium deoxycholate, 10 mM tris(2-carboxyethyl)phosphine, and 40 mM chloroacetamide and incubated for 10 min at 95 °C at 1,000 rpm (Thermomixer, Eppendorf, Hamburg, Germany). Each sample was processed for proteomics analysis following the solid-phase-enhanced sample-preparation (SP3) protocol as described [98]. Enzymatic digestion was performed by adding Trypsin/LysC (2 µg) and incubating overnight at 37 °C at 1,000 rpm. Protein identification and quantitation was performed by nanoLC-MS/MS as previously described [99], using an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA).

2.3.3.2. Data analysis

The raw data were processed using Proteome Discoverer 2.5.0.400 software (ThermoFisher Scientific, Waltham, MA, USA) and searched against the UniProt database for the *S. cerevisiae* Proteome 2020_03 (6049 entries) together with a common contaminant database from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry, Munich, Germany). The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragment ions. The maximum allowed missing cleavage sites was set to 2. Cysteine carbamidomethylation was defined as a constant modification. Methionine oxidation, asparagine and glutamine deamidation, serine, threonine and tyrosine phosphorylation, peptide N-terminus pyro-glutamine, protein N-terminus acetylation, Met-loss, and Met-loss + acetylation, were defined as variable modifications. Peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target FDR 1%, validation based on q-value. The ptmRS node was used for localization of modification sites. Protein label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performed at the processing step with the following parameters: peptides to use unique plus razor, precursor abundance based on intensity, and normalization based on the total peptide amount of annotated vacuolar membrane proteins. The percentage of phosphorylation for each

residue was manually calculated by the ratio between the amount of phosphorylated peptide and the total peptide amount.

To identify group differences concerning protein levels or the percentage of phosphorylation, first homogeneity of variances was assessed with the Levene test. Afterwards, pairwise comparisons with the Student t-test were performed. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 26. The level of significance was set at $\alpha = 0.05$. Statistical analysis of the overrepresentation of functional groups was performed by using the Gene Ontology database (1 July 2022 and 10.5281/zenodo.6799722).

2.3.4. Analysis of the AP-3 pathway

Cells expressing pGNS416-*GFP-NYV1-SNC1* (a gift from Christian Ungermann, Osnabrück University, Germany) [60] were grown to exponential phase in SC medium and treated with 0.024 mM FM4-64 (Molecular Probes, Eugene, OR, USA) for 1 h. After washing, cells were observed by fluorescence microscopy (AxioImager Z1, Carl Zeiss, Oberkochen, Germany). Output final images were obtained using ImageJ 1.45v software [100].

2.3.5. Western blotting

Yeast cells were grown to exponential or PDS phase in SC medium and collected by centrifugation. As positive control of TORC1-mediated phosphorylation of ribosomal protein S6 (Rps6 in yeast) at S232/233 (S235/236 in human S6) and carboxypeptidase Y (CPY) maturation, wild type cells grown to exponential phase were treated with rapamycin (200 ng mL⁻¹) for 4 h. For protein extraction, cells were incubated in 0.1M NaOH for 5 min at room temperature and harvested by centrifugation at 13,000 rpm (4 °C) for 15 min. The pellet was suspended in modified Laemmli buffer [62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and incubated 5 min at 95 °C. After centrifugation at 13,000 rpm (4 °C) for 3 min, the supernatant was collected, and protein content was estimated by Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA), using bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) as a standard. To analyze CPY and Apel levels or Rps6 phosphorylation at S232/233, protein samples (20 µg) were mixed with 5% (v/v) 2-mercaptoethanol before loading, separated by SDS-PAGE using 10% polyacrylamide gels (Grisp, Porto, Portugal), and blotted onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). Immunodetection was performed using anti-CPY (1:3,000; Invitrogen, Waltham, MA, USA), anti-Apel (1:200; Santa Cruz Biotechnology, Dallas, TX, USA), or anti-phospho-Rps6 (S235/236) (1:5,000; Cell Signaling, Danvers, MA, USA) as primary antibodies, and anti-rabbit IgG-peroxidase (1:5,000; Sigma Aldrich, St.

Louis, MO, USA) or anti-goat IgG-peroxidase (1:5,000; Sigma Aldrich, St. Louis, MO, USA) as secondary antibodies. To analyze the autophagic flux, cells expressing pRS416-*GFP-ATG8* were grown to exponential and PDS phases in SC medium or SC medium supplemented with 80 μ M of BPS (disodium salt hydrate; Sigma Aldrich, St. Louis, MO, USA). As a positive control, wild type cells grown to exponential phase were treated with rapamycin (200 ng mL⁻¹) for 4 h. Protein samples (20 μ g) were mixed with 5% (v/v) 2-mercaptoethanol before loading, separated by SDS-PAGE using 12.5% polyacrylamide gels, and blotted onto a nitrocellulose membrane. The membranes were incubated with the primary antibody anti-GFP (1:5,000; Roche, Basel, Switzerland) and with the secondary antibody anti-mouse IgG-peroxidase (1:5,000; Molecular Probes, Eugene, OR, USA). As loading control, Pgk1 levels were analyzed using anti-Pgk1 (1:30,000; Invitrogen, Waltham, MA, USA) as the primary antibody and anti-mouse IgG-peroxidase (1:10,000; Molecular Probes, Eugene, OR, USA) as the secondary antibody. The TORC1-dependent C-terminal phosphorylation of Sch9 was analyzed by Western blotting, using cells transformed with pRS416-*SCH9-5HA*. NTCB (2-nitro-5-thiocyanatobenzoic acid)-chemical fragmentation analysis [101] and immunodetection [102] were performed as described. Immunoblot was revealed by chemiluminescence (ECL, Advansta, San Jose, CA, USA).

2.3.6. Iron Levels

Yeast cells (6×10^8 cells mL⁻¹) were grown in SC medium to exponential and/or PDS phases and total iron levels were quantified using a colorimetric assay, as previously described [75].

2.3.7. β -galactosidase assay

Wild type and *ncr1* Δ cells were transformed with p*CTH2*-LacZ, a β -galactosidase reporter construct containing the consensus Aft1 binding sequences from *CTH2* promoter (a gift from Dennis Thiele, Duke University Medical Center, Durham, NC, USA) [103]. Cells were grown to exponential phase in SC medium, treated or not with 20 μ M of BPS for 4 h, and β -galactosidase activity was assayed as previously described [75], using 160 μ g of total protein or 20 μ g of total protein for samples treated with BPS.

2.3.8. Chronological lifespan

Chronological lifespan (CLS) was assessed as previously described [104]. Briefly, overnight cultures in SC medium were diluted to an OD_{600nm}=0.2 in SC or SC + 80 μ M BPS. After 24 h (considered time zero, t₀), cells were kept in culture medium at 26 °C, and cell viability at indicated days was determined by standard dilution plate counts on YPD medium

containing 1.5% (w/v) agar. Colonies were counted after 2-3 days of incubation at 26 °C. Cellular viability was expressed as the percentage of the colony-forming units (CFUs) in relation to t0.

2.3.9. Oxidative stress resistance

For oxidative stress resistance analysis, cells were grown to PDS phase in SC medium supplemented or not with 80 µM BPS and exposed to 5 mM H₂O₂ (Merck, Darmstadt, Germany) for 1 h. Cell viability was determined as described above and expressed as the percentage of the CFUs (treated cells vs non-stressed cells).

2.3.10. Intracellular oxidation

Cells were grown to the PDS (48 after log) phase in SC medium supplemented or not with 80 µM BPS and the levels of intracellular ROS were detected with dihydrorhodamine (DHR) 123 (Molecular Probes, Eugene, OR, USA) by flow cytometry. A total of 3×10^7 cells were treated with 6 µl of DHR 123 (stock solution at 2.5 mg mL⁻¹; prepared in DMSO) and incubated for 30 min at 26 °C in the dark. Cells were washed twice with phosphate-buffered saline (PBS) solution and resuspended in PBS. Fluorescence of DHR-positive cells was measured for 80,000 events on the FL-1 channel (533/30), using the BD Accuri C6 Flow cytometer and the FlowJow software (v 10.8.1).

2.3.11. Oxygen consumption

Cells were grown to the PDS phase in SC medium, and the oxygen consumption rate was measured for 1×10^7 cells in PBS, using a Clark-type oxygen electrode coupled to an Oxygraph plus system (Hansatech, Norfolk, UK). Data were analyzed using the OxyTrace + software v1.0.48.

2.3.12. Statistical analysis

Data were analyzed in GraphPad Prism Software v9.41. Values were compared by one-way ANOVA, two-way ANOVA or by Student's t-test * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

2.4. Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [105] partner repository with the dataset identifier PXD039881.

2.5. Acknowledgments

We are grateful to Dennis Thiele (Duke University Medical Center, Durham, NC, USA), Christian Ungermann (Osnabrück University, Germany), and Paula Ludovico (ICVS, Universidade do Minho, Portugal) for generously providing plasmids used in this study. The mass spectrometry-based approach was performed at the i3S Proteomics Scientific Platform with the assistance of Hugo Osório. We also would like to acknowledge the support of the i3S Scientific Platform Advanced Light Microscopy (member of the national infrastructure PPBI - Portuguese Platform of Bioimaging PPBI-POCI-01-0145-FEDER-022122).

2.6. Supplementary material

2.6.1 Supplementary figures

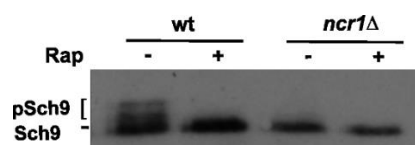


Figure S1. TORC1-mediated Sch9 phosphorylation decreases in *ncr1Δ* cells. Wild type (wt) and *ncr1Δ* cells transformed with pRS416-*SCH9-5HA* plasmid were grown to exponential phase in SC medium and protein extracts treated with NTCB (2-nitro-5-thiocyanatobenzoic acid) were analyzed by immunoblotting using anti-HA antibody. A representative experiment is shown. Cells were treated with rapamycin (Rap) or DMSO as control.

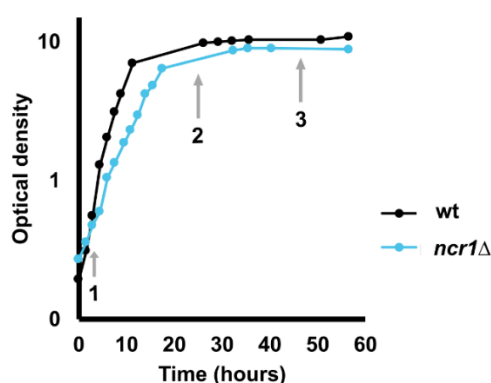


Figure S2. Growth curves of wild type (wt) and *ncr1Δ* cells. Cells were grown in SC media and samples were taken to monitor the OD_{600nm} overtime. Data are the mean values of three independent experiments. Arrows represent the time points where samples were taken for experiments at exponential phase (1) and post-diauxic shift (PDS) phase - 24 h (2) or 48h (3) after exponential phase.

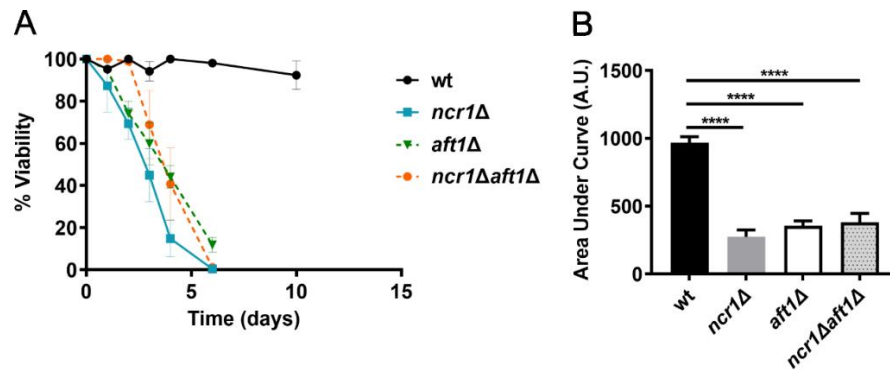


Figure S3. *AFT1* deletion does not have any impact on the chronological lifespan of *ncr1Δ* cells. (A) Wild type (wt), *ncr1Δ*, *aft1Δ* and *ncr1Δaft1Δ* cells were grown to post-diauxic shift (PDS) phase in SC medium and maintained in this medium overtime. Cellular viability was expressed as the percentage of the colony-forming units in relation to day 0. Data are the mean $\pm$ SEM of at least three independent experiments. (B) The area under each lifespan curve was computed using GraphPad Prism 8 in arbitrary units (A.U.). Data are the mean $\pm$ SEM. ****, $p \leq 0.0001$ (one-way ANOVA).

2.6.2 Supplementary tables

Table S1 - Levels of vacuolar membrane proteins in wild type and *ncr1*Δ samples

Levels of vacuolar membrane proteins in wild type and <i>ncr1</i> Δ samples												
Accession	Gene Symbol	Gene ID	Abundance (normalized) wild type				Abundance (normalized) <i>ncr1</i> Δ				Ratio <i>ncr1</i> Δ/wt	p-value (Student's t-test)
			Sample 1	Sample 2	Sample 3	Mean	Sample 1	Sample 2	Sample 3	Mean		
P32386	YBT1	D6VXW0; P32386; Q07882; sce:YLL048C	9.73E+09	1.03E+10	1.16E+10	1.05E+10	8.21E+09	8.62E+09	7.80E+09	8.21E+09	0.8	0.017
P39109	YCF1	D6VSB9; P39109; Q03905; sce:YDR135C	6.10E+09	6.99E+09	7.64E+09	6.91E+09	5.93E+09	6.17E+09	5.67E+09	5.92E+09	0.9	0.103
P39968	VAC8	D3DLN6; P39968; Q549T0; sce:YEL013W	5.62E+09	4.10E+09	6.47E+09	5.40E+09	4.22E+09	5.08E+09	4.07E+09	4.45E+09	0.8	0.283
P32563	VPH1	D6W2X0; P32563; sce:YOR270C	1.57E+10	1.72E+10	1.98E+10	1.76E+10	1.73E+10	1.69E+10	1.63E+10	1.68E+10	1.0	0.565
P35169	TOR1	D6VWN7; P35169; sce:YJR066W	6.11E+08	6.28E+08	9.66E+08	7.35E+08	5.42E+08	3.91E+08	9.68E+08	6.34E+08	0.9	0.651
P38929	PMC1	D6VUD2; P38929; sce:YGL006W	2.32E+09	2.65E+09	2.76E+09	2.58E+09	3.08E+09	3.10E+09	3.21E+09	3.13E+09	1.2	0.017
Q02725	VTC3	D6W3Z3; Q02725; sce:YPL019C	3.48E+09	1.92E+09	1.45E+09	2.28E+09	1.44E+09	1.39E+09	1.48E+09	1.44E+09	0.6	0.302
P16140	VMA2	D6VQC4; P16140; P32123; sce:YBR127C	1.09E+09	2.10E+09	7.41E+08	1.31E+09	2.56E+09	2.91E+09	3.31E+09	2.92E+09	2.2	0.025
P17255	VMA1	D6VRG7; O74301; P17255; Q9Y7W5; sce:YDL185W	1.18E+09	1.77E+09	6.35E+08	1.19E+09	1.07E+09	1.59E+09	1.51E+09	1.39E+09	1.2	0.621
P11491	PHO8	D6VTA4; E9P949; P11491; Q03374; sce:YDR481C	2.91E+09	2.75E+09	2.43E+09	2.70E+09	2.81E+09	2.80E+09	2.43E+09	2.68E+09	1.0	0.932

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

P14772	BPT1	D6VXY7; P14772; sce:YLL015W	1.20E+09	1.26E+09	1.80E+09	1.42E+09	1.21E+09	1.40E+09	1.45E+09	1.35E+09	1.0	0.768
P25371	ADP1	D6VR20; P25371; sce:YCR011C	1.51E+09	1.57E+09	2.10E+09	1.73E+09	2.02E+09	2.50E+09	2.29E+09	2.27E+09	1.3	0.081
Q12697	YPK9	D6W2Y9; Q12697; sce:YOR291W	1.18E+09	1.43E+09	1.75E+09	1.45E+09	8.88E+08	8.02E+08	1.26E+09	9.85E+08	0.7	0.095
P22855	AMS1	D6VTZ5; P22855; sce:YGL156W	1.64E+08	2.79E+08	8.89E+07	1.78E+08	1.98E+09	2.40E+09	2.87E+09	2.42E+09	13.6	0.001
P18962	DAP2	D3DKX5; E9P957; P18962; sce:YHR028C	2.10E+09	2.18E+09	2.39E+09	2.22E+09	2.40E+09	2.29E+09	2.40E+09	2.37E+09	1.1	0.203
P32600	TOR2	D6VX00; P32600; sce:YKL203C	1.65E+08	1.71E+08	4.04E+08	2.47E+08	1.90E+08	1.41E+08	2.83E+08	2.05E+08	0.8	0.661
P27514	PHO91	D6W118; P27514; sce:YNR013C	1.68E+09	1.35E+09	1.11E+09	1.38E+09	1.09E+09	1.22E+09	8.51E+08	1.05E+09	0.8	0.175
P53925	YNL115C	B0KZS0; D6W167; P53925; Q6B2V3; sce:YNL115C	1.18E+09	1.42E+09	1.29E+09	1.30E+09	2.83E+09	3.21E+09	1.95E+09	2.66E+09	2.0	0.023
P47075	VTC4	D6VWG5; P47075; Q7Z872; Q86ZR4; sce:YJL012C	2.67E+09	1.68E+09	1.33E+09	1.89E+09	1.16E+09	1.85E+09	1.61E+09	1.54E+09	0.8	0.471
Q12324	YVC1	D6W2E9; Q08500; Q12324; Q7LGN3; sce:YOR087W	7.86E+08	7.80E+08	9.68E+08	8.45E+08	6.42E+08	8.43E+08	9.83E+08	8.23E+08	1.0	0.860
P20107	ZRC1	D6W070; P20107; sce:YMR243C	2.88E+09	2.75E+09	2.38E+09	2.67E+09	1.99E+09	1.73E+09	1.73E+09	1.82E+09	0.7	0.008
P43585	VTC2	D6VTM5; P43585; sce:YFL004W	2.19E+09	1.94E+09	1.91E+09	2.01E+09	1.90E+09	6.12E+08	2.15E+09	1.55E+09	0.8	0.440
P40528	SYG1	D6VVN4; P40528; P40964; sce:YIL047C	6.47E+08	5.15E+08	3.74E+08	5.12E+08	5.38E+08	6.42E+08	5.85E+08	5.88E+08	1.1	0.415
P32366	VMA6	D6VZ81; P32366; sce:YLR447C	1.28E+09	1.99E+09	7.39E+08	1.34E+09	6.39E+08	6.39E+08	9.48E+08	7.42E+08	0.6	0.190
P42839	VNX1	D6W0M5; P42839; sce:YNL321W	6.59E+08	6.54E+08	5.01E+08	6.05E+08	6.62E+08	6.95E+08	6.65E+08	6.74E+08	1.1	0.311
P38873	KOG1	D3DLD4; P38873; sce:YHR186C	1.10E+08	1.42E+08	2.97E+08	1.83E+08	3.27E+08	1.85E+08	2.94E+08	2.69E+08	1.5	0.299
P38225	FAT1	D6VQ41; O60021; P38225; Q86ZS3; sce:YBR041W	2.33E+08	3.22E+08	7.43E+08	4.33E+08	1.29E+09	8.92E+08	1.52E+09	1.23E+09	2.9	0.029
P38244	PFF1	D6VQ74; P38244; P38245; Q86ZS2; sce:YBR074W	1.70E+09	1.78E+09	2.05E+09	1.85E+09	3.81E+09	3.27E+09	3.05E+09	3.38E+09	1.8	0.004
Q12296	MAM3	D6W207; Q12296; sce:YOL060C	5.93E+08	3.91E+08	5.95E+08	5.26E+08	4.60E+08	5.81E+08	6.61E+08	5.67E+08	1.1	0.671
P38966	VTC5	D6VS76; P38966; Q03192; sce:YDR089W	1.65E+09	1.33E+09	1.08E+09	1.35E+09	6.32E+08	6.58E+08	5.92E+08	6.27E+08	0.5	0.012

P02994	TEF1; TEF2	D6VQB6; P02994; Q7Z7U8; Q7Z8Q8; Q7Z8Q9; sce:YBR118W; sce:YPR080W	1.18E+09	9.08E+08	1.25E+09	1.11E+09	1.86E+09	1.39E+09	1.23E+09	1.49E+09	1.3	0.157
Q06708	VAC14	D6VZ21; Q06708; sce:YLR386W	1.11E+08	1.43E+08	2.59E+08	1.71E+08	1.80E+08	2.00E+08	3.86E+08	2.55E+08	1.5	0.350
P21576	VPS1	D6VXT7; P21576; sce:YKR001C	2.89E+08	2.36E+08	3.99E+08	3.08E+08	2.54E+08	2.78E+08	2.57E+08	2.63E+08	0.9	0.407
P22219	VPS15	D6VQ98; P22219; sce:YBR097W	4.01E+09	4.15E+09	1.49E+09	3.21E+09	4.62E+09	3.13E+09	4.35E+09	4.03E+09	1.3	0.450
P38329	VHC1	D6VQN1; P38329; sce:YBR235W	3.29E+08	3.12E+08	2.59E+08	3.00E+08	8.78E+08	5.67E+08	7.11E+08	7.19E+08	2.4	0.011
P39962	YCK3	D3DM29; P39962; sce:YER123W	6.36E+08	5.22E+08	4.82E+08	5.47E+08	1.71E+08	2.76E+08	2.12E+08	2.20E+08	0.4	0.004
P53273		D6VUQ7; P53273; sce:YGR125W	5.90E+08	4.26E+08	4.14E+08	4.77E+08	3.13E+08	3.19E+08	2.95E+08	3.09E+08	0.6	0.096
P37296	STV1	D6VZM9; P37296; sce:YMR054W	2.24E+08	2.65E+08	2.98E+08	2.63E+08	2.58E+08	2.89E+08	2.61E+08	2.70E+08	1.0	0.783
P12868	PEP5	D6W057; P12868; sce:YMR231W	1.41E+08	1.20E+08	2.17E+08	1.59E+08	1.27E+08	1.41E+08	1.61E+08	1.43E+08	0.9	0.627
P06780	RHO1	D6W4G6; P06780; sce:YPR165W	1.34E+09	1.06E+09	9.60E+08	1.12E+09	1.00E+09	1.11E+09	1.40E+09	1.17E+09	1.0	0.774
P47818	CCC1	D6VYM0; P47818; sce:YLR220W	1.46E+09	1.70E+09	1.46E+09	1.54E+09	1.21E+09	1.70E+09	1.04E+09	1.31E+09	0.9	0.354
P43561	FET5	D6VTI9; P43561; sce:YFL041W	1.23E+09	1.57E+09	9.16E+08	1.24E+09	1.86E+09	1.83E+09	2.11E+09	1.93E+09	1.6	0.029
P10592	SSA2	D6VXY0; P10592; sce:YLL024C	3.46E+08	3.14E+08	2.36E+08	2.99E+08	4.17E+08	3.34E+08	3.28E+08	3.60E+08	1.2	0.231
P47082	AVT1	D6VWH5; P47082; sce:YJR001W	3.12E+08	3.02E+08	3.40E+08	3.18E+08	2.47E+08	1.83E+08	2.87E+08	2.39E+08	0.8	0.072
Q12078	SMF3	D6VY36; Q12078; sce:YLR034C	1.07E+09	1.09E+09	1.16E+09	1.11E+09	3.12E+09	2.42E+09	2.10E+09	2.54E+09	2.3	0.009
P41807	VMA13	D6W446; P41807; sce:YPR036W	1.14E+08	2.03E+08	1.53E+08	1.57E+08	1.46E+08	1.24E+08	2.12E+08	1.60E+08	1.0	0.925
P38310	FTH1	D6VQK4; P38310; Q9URQ1; sce:YBR207W	2.41E+08	2.60E+08	2.51E+08	2.51E+08	5.38E+08	4.44E+08	5.67E+08	5.16E+08	2.1	0.017
P43555	EMP47	D6VTI2; P43555; sce:YFL048C	2.57E+08	2.95E+08	1.73E+08	2.42E+08	2.09E+08	3.19E+08	3.03E+08	2.77E+08	1.1	0.518
Q07468	VAM6	D6VRS2; Q07468; sce:YDL077C	1.04E+08	8.77E+07	1.89E+08	1.27E+08	9.95E+07	1.17E+08	1.41E+08	1.19E+08	0.9	0.830
P10591	SSA1	D6VPL2; P10591; sce:YAL005C	2.52E+07	2.18E+07	1.01E+07	1.90E+07	5.15E+07	4.98E+07	4.03E+07	4.72E+07	2.5	0.008

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P19524	MYO2	D6W323; P19524; sce:YOR326W	1.90E+08	1.50E+08	1.17E+08	1.52E+08	6.99E+07	6.54E+07	8.91E+07	7.48E+07	0.5	0.025
P27809	KRE2	D6VTA6; P27809; sce:YDR483W	2.43E+08	2.20E+08	1.27E+08	1.97E+08	9.75E+07	1.01E+08	1.03E+08	1.01E+08	0.5	0.113
P53134	YGL114W	D6VU33; P53134; sce:YGL114W	2.25E+08	2.31E+08	2.27E+08	2.28E+08	3.18E+08	3.89E+08	2.80E+08	3.29E+08	1.4	0.033
Q04119	PPN1	D6VT77; Q04119; sce:YDR452W	2.46E+08	3.12E+08	5.26E+07	2.04E+08	1.93E+08	2.66E+08	3.97E+08	2.85E+08	1.4	0.450
Q04602	VBA4	D6VSA4; Q04602; sce:YDR119W	4.85E+08	3.59E+08	3.09E+08	3.84E+08	2.69E+08	2.63E+08	2.95E+08	2.76E+08	0.7	0.111
P27614	CPS1	D6VW16; P27614; sce:YJL172W	9.86E+08	8.77E+08	2.29E+08	6.97E+08	1.28E+09	1.40E+09	9.74E+08	1.22E+09	1.7	0.124
P34240	ZRT3	D6VX25; P34240; sce:YKL175W	2.73E+08	2.71E+08	3.94E+08	3.13E+08	2.01E+08	2.78E+08	3.34E+08	2.71E+08	0.9	0.496
P35724	MNR2	D6VXM3; P35724; sce:YKL064W	1.66E+08	1.57E+08	1.36E+08	1.53E+08	1.15E+08	1.59E+08	1.17E+08	1.30E+08	0.9	0.248
Q06538	CSC1	D6VYN9; Q06538; sce:YLR241W	5.62E+08	5.80E+08	6.53E+08	5.98E+08	3.90E+08	4.85E+08	5.51E+08	4.75E+08	0.8	0.086
P39925	AFG3	D3DLR5; P39925; sce:YER017C	2.23E+08	1.86E+08	1.11E+08	1.73E+08	8.20E+07	7.47E+07	1.06E+08	8.75E+07	0.5	0.066
P31412	VMA5	D6VXK7; P31412; sce:YKL080W	4.98E+07	1.36E+08	4.88E+07	7.81E+07	4.72E+07	1.10E+08	2.43E+08	1.33E+08	1.7	0.439
Q05521	DPP1	D6VSR4; Q05521; sce:YDR284C	5.75E+08	5.87E+08	7.58E+08	6.40E+08	6.31E+08	4.40E+08	5.64E+08	5.45E+08	0.9	0.307
Q12255	NYV1	D6VY93; Q12255; sce:YLR093C	4.50E+08	3.46E+08	4.07E+08	4.01E+08	1.46E+08	1.52E+08	1.91E+08	1.63E+08	0.4	0.002
Q03823		D6VZY2; Q03823; sce:YMR160W	1.01E+08	9.47E+07	1.19E+08	1.05E+08	1.37E+08	1.08E+08	1.48E+08	1.31E+08	1.2	0.134
Q12013	AKR2	D6W2A0; Q12013; sce:YOR034C	1.55E+08	9.13E+07	1.68E+08	1.38E+08	6.00E+07	6.93E+07	8.59E+07	7.17E+07	0.5	0.056
Q02205	MEH1	D6VX73; Q02205; sce:YKR007W	9.67E+07	4.78E+07	1.24E+08	8.96E+07	6.38E+07	3.73E+07	2.30E+07	4.14E+07	0.5	0.130
Q03441	RMD1	D6VRY7; Q03441; Q05210; sce:YDL001W	2.26E+08	2.25E+08	2.55E+08	2.35E+08	1.84E+08	2.08E+08	2.53E+08	2.15E+08	0.9	0.420
Q12209	FRE8	D6VY49; Q12209; sce:YLR047C	1.36E+08	1.27E+08	1.10E+08	1.24E+08	2.20E+08	1.63E+08	2.76E+08	2.20E+08	1.8	0.047
Q99385	VCX1	D6VRM2; O13580; O13581; Q99385; sce:YDL128W	8.52E+08	9.21E+08	1.03E+09	9.36E+08	6.86E+08	7.71E+08	8.73E+08	7.77E+08	0.8	0.103
Q04338	VTI1	D6W022; Q04338; sce:YMR197C	6.90E+08	5.50E+08	5.27E+08	5.89E+08	4.30E+08	6.70E+08	3.17E+08	4.72E+08	0.8	0.371

P06105	SCP160	D6VWA3; P06105; sce:YJL080C	1.06E+08	6.76E+07	8.04E+07	8.45E+07	5.56E+07	4.21E+07	4.09E+07	4.62E+07	0.5	0.034
Q04301	VBA1	D6VZR1; Q04301; sce:YMR088C	2.33E+08	2.64E+08	2.48E+08	2.49E+08	2.25E+08	1.95E+08	2.32E+08	2.17E+08	0.9	0.097
P46989	ATG27	D6VW11; P46989; Q86ZR9; sce:YJL178C	1.51E+09	1.48E+09	1.95E+09	1.65E+09	1.52E+09	1.40E+09	1.07E+09	1.33E+09	0.8	0.197
P22203	VMA4	D6W328; P22203; sce:YOR332W	2.77E+08	4.51E+08	1.60E+08	2.96E+08	3.55E+08	4.65E+08	3.98E+08	4.06E+08	1.4	0.290
P25351	YCR023C	D6VR32; P25351; Q8NKJ7; sce:YCR023C	1.65E+08	1.94E+08	1.87E+08	1.82E+08	2.33E+08	3.19E+08	2.19E+08	2.57E+08	1.4	0.080
P22543	VPS34	D6VYN8; P22543; sce:YLR240W	7.49E+07	7.35E+07	9.46E+07	8.10E+07	7.81E+07	7.78E+07	9.66E+07	8.42E+07	1.0	0.749
P32798	COT1	D6W314; P32798; sce:YOR316C	6.42E+08	5.31E+08	6.10E+08	5.94E+08	7.88E+08	6.61E+08	8.19E+08	7.56E+08	1.3	0.051
P38164	SEA4	D6VPP9; P38164; Q86ZS4; sce:YBL104C	3.44E+07	2.95E+07	7.61E+07	4.67E+07	1.17E+08	7.37E+07	5.95E+07	8.34E+07	1.8	0.182
P25568	ATG22	D6VQX7; P25568; sce:YCL038C	2.04E+08	2.24E+08	2.00E+08	2.09E+08	2.58E+08	2.77E+08	2.90E+08	2.75E+08	1.3	0.005
P53285	VPS62	D6VUS1; P53285; sce:YGR141W	5.31E+08	4.03E+08	4.34E+08	4.56E+08	4.77E+08	3.58E+08	3.46E+08	3.94E+08	0.9	0.331
Q12256	TPO4	D6W2X3; Q12256; sce:YOR273C	2.07E+08	2.60E+08	1.17E+08	1.95E+08	2.76E+08	2.95E+08	2.20E+08	2.64E+08	1.4	0.221
P38959	VPS41	D6VS67; P38959; P87334; Q12011; sce:YDR080W	6.27E+07	5.83E+07	8.12E+07	6.74E+07	7.19E+07	7.34E+07	6.92E+07	7.15E+07	1.1	0.620
P32602	SEC17	D6VPU9; P32602; sce:YBL050W	1.05E+08	6.88E+07	7.09E+07	8.16E+07	1.71E+08	1.59E+08	1.22E+08	1.51E+08	1.9	0.021
Q04991	FMP42	D6W046; Q04991; sce:YMR221C	8.64E+08	1.01E+09	8.82E+08	9.20E+08	1.10E+09	1.33E+09	6.99E+08	1.04E+09	1.1	0.550
P27801	PEP3	D6VYE3; P27801; sce:YLR148W	9.32E+07	1.08E+08	1.89E+08	1.30E+08	8.67E+07	1.03E+08	1.20E+08	1.03E+08	0.8	0.442
Q03516	RSN1	D6W092; Q03516; sce:YMR266W	4.58E+08	4.07E+08	1.23E+08	3.29E+08	2.06E+08	1.19E+08	2.13E+08	1.79E+08	0.5	0.239
P32939	YPT7	D6VZH4; P32939; sce:YML001W	1.29E+09	1.42E+09	1.83E+09	1.51E+09	1.32E+09	1.79E+09	1.53E+09	1.55E+09	1.0	0.868
Q07895		D6VY03; Q07895; sce:YLR001C	2.82E+08	3.34E+08	4.90E+08	3.68E+08	3.63E+08	2.76E+08	4.51E+08	3.64E+08	1.0	0.955
Q06247		D6VYH7; Q06247; sce:YLR173W	1.13E+08	1.24E+08	1.35E+08	1.24E+08	1.35E+08	1.35E+08	1.29E+08	1.33E+08	1.1	0.233
Q08227	INP54	D6W202; Q08227; sce:YOL065C	2.29E+08	2.12E+08	1.61E+08	2.00E+08	2.05E+08	1.97E+08	2.52E+08	2.18E+08	1.1	0.550

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

P32610	VMA8	D3DLJ9; P32610; sce:YEL051W	1.32E+08	1.10E+08	7.58E+07	1.06E+08	1.01E+08	1.17E+08	1.33E+08	1.17E+08	1.1	0.594
P36018	YPT52	D6VX79; P36018; sce:YKR014C	1.83E+08	2.00E+08	1.67E+08	1.83E+08	1.41E+08	1.13E+08	1.27E+08	1.27E+08	0.7	0.010
Q12407		D6VRF5; Q12407; sce:YDL199C	6.49E+07	8.23E+07	6.27E+07	7.00E+07	6.83E+07	6.45E+07	6.07E+07	6.45E+07	0.9	0.452
Q03308	VPS16	D6W3W9; Q03078; Q03308; sce:YPL045W	5.26E+07	4.05E+07	7.25E+07	5.52E+07	3.70E+07	2.99E+07	4.45E+07	3.71E+07	0.7	0.152
Q12241	VAM3	D6W2G5; Q12241; sce:YOR106W	1.82E+08	9.47E+07	1.25E+08	1.34E+08	4.58E+07	5.03E+07	5.67E+07	5.10E+07	0.4	0.033
Q07824	TPO1	D6VXX6; Q07824; sce:YLL028W	1.33E+08	1.38E+08	3.64E+07	1.02E+08	1.11E+08	8.57E+07	1.72E+08	1.23E+08	1.2	0.645
Q12301	YDL180W	D6VRH2; Q12301; sce:YDL180W	9.81E+07	8.03E+07	8.21E+07	8.68E+07	4.72E+07	6.07E+07	5.52E+07	5.44E+07	0.6	0.009
P38130	KTR3	D6VQK3; E9P931; P38130; sce:YBR205W	2.24E+08	2.06E+08	9.50E+07	1.75E+08	1.97E+08	2.12E+08	2.22E+08	2.10E+08	1.2	0.477
P34756	FAB1	D6VTQ0; P34756; sce:YFR019W	1.72E+07	2.30E+07	3.58E+07	2.54E+07	1.54E+07	1.73E+07	2.41E+07	1.89E+07	0.7	0.353
P40482	SEC24	D6VVH8; P40482; sce:YIL109C	4.22E+07	5.05E+07	5.72E+07	5.00E+07	8.02E+07	4.01E+07	5.80E+07	5.94E+07	1.2	0.489
P33311	MDL2	D6W3A0; P33311; Q08983; sce:YPL270W	1.27E+08	9.17E+07	4.82E+07	8.89E+07	1.85E+07	1.77E+07	3.95E+07	2.52E+07	0.3	0.056
P42951	LSB6	D6VW84; P42951; sce:YJL100W	7.44E+07	6.84E+07	6.95E+07	7.08E+07	9.44E+07	1.12E+08	1.00E+08	1.02E+08	1.4	0.004
P32912	VAM7	D6VTU3; P32912; sce:YGL212W	1.61E+08	1.23E+08	1.00E+08	1.28E+08	1.40E+08	1.51E+08	7.72E+07	1.23E+08	1.0	0.859
Q12473	FRE6	D6VXV7; Q12473; sce:YLL051C	1.33E+08	1.17E+08	1.63E+08	1.37E+08	2.41E+08	1.40E+08	2.23E+08	2.01E+08	1.5	0.133
P25356	BPH1	D6VR42; P25356; Q02396; Q07348; sce:YCR032W	3.45E+07	3.12E+07	4.16E+07	3.57E+07	4.20E+07	3.40E+07	9.42E+06	2.85E+07	0.8	0.519
P32802	EMP70	D6VY83; P32802; Q12101; sce:YLR083C	1.10E+08	1.13E+08	6.95E+07	9.72E+07	3.01E+07	2.46E+07	3.53E+07	3.00E+07	0.3	0.035
Q12003	ENV7	D6W3D4; Q12003; Q7LGU7; sce:YPL236C	6.89E+07	8.05E+07	1.05E+08	8.48E+07	8.67E+07	7.64E+07	1.26E+08	9.65E+07	1.1	0.562
Q02948	VPS30	D6W3P7; Q02948; sce:YPL120W	3.83E+07	3.49E+07	5.48E+07	4.27E+07	6.49E+07	7.49E+07	6.37E+07	6.78E+07	1.6	0.024
P32343	SSH4	D6VX71; P32343; sce:YKL124W	8.87E+07	9.58E+07	1.00E+08	9.49E+07	7.66E+07	9.13E+07	6.61E+07	7.80E+07	0.8	0.103
P27810	KTR1	D6W2G0; P27810; sce:YOR099W	2.99E+08	2.56E+08	1.40E+08	2.32E+08	1.13E+08	1.65E+08	1.22E+08	1.34E+08	0.6	0.121

P47170	IML1	D6VWV7; P47170; sce:YJR138W	2.96E+07	2.74E+07	3.45E+07	3.05E+07	1.37E+07	1.85E+07	2.38E+07	1.87E+07	0.6	0.030
Q07376	MCH1	D6VRU2; Q07376; sce:YDL054C	2.20E+08	1.82E+08	1.97E+08	2.00E+08	1.20E+08	1.08E+08	1.57E+08	1.28E+08	0.6	0.018
P31381	FUN26	D6VPJ6; P31381; sce:YAL022C	1.31E+08	1.04E+08	1.16E+08	1.17E+08	9.08E+07	9.55E+07	8.62E+07	9.08E+07	0.8	0.033
P42837	FIG4	D6W0M2; P42837; sce:YNL325C	9.78E+07	2.05E+08	1.00E+08	1.34E+08	2.30E+08	3.31E+08	2.36E+08	2.66E+08	2.0	0.053
P32867	SSO1	D6W3D8; P32867; sce:YPL232W	1.32E+08	5.91E+07	2.60E+07	7.22E+07	1.92E+07	4.42E+06	3.06E+07	1.81E+07	0.3	0.167
Q05902	ECM38	D6VYU3; Q05902; sce:YLR299W	2.46E+07	3.54E+07	1.09E+07	2.37E+07	3.95E+07	5.51E+07	7.99E+07	5.82E+07	2.5	0.066
P40152	PPN2	D6W0X3; P40152; sce:YNL217W	1.54E+08	1.60E+08	1.50E+08	1.55E+08	2.22E+08	1.80E+08	2.25E+08	2.09E+08	1.4	0.059
P53879	RHO5	D6W106; P53879; sce:YNL180C	6.07E+07	5.11E+07	2.54E+07	4.57E+07	1.42E+07	2.25E+07	1.59E+07	1.75E+07	0.4	0.060
P21801	SDH2	D6VXW5; P21801; sce:YLL041C	5.90E+07	6.39E+07	1.83E+07	4.71E+07	2.48E+07	2.40E+07	1.66E+07	2.18E+07	0.5	0.220
P36051	MCD4	D6VX34; P36051; sce:YKL165C	2.02E+08	2.53E+08	2.58E+08	2.37E+08	1.36E+08	1.26E+08	1.90E+08	1.51E+08	0.6	0.031
Q12010	YPQ1	D6W1X6; Q12010; sce:YOL092W	4.92E+08	7.11E+08	5.54E+08	5.86E+08	6.07E+08	5.09E+08	4.34E+08	5.17E+08	0.9	0.449
Q06451	TPO3	D6W4F2; Q06451; sce:YPR156C	1.82E+08	2.19E+08	3.98E+07	1.47E+08	1.41E+08	9.23E+07	1.90E+08	1.41E+08	1.0	0.925
P53191	PIB2	D6VUB4; E9P8Z2; P53191; sce:YGL023C	2.05E+07	2.01E+07	2.11E+07	2.06E+07	3.18E+07	5.43E+07	1.84E+07	3.48E+07	1.7	0.247
P42836	PFA3	D6W0M1; P42836; sce:YNL326C	8.15E+07	9.23E+07	6.57E+07	7.98E+07	5.23E+07	4.17E+07	6.78E+07	5.39E+07	0.7	0.075
P40501	AVT7	D6VVJ9; P40501; sce:YIL088C	2.96E+08	4.12E+08	4.66E+08	3.91E+08	4.18E+08	3.75E+08	4.29E+08	4.07E+08	1.0	0.779
P50945	MIC27	D6W179; P50945; Q45U03; sce:YNL100W	3.57E+08	2.82E+08	1.04E+08	2.48E+08	1.27E+07	1.84E+07	1.65E+07	1.59E+07	0.1	0.091
Q03897	MTC5	D6VSB4; Q03897; sce:YDR128W	3.70E+07	2.74E+07	2.42E+07	2.95E+07	2.67E+07	3.17E+07	3.40E+07	3.08E+07	1.0	0.792
Q12116	TMS1	D6VS91; Q12116; sce:YDR105C	3.33E+07	3.19E+07	2.61E+07	3.04E+07	2.07E+07	3.07E+07	3.19E+07	2.78E+07	0.9	0.559
Q03730		D6VZF6; Q03730; Q6B2K7; sce:YML018C	1.64E+08	1.03E+08	1.05E+08	1.24E+08	8.90E+07	1.20E+08	6.82E+07	9.25E+07	0.7	0.275
P38146	YPT10	D6VQR1; P38146; sce:YBR264C	1.28E+08	1.27E+08	1.35E+08	1.30E+08	1.79E+08	1.82E+08	1.81E+08	1.81E+08	1.4	0.000

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

Q12096	GNT1	D6W318; Q12096; sce:YOR320C	4.59E+07	5.08E+07	3.12E+07	4.26E+07	3.21E+07	3.74E+07	3.38E+07	3.44E+07	0.8	0.250
P32335	MSS51	D6VYK4; P32335; sce:YLR203C	1.59E+07	1.77E+07	7.90E+06	1.38E+07	6.24E+06	6.84E+06	5.84E+06	6.31E+06	0.5	0.128
P53283	TPO2	D6VUR9; P53283; sce:YGR138C	8.84E+07	1.28E+08	3.13E+07	8.26E+07	3.09E+07	1.72E+07	4.12E+07	2.98E+07	0.4	0.142
P38142	VVS1	D6VQN7; P38142; Q6B2Y5; sce:YBR241C	1.23E+08	1.44E+08	8.98E+07	1.19E+08	4.69E+08	4.70E+08	4.72E+08	4.70E+08	4.0	0.000
Q02887	ATG21	D6W3R6; Q02887; sce:YPL100W	1.53E+07	8.59E+06	2.96E+07	1.78E+07	1.09E+07	1.14E+07	1.13E+07	1.12E+07	0.6	0.394
P50079	HSV2	D6VV05; P50079; sce:YGR223C	3.75E+07	1.98E+07	7.26E+07	4.33E+07	3.40E+07	2.40E+07	3.53E+07	3.11E+07	0.7	0.488
Q12042		D6W3K6; Q12042; sce:YPL162C	8.25E+07	7.91E+07	8.76E+07	8.31E+07	6.94E+07	2.60E+07	6.30E+07	5.28E+07	0.6	0.151
P20795	VPS33	D6VZ31; P20795; sce:YLR396C	7.75E+07	9.92E+07	4.22E+07	7.29E+07	1.74E+08	2.77E+08	1.91E+08	2.14E+08	2.9	0.017
Q04162		D6VT21; Q04162; sce:YDR387C	1.66E+07	1.89E+07	2.56E+07	2.04E+07	2.39E+07	2.96E+07	2.48E+07	2.61E+07	1.3	0.151
P34110	VPS35	D6VW33; P34110; sce:YJL154C	8.70E+07	6.01E+07	2.53E+07	5.75E+07	6.51E+07	1.71E+07	8.08E+07	5.43E+07	0.9	0.910
P41318	LST8	D6W1H1; P41318; sce:YNL006W	2.47E+07	2.16E+07	2.36E+07	2.33E+07	3.75E+07	5.99E+07	2.41E+07	4.05E+07	1.7	0.176
P40335	PEP8	D6VWC9; E9P910; P40335; sce:YJL053W	1.08E+07	1.13E+07	1.06E+07	1.09E+07	6.13E+06	9.08E+06	6.64E+06	7.28E+06	0.7	0.019
P33894	STE13	D6W2S4; P33894; Q9URF1; sce:YOR219C	2.29E+07	2.41E+07	2.11E+07	2.27E+07	1.01E+07	1.79E+07	1.88E+07	1.56E+07	0.7	0.070
P53950	VAC7	D6W1C5; P53950; sce:YNL054W	6.39E+07	5.16E+07	3.61E+07	5.05E+07	6.28E+07	4.94E+07	4.29E+07	5.17E+07	1.0	0.912
P32854	PEP12	D6W2A3; P32854; sce:YOR036W	4.24E+07	2.45E+07	2.85E+07	3.18E+07	1.67E+07	7.41E+06	6.08E+06	1.01E+07	0.3	0.027
Q04934	IVY1	D6VSL1; Q04934; sce:YDR229W	9.67E+06	1.18E+07	2.43E+07	1.53E+07	1.11E+08	7.47E+07	5.00E+07	7.86E+07	5.2	0.026
Q05919	VPS38	D6VYZ7; Q05919; Q6B1J7; sce:YLR360W	1.39E+07	1.35E+07	3.46E+07	2.07E+07	1.66E+07	1.51E+07	2.22E+07	1.80E+07	0.9	0.730
P39548	YAR028W	D6VPN0; P39548; sce:YAR028W	4.53E+07	3.74E+07	2.21E+07	3.49E+07	5.76E+07	7.61E+07	6.46E+07	6.61E+07	1.9	0.023
P30605	ITR1	D6VTB9; P30605; sce:YDR497C	1.31E+08	7.26E+07	5.01E+07	8.45E+07	5.38E+07	5.64E+07	4.23E+07	5.08E+07	0.6	0.241
P53011	SEH1	D6VU45; P53011; sce:YGL100W	1.44E+08	1.47E+08	7.75E+07	1.23E+08	1.97E+08	7.74E+07	4.10E+08	2.28E+08	1.9	0.351

P40074	AVT6	D3DM25; P40074; sce:YER119C	1.37E+08	1.16E+08	1.04E+08	1.19E+08	1.63E+08	2.20E+08	1.37E+08	1.73E+08	1.5	0.107
P25515	VMA3	D3DLM1; P25515; sce:YEL027W	2.49E+08	2.19E+08	7.63E+08	4.11E+08	1.42E+08	1.98E+08	5.49E+08	2.96E+08	0.7	0.628
P53388	DIP5	D6W3A5; P53388; sce:YPL265W	8.10E+07	7.15E+07	3.38E+07	6.21E+07	9.34E+07	5.98E+07	7.94E+07	7.75E+07	1.2	0.426
P40570	VLD1	D6VVU4; P40570; sce:YIR014W	4.33E+07	5.00E+07	5.76E+07	5.03E+07	5.49E+07	7.48E+07	6.66E+07	6.54E+07	1.3	0.100
P47159		D6VWU3; P47159; Q6Q5M7; sce:YJR124C	6.41E+07	6.43E+07	9.70E+07	7.51E+07	6.34E+07	5.87E+07	6.86E+07	6.36E+07	0.8	0.403
Q03212	EAR1	D6VZZ3; Q03212; Q03830; sce:YMR171C	2.42E+07	2.43E+07	2.19E+07	2.34E+07	6.87E+06	1.85E+07	7.68E+06	1.10E+07	0.5	0.075
Q06810	OPY2	D6W479; Q06810; Q6B149; sce:YPR075C	3.31E+07	1.54E+07	2.09E+07	2.31E+07	4.48E+06	1.06E+07	5.42E+06	6.84E+06	0.3	0.043
P50944	AVT4	D6W178; P50944; sce:YNL101W	5.55E+07	4.59E+07	3.45E+07	4.53E+07	4.92E+07	4.53E+07	2.78E+07	4.08E+07	0.9	0.641
P19073	CDC42	D6VYN1; P19073; Q05978; sce:YLR229C	7.89E+08	7.53E+08	3.20E+08	6.21E+08	8.37E+08	9.58E+08	9.46E+08	9.14E+08	1.5	0.185
P21560	CBP3	D6W3F5; P21560; sce:YPL215W	6.84E+07	6.47E+07	1.39E+07	4.90E+07	1.80E+07	2.54E+07	1.75E+07	2.03E+07	0.4	0.242
P17064	FCY2	D3DLV9; P17064; sce:YER056C	1.26E+08	1.29E+08	5.15E+07	1.02E+08	8.02E+07	6.13E+07	1.10E+08	8.37E+07	0.8	0.559
P38742	NPR3	D3DKU5; P38742; sce:YHL023C	7.05E+06	5.28E+06	1.09E+07	7.73E+06	3.62E+06	5.26E+06	6.13E+06	5.00E+06	0.6	0.205
P48558	BXI1	D6W0P0; P48558; Q6B2Q1; sce:YNL305C	1.27E+08	1.20E+08	9.88E+07	1.15E+08	6.41E+07	5.87E+07	1.07E+08	7.65E+07	0.7	0.089
Q07549	SNA4	D6VRM7; Q07549; sce:YDL123W	3.84E+08	1.83E+08	9.64E+07	2.21E+08	1.55E+08	1.62E+08	1.50E+08	1.56E+08	0.7	0.523
P38270	ATG14	D6VQC5; P38270; sce:YBR128C	8.07E+06	7.96E+06	1.49E+07	1.03E+07	1.64E+07	8.93E+06	1.42E+07	1.32E+07	1.3	0.418
Q08921	TCO89	D6W3I8; Q08921; sce:YPL180W	1.10E+07	7.98E+06	1.53E+07	1.14E+07	1.42E+07	4.94E+07	1.14E+07	2.50E+07	2.2	0.382
Q08281	RTC1	D6W1T1; Q08281; Q92271; sce:YOL138C	1.53E+07	2.21E+07	2.49E+07	2.08E+07	2.19E+07	2.28E+07	2.12E+07	2.20E+07	1.1	0.700
Q08157		D6W247; Q08157; sce:YOL019W	3.04E+07	2.40E+07	8.84E+06	2.11E+07	2.78E+07	1.77E+07	2.62E+07	2.39E+07	1.1	0.717
Q12016	VPS68	D6W1T9; Q12016; sce:YOL129W	3.07E+07	9.23E+07	8.19E+07	6.83E+07	4.88E+07	1.64E+07	1.15E+08	6.02E+07	0.9	0.827
Q00582	GTR1	D6W0G3; Q00582; sce:YML121W	9.23E+06	9.50E+06	1.86E+07	1.24E+07	1.03E+07	2.56E+06	1.50E+07	9.30E+06	0.7	0.546

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

P36062	AVT3	D6VX50; P36062; sce:YKL146W	1.51E+08	1.98E+08	2.05E+08	1.85E+08	1.72E+08	1.28E+08	1.45E+08	1.49E+08	0.8	0.160
P25625	PER1	D6VR53; P25625; sce:YCR044C	8.62E+06	2.99E+06	6.20E+06	5.93E+06	1.30E+06	1.48E+06	2.42E+06	1.73E+06	0.3	0.065
P46996		D6VW24; P46996; sce:YJL163C	1.19E+07	1.44E+07	1.39E+07	1.34E+07	1.57E+07	1.43E+07	1.52E+07	1.51E+07	1.1	0.119
Q08234	YOL075C	D6W1Z3; Q08233; Q08234; sce:YOL075C	1.00E+07	9.76E+06	3.34E+06	7.71E+06	2.51E+06	1.51E+06	5.29E+06	3.10E+06	0.4	0.135
P40046	VTC1	D3DLX7; P40046; sce:YER072W	1.20E+09	7.89E+08	6.80E+08	8.90E+08	7.54E+08	8.71E+08	5.35E+08	7.20E+08	0.8	0.415
P39923	NPR2	D3DLI8; P39923; Q6B264; sce:YEL062W	9.35E+06	3.77E+06	2.76E+07	1.36E+07	9.29E+06	1.21E+07	1.12E+07	1.09E+07	0.8	0.746
Q12424		D6VRE8; Q12424; sce:YDL206W	7.10E+06	8.24E+06	7.95E+06	7.76E+06	2.30E+07	6.48E+06	7.20E+06	1.22E+07	1.6	0.495
Q08299	ENB1	D6W1R2; Q08299; sce:YOL158C	2.95E+07	2.17E+07	5.92E+06	1.91E+07	9.09E+07	3.87E+07	1.00E+08	7.66E+07	4.0	0.047
P38247	SLM4	D6VQ76; P38247; sce:YBR077C	9.16E+06	9.48E+06	8.20E+06	8.95E+06	1.50E+07	2.71E+07	2.06E+07	2.09E+07	2.3	0.027
P48836	VMA10	D3DKY7; P48836; sce:YHR039C-A	2.01E+07	4.50E+07	1.02E+07	2.51E+07	1.91E+07	6.85E+07	5.21E+07	4.66E+07	1.9	0.294
P38090	AGP2	D6VQC9; P38090; sce:YBR132C	4.23E+07	3.48E+07	2.01E+07	3.24E+07	6.04E+07	3.56E+07	3.76E+07	4.45E+07	1.4	0.305
P53059	MNT2	D6VV78; P53059; sce:YGL257C	6.86E+06	8.13E+06	7.29E+06	7.43E+06	3.88E+06	1.62E+05	2.93E+06	2.33E+06	0.3	0.012
P53290	GTR2	D6VUU7; P53290; sce:YGR163W	5.22E+06	7.21E+06	1.35E+07	8.65E+06	6.08E+06	3.49E+06	5.86E+06	5.14E+06	0.6	0.254
P14359	SNA3	D6VW35; P14359; Q6B236; sce:YJL151C	8.22E+06	2.41E+06	3.58E+05	3.66E+06	3.82E+05	1.78E+06	4.05E+06	2.07E+06	0.6	0.572
P23643	VPS3	D6VTB7; P23643; sce:YDR495C	3.98E+06	5.00E+06	5.78E+06	4.92E+06	4.23E+06	5.16E+06	7.18E+06	5.52E+06	1.1	0.583
P32842	VMA11	D6W3D6; P32365; P32842; sce:YPL234C	2.62E+08	2.17E+08	4.42E+08	3.07E+08	1.95E+08	1.78E+08	2.51E+08	2.08E+08	0.7	0.243
Q02896	YDC1	D6W3T0; Q02896; sce:YPL087W	2.61E+07	3.05E+07	2.46E+07	2.71E+07	3.40E+07	5.96E+07	5.75E+07	5.04E+07	1.9	0.098
P53947	YNL058C	D6W1C1; P53947; sce:YNL058C	1.11E+07	7.00E+06	4.53E+06	7.55E+06	1.01E+06	1.11E+06	1.41E+06	1.18E+06	0.2	0.030
Q12284	ERV2	D6W448; Q12284; sce:YPR037C	2.83E+07	2.92E+07	1.69E+07	2.48E+07	1.65E+07	4.17E+07	1.55E+07	2.46E+07	1.0	0.983
P13856	RSR1	D6VUT2; P13856; sce:YGR152C	2.80E+07	2.21E+07	1.85E+07	2.29E+07	1.48E+07	1.49E+07	1.95E+07	1.64E+07	0.7	0.109

P47008	SFH5	D6VW40; P47008; Q6Q535; sce:YJL145W	7.14E+06	5.44E+06	3.85E+06	5.47E+06	4.06E+06	6.19E+06	7.40E+06	5.88E+06	1.1	0.779
P39547	UIP3	D6VPM9; P39547; sce:YAR027W	4.00E+07	3.04E+07	1.23E+07	2.76E+07	1.06E+07	1.48E+07	1.23E+07	1.26E+07	0.5	0.141
P38301	GDT1	D6VQI2; E9PAD4; P38301; Q6Q545; sce:YBR187W	4.75E+07	3.66E+07	1.28E+07	3.23E+07	2.39E+07	3.14E+07	1.98E+07	2.50E+07	0.8	0.537
Q04562	TMN2	D6VS93; Q04562; sce:YDR107C	4.63E+06	4.34E+06	1.84E+06	3.60E+06	1.89E+06	2.11E+06	2.93E+06	2.31E+06	0.6	0.241
P40857	PHS1	D6VW87; P40857; sce:YJL097W	4.06E+07	3.69E+07	2.12E+07	3.29E+07	2.09E+07	2.46E+07	2.65E+07	2.40E+07	0.7	0.223
P53918	ESBP6	D6W158; P53918; sce:YNL125C	4.08E+06	3.45E+06	2.77E+05	2.60E+06	8.30E+05	6.57E+05	1.27E+06	9.20E+05	0.4	0.288
P38182	ATG8	D6VPS5; P38182; sce:YBL078C	2.33E+07	2.79E+07	3.70E+07	2.94E+07	2.97E+07	3.33E+07	3.47E+07	3.26E+07	1.1	0.502
Q12057	PIN2	D6W2G4; Q12057; sce:YOR104W	3.03E+06	1.30E+06	6.47E+05	1.66E+06	2.76E+05	2.85E+05	3.41E+05	3.01E+05	0.2	0.196
P11792	SCH9	D3DLF4; P11792; sce:YHR205W	1.88E+06	1.38E+06	3.03E+06	2.10E+06	4.24E+05	2.78E+06	1.97E+06	1.72E+06	0.8	0.682
P53142	VPS73	D6VU42; P53142; sce:YGL104C	2.85E+06	2.09E+06	1.85E+06	2.27E+06		2.37E+05	2.29E+05	2.33E+05	0.1	0.014
P25591	VAC17	D6VQV5; P25590; P25591; sce:YCL063W	2.15E+06	8.64E+05	1.29E+06	1.44E+06	2.87E+06	5.35E+06	2.59E+06	3.60E+06	2.5	0.086
P47040	YHC3	D6VWC3; O60004; P47040; Q6B1E1; sce:YJL059W	1.64E+07	1.73E+07	1.48E+07	1.62E+07	1.37E+07	1.58E+07	1.65E+07	1.53E+07	0.9	0.490
P53262	VOA1	D6VUN8; P53262; sce:YGR106C	6.81E+07	7.12E+07	8.27E+07	7.40E+07	7.68E+07	1.84E+07	8.24E+07	5.92E+07	0.8	0.548
Q04121	NHX1	D6VT80; Q04121; sce:YDR456W	4.08E+06	2.73E+06	1.22E+06	2.68E+06		2.12E+06		2.12E+06	0.8	0.765
Q06328	YPQ2	D6VSY2; Q06328; sce:YDR352W	2.00E+06	3.04E+06	8.91E+05	1.97E+06	2.19E+06		1.92E+06	2.06E+06	1.0	0.926
Q03446	RCR2	D6VRZ0; Q03446; sce:YDR003W	1.48E+07	1.15E+07	4.55E+06	1.03E+07	1.08E+07	8.01E+06	1.15E+07	1.01E+07	1.0	0.961
P40514		D6VVL7; P40514; Q6B2R2; sce:YIL067C	4.34E+06	3.48E+06	1.21E+06	3.01E+06	2.06E+06	1.86E+06	2.34E+06	2.09E+06	0.7	0.382
P38778	SMF2	D3DKZ8; P38778; sce:YHR050W	5.54E+06	6.06E+06	1.95E+06	4.52E+06	3.62E+06	5.09E+06	5.68E+06	4.80E+06	1.1	0.853
P38735	VMR1	D3DKT3; P38735; sce:YHL035C		1.05E+06		1.05E+06					**	**
Q04491	SEC13	D6VYK9; Q04491; sce:YLR208W	6.65E+05	2.16E+06	8.28E+04	9.68E+05	4.23E+06	2.34E+06	7.33E+06	4.63E+06	4.8	0.081

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

P53838	BOR1	D6W0R9; P53838; sce:YNL275W	7.68E+06	9.45E+06	3.45E+06	6.86E+06	6.62E+06	2.70E+06	7.80E+06	5.71E+06	0.8	0.649
P33300	SUR1	D6W3V7; P33300; sce:YPL057C	9.80E+06	9.47E+06	2.59E+06	7.28E+06	6.94E+06	3.73E+06	3.64E+06	4.77E+06	0.7	0.386
P38731	ARN1	D3DKS9; P38731; sce:YHL040C	3.93E+05	4.58E+05	2.18E+05	3.56E+05	8.72E+06	3.19E+06	1.02E+07	7.36E+06	20.7	0.081
Q3E7B6	VMA9	D6VR05; Q3E7B6; sce:YCL005W-A	1.31E+09	9.54E+08	1.34E+09	1.20E+09	1.11E+09	8.54E+08	5.55E+08	8.41E+08	0.7	0.151
P38287	CSH1	D6VQF6; P38287; sce:YBR161W	1.86E+06	2.18E+06	1.10E+06	1.71E+06	1.60E+06	3.32E+05	1.80E+06	1.24E+06	0.7	0.449
P25621	FEN2	D6VR38; P25621; sce:YCR028C	4.16E+06	3.48E+06	5.51E+05	2.73E+06	4.37E+06	3.56E+06	3.54E+06	3.82E+06	1.4	0.392
P39111	VMA7	D6VUF5; P39111; sce:YGR020C	2.47E+05	8.35E+05		5.41E+05	1.39E+06	1.31E+05	6.61E+05	7.29E+05	1.3	0.743
P38279	RTC2	D6VQE2; P38279; sce:YBR147W			1.04E+05	1.04E+05	1.57E+06	2.08E+06	3.63E+05	1.34E+06	12.9	0.350
P40343	VPS27	D6W1I1; P40343; sce:YNR006W	4.73E+05	8.33E+05	1.58E+05	4.88E+05	1.13E+06	5.95E+05	2.33E+05	6.52E+05	1.3	0.641
P38724	ARN2	D3DKS4; P38724; sce:YHL047C					6.79E+06	3.40E+06	3.64E+06	4.61E+06	*	*
Q06651	PIB1	D6VSU2; Q06651; Q6Q5S5; sce:YDR313C	3.11E+06		1.78E+06	2.44E+06	3.92E+05	1.73E+06	1.72E+05	7.66E+05	0.3	0.128
P38753	HSE1	D3DKR2; P38753; sce:YHL002W		3.89E+05		3.89E+05					**	**
Q04272	VPS20	D6VZQ1; Q04272; sce:YMR077C	2.73E+06	2.37E+06	1.43E+06	2.18E+06	2.80E+06	3.85E+06	1.80E+06	2.82E+06	1.3	0.414
P53882	TDA7	D6W109; P53882; sce:YNL176C	9.49E+06	1.76E+06	1.33E+06	4.20E+06	5.92E+06	1.86E+06	1.27E+06	3.02E+06	0.7	0.717
P38356	BSD2	D6VQT5; P38356; sce:YBR290W	1.09E+06	1.35E+06	2.22E+05	8.88E+05	4.28E+05	7.05E+05	7.61E+05	6.32E+05	0.7	0.511
P38273	CCZ1	D6VQC8; P38273; sce:YBR131W	1.92E+07		1.31E+07	1.62E+07		3.46E+05		3.46E+05	0.0	0.203
P47041	BIT61	D6VWC4; P47041; sce:YJL058C	4.80E+05	5.19E+05	1.03E+06	6.76E+05		7.53E+05	4.39E+05	5.96E+05	0.9	0.775
P43601	ATG18	D6VTQ1; P43601; sce:YFR021W	3.49E+05	3.73E+05	1.11E+05	2.78E+05	1.93E+05	5.23E+05	5.76E+05	4.30E+05	1.5	0.355
P39980	SIT1	D3DLI5; E9P951; P39980; sce:YEL065W	1.20E+06	1.41E+06	2.44E+05	9.53E+05	2.11E+06	5.26E+06	7.44E+06	4.93E+06	5.2	0.066

* - proteins only detected in *ncr1*Δ samples; ** - protein only detected in wild type samples

Table S2 – Phosphorylation residues identified in wild type and *ncr1*Δ samples and respective percentage of phosphorylation.

Phosphorylation residues identified in wild type and <i>ncr1</i> Δ samples and respective percentage of phosphorylation												
Master Protein Accessions	Gene Symbol	Modifications in Master Proteins	% Phosphorylation - wild type				% Phosphorylation <i>ncr1</i> Δ				Ratio <i>ncr1</i> Δ/wt	p-value (Student's t-test)
			Sample 1	Sample 2	Sample 3	Mean	Sample 1	Sample 2	Sample 3	Mean		
Q99385	VCX1	Q99385 1xAcetyl [N-Term]; 1xPhospho [S13(100)]	3.85	5.46	5.13	4.81	1.80	0.00	2.49	1.43	0.30	0.019
Q12016	VPS68	Q12016 1xAcetyl [N-Term]; 1xPhospho [S8(100)]	26.23	6.17	4.11	12.17	1.15	2.25	4.86	2.75	0.23	0.313
P40501	AVT7	P40501 1xPhospho [T4(99.1)]	0.60	0.38	0.66	0.55	2.91	4.10	0.72	2.58	4.71	0.111
P32366	VMA6	P32366 1xPhospho [Y5(100)]	0.00	1.17	6.11	2.43	6.48	0.00	0.00	2.16	0.89	0.930
Q12296	MAM3	Q12296 1xPhospho [S439(100)]	4.75	3.33	5.12	4.40	8.23	13.24	8.84	10.10	2.30	0.027
Q04602	VBA4	Q04602 1xPhospho [S106(100)]	9.24	0.00	3.03	4.09	0.00	1.86	0.00	0.62	0.15	0.281
Q12324	YVC1	Q12324 1xPhospho [T636(100)]	10.38	7.98	7.07	8.48	14.25	8.20	10.54	11.00	1.30	0.280
Q03516	RSN1	Q03516 1xPhospho [S933(100)]	100.00	100.00	0.00	66.67	0.00	0.00	100.00	33.33	0.50	0.519
P38966	YDR089W	P38966 1xPhospho [S612(100)]	10.65	16.32	7.18	11.38	0.00	0.00	11.02	3.67	0.32	0.165
P39968	VAC8	P39968 1xPhospho [S16(100)]	67.74	65.66	56.37	63.26	45.21	49.21	57.18	50.53	0.80	0.062
P38310	FTH1	P38310 1xPhospho [S453(98.9)]	0.97	0.47	0.32	0.59	3.44	5.50	2.00	3.65	6.20	0.042
P42839	VNX1	P42839 1xPhospho [T26(100)]	0.95	1.40	0.29	0.88	0.00	0.50	1.97	0.82	0.94	0.937
P43585	VTC2	P43585 2xPhospho [S615(100); S616(100)]	2.66	0.00	0.00	0.89	0.00	0.00	0.00	0.00	**	0.423
P43585	VTC2	P43585 1xPhospho [S196(100)]	31.96	28.92	29.82	30.23	0.00	20.02	44.88	21.63	0.72	0.545
P47818	CCC1	P47818 1xPhospho [Y173(100)]	0.00	0.00	0.00	0.00	0.29	0.49	0.20	0.33	*	0.062
P32563	VPH1	P32563 1xPhospho [S160(98.9)]	2.98	2.33	1.65	2.32	0.15	0.00	0.00	0.05	0.02	0.004

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

P47818	CCC1	P47818 1xPhospho [T32(99.4)]	2.32	2.60	2.90	2.61	4.51	4.64	5.17	4.77	1.83	0.001
P38244	PFF1	P38244 1xPhospho [S608(100)]	7.59	6.95	2.70	5.75	0.00	0.47	1.47	0.65	0.11	0.033
P38929	PMC1	P38929 1xPhospho [S1164(99.3)]	6.50	15.36	0.00	7.29	9.49	5.71	11.14	8.78	1.20	0.768
Q04991	FMP42	Q04991 1xPhospho [S238(100)]	20.41	11.82	14.11	15.45	4.25	3.20	14.26	7.24	0.47	0.133
Q04602	VBA4	Q04602 1xPhospho [S106(100)]	77.36	74.78	63.13	71.76	0.00	60.47	0.00	20.16	0.28	0.119
Q06451	TPO3	Q06451 1xPhospho [S101(100)]	0.00	0.00	4.62	1.54	0.00	36.84	47.29	28.04	18.20	0.205
Q12296	MAM3	Q12296 1xPhospho [T519(99.7)]	0.00	0.00	0.00	0.00	0.00	11.54	2.76	4.77	*	0.304
Q12296	MAM3	Q12296 2xPhospho [T519(98.4); S527(100)]	12.86	13.01	25.58	17.15	0.00	8.94	11.35	6.77	0.39	0.129
Q12296	MAM3	Q12296 3xPhospho [S523(98.7); S527(100); T/S]	10.52	33.37	70.04	37.98	15.05	14.13	24.69	17.96	0.47	0.320
P40528	SYG1	P40528 1xPhospho [S44(99)]	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.18	*	0.423
Q12697	YPK9	Q12697 1xPhospho [S1120(98.7)]	10.77	0.00	0.00	3.59	0.00	0.00	2.90	0.97	0.27	0.545
Q12697	YPK9	Q12697 2xPhospho [S1117(100); S1120(98.6)]	2.64	1.44	0.45	1.51	0.00	0.00	0.00	0.00	**	0.076
P43585	VTC2	P43585 1xPhospho [S657(100)]	61.75	55.84	47.91	55.17	63.90	59.97	68.44	64.11	1.16	0.130
P39109	YCF1	P39109 1xPhospho [S903(100)]	83.44	89.52	84.87	85.94	80.49	89.09	72.17	80.58	0.94	0.362
Q02725	VTC3	Q02725 1xPhospho [S198(100)]	10.17	4.80	2.97	5.98	1.75	2.94	1.70	2.13	0.36	0.155
P32386	YBT1	P32386 1xPhospho [S81(98.8)]	4.00	3.61	0.00	2.54	0.00	0.00	0.00	0.00	**	0.185
Q12256	TPO4	Q12256 1xPhospho [S44(99.5)]	16.63	18.47	0.00	11.70	0.00	0.00	0.00	0.00	**	0.185
P35724	MNR2	P35724 1xPhospho [S576(100)]	24.77	15.51	6.20	15.49	2.32	4.26	7.19	4.59	0.30	0.121
P39962	YCK3	P39962 1xPhospho [S48(100)]	41.81	29.07	12.30	27.72	0.00	0.00	0.00	0.00	**	0.032
Q07376	MCH1	Q07376 1xPhospho [S255(100)]	4.72	7.41	3.49	5.21	0.00	4.70	1.03	1.91	0.37	0.147
P20107	ZRC1	P20107 1xPhospho [S425(99.4)]	3.04	4.13	3.64	3.60	0.00	1.39	3.76	1.72	0.48	0.174
P32386	YBT1	P32386 2xPhospho [S1519(100); S/T]	0.77	0.00	0.00	0.26	0.13	1.27	0.00	0.47	1.81	0.683
P32386	YBT1	P32386 1xPhospho [S1516(99.6)]	0.00	0.22	0.10	0.11	0.00	0.00	0.26	0.09	0.81	0.861
P38966	YDR089W	P38966 1xPhospho [S327(100)]	13.39	10.81	4.46	9.55	9.29	0.00	0.00	3.10	0.32	0.189
Q12296	MAM3	Q12296 1xPhospho [S501(100)]	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	1.00	-
P42839	VNX1	P42839 1xPhospho [S110(100)]	45.56	45.43	45.33	45.44	47.71	55.39	42.99	48.69	1.07	0.418
P42839	VNX1	P42839 1xPhospho [S121(99.6)]	4.42	3.50	2.37	3.43	3.18	5.03	5.13	4.45	1.30	0.306
P32386	YBT1	P32386 1xPhospho [S462(100)]	100.00	92.29	65.97	86.09	84.19	84.30	94.98	87.83	1.02	0.881

Q06708	VAC14	Q06708 1xPhospho [S819(100)]	100.00	9.16	0.00	36.39	100.00	3.02	6.69	36.57	1.01	0.997
P39109	YCF1	P39109 3xPhospho [S872(96.2); S873(96.2); S]	0.00	0.66	0.00	0.22	0.00	0.32	0.58	0.30	1.36	0.787
P39109	YCF1	P39109 1xPhospho [S877(98.6)]	5.22	1.32	1.84	2.79	0.17	0.60	1.73	0.83	0.30	0.208
P39109	YCF1	P39109 3xPhospho [S873(99.3); S]	0.50	0.35	0.00	0.28	0.00	0.18	0.00	0.06	0.21	0.235
P39109	YCF1	P39109 2xPhospho [S877(99); S878(99)]	2.56	1.61	0.56	1.58	0.40	0.73	0.86	0.66	0.42	0.199
Q02725	VTC3	Q02725 1xPhospho [S274(100)]	4.91	5.35	4.71	4.99	2.61	1.72	2.50	2.28	0.46	0.001
Q02725	VTC3	Q02725 2xPhospho [S267(99.1); S]	0.65	1.08	0.00	0.58	0.00	0.00	0.00	0.00	**	0.140
P32386	YBT1	P32386 2xPhospho [S940(100); S944(100)]	79.31	65.12	100.00	81.48	89.09	86.77	88.86	88.24	1.08	0.542
P32386	YBT1	P32386 3xPhospho [S936(99.5); S940(100); S944(100)]	20.69	34.88	0.00	18.52	10.91	13.23	11.14	11.76	0.63	0.542
P32386	YBT1	P32386 2xPhospho [S955(100); S/T]	55.37	61.61	23.93	46.97	45.98	53.85	54.46	51.43	1.10	0.728
Q02725	VTC3	Q02725 1xPhospho [S285(100)]	5.01	0.16	1.51	2.23	0.00	0.00	0.00	0.00	**	0.263
Q04991	FMP42	Q04991 1xPhospho [S269(100)]	0.60	1.22	0.04	0.62	0.51	0.06	0.84	0.47	0.75	0.732
Q04121	NHX1	Q04121 1xPhospho [S499(99.5)]	100.00	100.00	100.00	100.00	0.00	100.00	0.00	33.33	0.33	0.184
P35724	MNR2	P35724 1xPhospho [S461(100)]	7.10	7.31	0.00	4.80	39.76	45.15	0.00	28.30	5.89	0.238
P32798	COT1	P32798 1xPhospho [S225(100)]	14.68	17.69	10.36	14.24	3.04	4.22	4.53	3.93	0.28	0.009
P32939	YPT7	P32939 1xPhospho [T59(100)]	0.13	0.19	0.19	0.17	0.00	0.27	0.16	0.14	0.85	0.758
P14772	BPT1	P14772 2xPhospho [T906(99.5); T916(100)]	18.05	28.39	18.30	21.58	0.00	45.44	51.29	32.24	1.49	0.581
P32798	COT1	P32798 1xPhospho [S407(100)]	1.81	1.12	1.68	1.54	0.00	0.49	0.25	0.24	0.16	0.007
Q12078	SMF3	Q12078 1xPhospho [T425(99.5)]	0.20	0.12	0.08	0.14	0.00	0.11	0.10	0.07	0.52	0.272
P39109	YCF1	P39109 1xPhospho [S914(99.1)]	10.71	6.15	0.00	5.62	0.00	3.73	2.96	2.23	0.40	0.363
P53273	YGR125W	P53273 1xPhospho [S42(100)]	0.00	0.00	1.90	0.63	0.00	0.00	0.00	0.00	**	0.423
P32386	YBT1	P32343 1xPhospho [S358(100)]	57.13	41.47	34.21	44.27	25.86	25.37	35.86	29.03	0.66	0.115
P32386	YBT1	P32386 1xPhospho [S654(100)]	70.79	68.62	15.94	51.78	100.00	100.00	100.00	100.00	1.93	0.115
P32563	VPH1	P32563 1xPhospho [T32(99)]	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.04	*	0.423
P40074	AVT6	P40074 1xPhospho [S344(100)]	5.58	4.11	7.00	5.56	0.00	0.61	0.00	0.20	0.04	0.003
Q03730	YML018C	Q03730 1xPhospho [S93(100)]	4.76	14.09	8.89	9.25	9.34	2.59	9.77	7.23	0.78	0.602
P32386	YBT1	P32386 2xPhospho [S1516(98.7); S1519(100)]	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00	**	-

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

P38873	KOG1	P38873 2xPhospho [S491(99.5); S494(100)]	100.00	0.00	0.00	33.33	0.00	0.00	100.00	33.33	1.00	1.000
P20107	ZRC1	P20107 1xPhospho [S393(100)]	2.39	2.74	1.62	2.25	1.31	1.80	1.67	1.59	0.71	0.144
P20107	ZRC1	P20107 2xPhospho [S396(100); S397(100)]	0.46	0.74	0.00	0.40	0.26	0.42	0.15	0.28	0.69	0.620
P47082	AVT1	P47082 1xPhospho [S187(100)]	0.94	0.91	0.00	0.62	0.00	0.00	0.00	0.00	**	0.184
P53273	YGR125W	P53273 1xPhospho [S185(99.4)]	0.92	1.06	0.87	0.95	0.00	0.00	0.00	0.00	**	0.004
P34240	ZRT3	P34240 1xPhospho [S262(100)]	33.71	21.68	26.83	27.41	15.04	22.89	22.53	20.15	0.74	0.169
P47818	CCC1	P47818 2xPhospho [S53(99.8); S/Y]	12.64	19.23	0.00	10.62	0.00	21.74	17.95	13.23	1.25	0.781
Q02725	VTC3	Q02725 2xPhospho [S621(100); S622(100)]	94.28	95.13	100.00	96.47	100.00	100.00	100.00	100.00	1.04	0.186
P27514	PHO91	P27514 1xPhospho [S312(99.4)]	0.00	0.00	0.00	0.00	28.28	0.00	29.88	19.39	*	0.184
P27514	PHO91	P27514 2xPhospho [S311(100); S312(100)]	16.71	26.68	3.75	15.72	14.19	35.32	25.78	25.10	1.60	0.357
Q05521	DPP1	Q05521 1xPhospho [S285(100)]	46.68	29.03	25.21	33.64	45.43	47.56	35.02	42.67	1.27	0.304
P43585	VTC2	P43585 1xPhospho [S616(100)]	4.49	3.81	10.07	6.12	8.33	0.00	5.59	4.64	0.76	0.663
P43585	VTC2	P43585 2xPhospho [S615(100); S616(100)]	93.28	93.30	84.65	90.41	91.67	97.32	91.68	93.56	1.03	0.412
P43585	VTC2	P43585 3xPhospho [S606(99); S615(99); S616(99)]	0.00	0.30	0.00	0.10	0.00	0.00	0.00	0.00	**	0.423
P43561	FET5	P43561 1xPhospho [Y391(100)]	13.62	9.15	8.46	10.41	1.64	1.72	1.33	1.56	0.15	0.031
Q04991	FMP42	Q04991 1xPhospho [S238(100)]	19.24	11.76	10.07	13.69	10.36	11.63	8.87	10.28	0.75	0.310
P32912	VAM7	P32912 1xPhospho [S233(99.6)]	2.73	1.93	2.37	2.34	1.40	0.70	0.00	0.70	0.30	0.024
P53273	YGR125W	P53273 2xPhospho [S152(100); S153(100)]	2.59	4.89	0.00	2.50	6.93	16.87	14.77	12.86	5.15	0.036
P32798	COT1	P32798 1xPhospho [T206(100)]	0.00	0.00	0.00	0.00	0.69	0.25	0.00	0.31	*	0.195
P11491	PHO8	P11491 1xPhospho [S17(99.7)]	1.68	0.90	55.55	19.38	0.58	0.64	0.45	0.56	0.03	0.407
P39109	YCF1	P39109 1xPhospho [S251(99.6)]	1.70	1.11	0.48	1.10	0.87	0.78	0.96	0.87	0.79	0.559
P25371	ADP1	P25371 1xPhospho [S659(100)]	3.93	0.00	2.88	2.27	9.04	6.69	9.09	8.27	3.65	0.013
P37296	STV1	P37296 1xPhospho [S223(100)]	78.65	69.02	0.00	49.22	100.00	47.04	37.65	61.56	1.25	0.715
P47818	CCC1	P47818 2xPhospho [S68(100); S71(100)]	1.02	1.01	0.29	0.77	1.63	1.98	2.21	1.94	2.51	0.017
P47818	CCC1	P47818 1xPhospho [S71(100)]	2.95	2.75	2.41	2.70	6.24	3.34	5.10	4.89	1.81	0.063
P53388	DIP5	P53388 1xPhospho [S22(100)]	28.31	27.84	0.00	18.72	0.00	0.00	0.00	0.00	**	0.184

Q04991	FMP42	Q04991 1xPhospho [S259(100)]	0.88	1.24	0.89	1.00	1.28	0.00	1.40	0.89	0.89	0.832
P39109	YCF1	P39109 3xPhospho [S908(100); T911(100); S914(100)]	83.86	88.74	0.00	57.53	87.85	87.13	100.00	91.66	1.59	0.358
P39109	YCF1	P39109 2xPhospho [S908(100); T/S]	16.14	11.26	100.00	42.47	12.15	12.87	0.00	8.34	0.20	0.358
P20107	ZRC1	P20107 1xPhospho [S387(100)]	12.34	20.19	0.00	10.84	7.79	0.00	16.93	8.24	0.76	0.751
P38329	VHC1	P38329 1xPhospho [S34(100)]	41.62	64.69	52.81	53.04	54.47	63.05	62.46	59.99	1.13	0.390
P34240	ZRT3	P34240 1xPhospho [S188(100)]	0.00	100.00	100.00	66.67	0.00	65.80	94.95	53.58	0.80	0.779
P27514	PHO91	P27514 1xPhospho [S295(99.7)]	33.16	28.63	87.25	49.68	22.86	30.93	19.34	24.38	0.49	0.310
P27514	PHO91	P27514 3xPhospho [S295(98.9); S311(100); S312(100)]	3.58	7.29	0.00	3.62	0.00	0.00	4.37	1.46	0.40	0.445
P20107	ZRC1	P20107 1xPhospho [T423(97.1)]	0.00	0.00	0.69	0.23	0.00	0.00	0.00	0.00	**	0.423
P53925	YNL115C	P53925 1xPhospho [S244(100)]	3.09	4.82	55.07	20.99	8.20	6.93	8.31	7.82	0.37	0.520
P53925	YNL115C	P53925 2xPhospho [S242(100); S244(99.6)]	0.84	0.62	10.50	3.98	0.47	0.19	0.27	0.31	0.08	0.376
P11491	PHO8	P11491 1xPhospho [S123(100)]	7.34	7.63	4.06	6.35	12.14	8.60	11.90	10.88	1.71	0.049
Q02725	VTC3	Q02725 1xPhospho [S43(98.8)]	2.60	4.72	1.72	3.01	0.00	0.00	0.00	0.00	**	0.077
P53273	YGR125W	P53273 1xPhospho [S140(100)]	19.07	32.96	29.63	27.22	46.25	51.24	40.20	45.90	1.69	0.024
P53925	YNL115C	P53925 1xPhospho [S56(100)]	1.18	0.73	0.52	0.81	0.80	0.45	1.07	0.77	0.95	0.897
Q02205	MEH1	Q02205 1xPhospho [S146(99.5)]	7.55	8.24	5.65	7.14	0.00	1.72	0.00	0.57	0.08	0.002
Q06451	TPO3	Q06451 1xPhospho [S132(100)]	71.75	73.67	70.34	71.92	37.12	23.46	33.33	31.30	0.44	0.001
P11491	PHO8	P11491 1xPhospho [S123(100)]	13.74	0.00	9.51	7.75	100.00	0.00	43.89	47.96	6.19	0.241
Q04602	VBA4	Q04602 1xPhospho [S62(100)]	0.72	0.00	0.00	0.24	0.00	0.00	0.00	0.00	**	0.423
P42836	PFA3	P42836 1xPhospho [S329(100)]	62.31	38.08	24.44	41.61	0.00	11.35	0.00	3.78	0.09	0.032
P56508	SNA2	P56508 1xPhospho [S70(99)]	20.11	17.72	9.32	15.72	11.99	15.31	11.03	12.78	0.81	0.451

* - phosphorylation only detected in *ncr1*Δ samples; ** - phosphorylation only detected in wild type samples

Table S3 - Gene Ontology (GO) enrichment analysis on biological processes

Gene Ontology (GO) enrichment analysis on biological processes-								
GO Biological process	Protein name	Description	Ratio protein abundance (<i>ncr1Δ</i> /wt)	Phosphosite	%phosphorylation (wt)	%phosphorylation (<i>ncr1Δ</i>)	Fold enrichment	P value
Vesicle fusion with the vacuole	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				> 100	5.43E-04
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
Vesicle fusion	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				13.98	7.16E-06
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85					
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	NYV1	v-SNARE component of the vacuolar SNARE complex; involved in vesicle fusion; inhibits ATP-dependent Ca(2 ⁺) transport activity of Pmc1p	0.41					
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Vesicle-mediated transport	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				3.54	1.53E-05
	SSA1	ATPase involved in protein folding, nuclear import, stress granule disassembly, and clathrin coat disassembly; (localizes to many places in the cell)	2.48					
	FTH1	High affinity iron transporter; involved in transport of intravacuolar stores of iron; indirect role in endocytosis	2.06	S453	0.6	3.6		
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85					

	VPS30	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy, Complex II is required for vacuolar protein sorting;	1.59					
	EMP70	Role in endosome-to-vacuole sorting	0.31					
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	YCK3	Palmitoylated vacuolar membrane-localized casein kinase I isoform	0.40	S48	27.7	0		
	NYV1	v-SNARE component of the vacuolar SNARE complex; involved in vesicle fusion; inhibits ATP-dependent Ca(2+) transport activity of Pmc1p	0.41					
	MYO2	Type V myosin motor involved in actin-based transport of cargos	0.49					
	PEP8	Endosomal retromer complex subunit involved in retrograde transport	0.67					
	YPT52	Endosomal Rab family GTPase	0.69					
	MEH1	Subunit of the EGO/GSE complex		S146	7.1	0.6		
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Vesicle organization	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				8.73	5.03E-06
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85					
	LSB6	Type II phosphatidylinositol 4-kinase	1.44					
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	NYV1	v-SNARE component of the vacuolar SNARE complex; involved in vesicle fusion; inhibits ATP-dependent Ca(2+) transport activity of Pmc1p	0.41					
	YPT52	Endosomal Rab family GTPase	0.69					
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Vacuole fusion	IVY1	Phospholipid-binding protein that interacts with both Ypt7p and Vps33p	5.15				26.37	2.12E-10

	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93					
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	NYV1	v-SNARE component of the vacuolar SNARE complex; involved in vesicle fusion; inhibits ATP-dependent Ca(2+) transport activity of Pmc1p	0.41					
	VTC5	Subunit of the vacuolar transporter chaperone (VTC) complex	0.46					
	PFA3	Palmitoyltransferase for Vac8p		S329	41.6	3.8		
	VTC3	Regulatory subunit of the vacuolar transporter chaperone (VTC) complex; (VTC complexes containing Vtc3 are mainly found on vacuoles)		S274	5.0	2.3		
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Vacuole organization	IVY1	Phospholipid-binding protein that interacts with both Ypt7p and Vps33p	5.15				14.65	9.94E-12
	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93					
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85					
	VPS30	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy, Complex II is required for vacuolar protein sorting	1.59					
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	NYV1	v-SNARE component of the vacuolar SNARE complex; involved in vesicle fusion; inhibits ATP-dependent Ca(2+) transport activity of Pmc1p	0.41					
	VTC5	Subunit of the vacuolar transporter chaperone (VTC) complex	0.46					
	MYO2	Type V myosin motor involved in actin-based transport of cargos	0.49					
	MEH1	Subunit of the EGO/GSE complex		S146	7.1	0.6		
	PFA3	Palmitoyltransferase for Vac8p		S329	41.6	3.8		

	VTC3	Regulatory subunit of the vacuolar transporter chaperone (VTC) complex		S274	5.0	2.3		
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Vacuolar transmembrane transport	VHC1	Vacuolar potassium:chloride symporter; localizes to vCLAMPs	2.39				45.57	5.26E-06
	ATG22	Vacuolar integral membrane protein required for efflux of amino acids during autophagic body breakdown in the vacuole	1.31					
	AVT6	Vacuolar aspartate and glutamate exporter		S344	5.6	0.2		
	YGR125W	Vacuolar uptake and storage of arginine; member of the APC (amino acid-polyamine-organocation) transporter superfamily 1		S185 S152; S153 S140	0.9 2.5 27.2	0.0 12.9 45.9		
Iron import into cell	ENB1	ENB1/ARN4 - Ferric enterobactin transmembrane transporter	4.02				43.95	9.79E-05
	FRE8	Putative ferric-chelate reductase	1.77					
	FET5	Ferroxidase involved in iron transport	1.56	Y391	10.4	1.6		
Iron ion homeostasis	ENB1	ENB1/ARN4 - Ferric enterobactin transmembrane transporter	4.02				10.09	3.96E-05
	SMF3	Metal ion transmembrane transporter involved in iron ion transport	2.30					
	FTH1	High affinity iron transporter; involved in transport of intravacuolar stores of iron; indirect role in endocytosis	2.06	S453	0.6	3.6		
	FRE8	Putative ferric-chelate reductase	1.77					
	FET5	Ferroxidase involved in iron transport	1.56	Y391	10.4	1.6		
	CCC1	Vacuolar Fe ²⁺ /Mn ²⁺ transporter		T32 S68 ; S71	2.6 0.8	4.8 1.9		
Manganese ion homeostasis	SMF3	Metal ion transmembrane transporter involved in iron ion transport	2.30				30.76	2.29E-04
	CCC1	Vacuolar Fe ²⁺ /Mn ²⁺ transporter		T32 S68 ; S71	2.6 0.8	4.8 1.9		
	MAM3	Required for Mg ²⁺ sequestration in vacuole		S439	4.4	10.1		

Calcium ion transport	VHC1	Vacuolar potassium:chloride symporter; localizes to vCLAMPs	2.39				27.34	2.73E-05
	PMC1	Vacuolar Ca ²⁺ ATPase involved in depleting cytosol of Ca ²⁺ ions	1.22					
	YBT1	Transporter of the ATP-binding cassette (ABC) family; negative regulator of vacuole fusion; regulates release of luminal Ca ²⁺ stores	0.78	S654 S1516 ; S1519	51.8 100.0	100 0		
	VCX1	Vacuolar membrane antiporter with Ca ²⁺ /H ⁺ and K ⁺ /H ⁺ exchange activity (transports calcium into the vacuole)		S13	4.8	1.4		
Calcium ion homeostasis	VMA2	V-type proton ATPase subunit B; Subunit B of V1 peripheral membrane domain of vacuolar H ⁺ -ATPase	2.23				22.79	5.04E-05
	PMC1	Vacuolar Ca ²⁺ ATPase involved in depleting cytosol of Ca ²⁺ ions	1.22					
	CCC1	Vacuolar Fe ²⁺ /Mn ²⁺ transporter		T32 S68 ; S71	2.6 0.8	4.8 1.9		
	VCX1	Vacuolar membrane antiporter with Ca ²⁺ /H ⁺ and K ⁺ /H ⁺ exchange activity (transports calcium into the vacuole)		S13	4.8	1.4		
Polyphosphate metabolic process	VTC5	Subunit of the vacuolar transporter chaperone (VTC) complex	0.46				21.97	5.29E-04
	VTC3	Regulatory subunit of the vacuolar transporter chaperone (VTC) complex		S274	5.0	2.3		
	VPH1	Subunit a of the vacuolar-ATPase V0 domain		S160	2.3	0.0		
Vesicle docking	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				17.09	1.84E-05
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	MYO2	Type V myosin motor involved in actin-based transport of cargos	0.49					
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Golgi organization	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85				11.72	5.02E-04
	LSB6	Type II phosphatidylinositol 4-kinase	1.44					
	YPT10	Rab family GTP-binding protein	1.39					

	MYO2	Type V myosin motor involved in actin-based transport of cargos	0.49					
Autophagy	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				6.65	2.68E-07
	SLM4		2.34					
	VMA2	V-type proton ATPase subunit B; Subunit B of V1 peripheral membrane domain of vacuolar H ⁺ -ATPase	2.23					
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF)	1.85					
	VPS30	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy, Complex II is required for vacuolar protein sorting	1.59					
	ATG22	Vacuolar integral membrane protein required for efflux of amino acids during autophagic body breakdown in the vacuole	1.31					
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	MEH1	Subunit of the EGO/GSE complex		S146	7.1	0.6		
	VTC3	Regulatory subunit of the vacuolar transporter chaperone (VTC) complex		S274	5.0	2.3		
	VTC5	Subunit of the vacuolar transporter chaperone (VTC) complex	0.46					
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Lysosomal microautophagy	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				14.65	1.35E-07
	SLM4		2.34					
	VPS30	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy, Complex II is required for vacuolar protein sorting	1.59					
	VTC5	Subunit of the vacuolar transporter chaperone (VTC) complex	0.46					
	VAM3	Syntaxin-like vacuolar t-SNARE	0.38					
	MEH1	Subunit of the EGO/GSE complex		S146	7.1	0.6		

Iron limitation restores autophagy and increases lifespan in the yeast model of Niemann–Pick Type C1

	VTC3	Regulatory subunit of the vacuolar transporter chaperone (VTC) complex		S274	5.0	2.3		
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		
Protein targeting to vacuole	VPS33	ATP-binding protein that is a subunit of the HOPS and CORVET complexes	2.93				5.97	5.87E-04
	VPS30	Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy, Complex II is required for vacuolar protein sorting	1.59					
	YPT52	Endosomal Rab family GTPase	0.69					
	PEP12	Target membrane receptor (t-SNARE)	0.32					
	VPS73	Putative transmembrane transporter involved in vacuolar protein targeting	0.10					
	VAM7	v-SNARE protein; functions with Vam3p in vacuolar protein trafficking		S233	2.3	0.7		

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CHAPTER III

Metabolic dysregulation in *ncr1* Δ cells: H₂S production and Snf1/AMPK activity

Abstract

Niemann-Pick type C is a rare and severe lysosomal storage disorder caused by loss of function of NPC1 or NPC2. This disease is characterized by the accumulation of cholesterol and other lipids in late endosomes/lysosomes of various tissues. *Saccharomyces cerevisiae* lacking Ncr1, orthologue of NPC1, recapitulates several phenotypes observed in NPC1 cells and has been used as a model system to explore the mechanisms underlying the disease. Previously, we identified several vacuolar membrane proteins differentially regulated in *ncr1* Δ cells and demonstrated that autophagy is disrupted, contributing to the premature aging of these cells. In this study, we identified transcription factors associated with sulfur metabolism as potential regulators of vacuolar proteins altered in *ncr1* Δ cells. Cells lacking Ncr1 exhibited a lower production of H₂S, a pro-longevity compound, but increasing H₂S did not suppress the shortened lifespan observed in *ncr1* Δ cells. Additionally, we found that Snf1/AMPK, required for autophagy induction, is not inhibited in the *ncr1* Δ mutant despite the activation in these cells of its negative regulator Sit4. Overall, our results suggest that reduced H₂S production is not a primary factor contributing to the premature aging observed in *ncr1* Δ cells, and that autophagy impairment in this mutant is not linked to the inhibition of Snf1.

Keywords: Ncr1; vacuoles; H₂S; autophagy; Snf1/AMPK; chronological aging

3.1 Introduction

Niemann-Pick type C (NPC) is a rare lysosomal storage disease (LSD) characterized by impaired intracellular lipid trafficking, leading to the aberrant accumulation of cholesterol and other lipids in various tissues, including the brain, liver, and spleen [1]. This accumulation results in progressive neurodegeneration and visceral organ dysfunction. NPC disease is an autosomal recessive disorder caused by loss of function mutations in either NPC1 or NPC2, with NPC1 mutations accounting for the majority of cases [1–3]. NPC1 is a transmembrane protein localized in late endosomes/lysosomes (LE/Lys) [4,5]. It plays a crucial role in exporting cholesterol and facilitating its transfer to various cellular compartments, including the endoplasmic reticulum, plasma membrane, trans-Golgi network, recycling endosomes, and mitochondria [6,7]. NPC2 is a small soluble glycoprotein located in the lumen of LE/Lys. It binds to cholesterol and facilitates its transfer to NPC1 [8,9].

Currently, there is no cure for NPC disease, and affected individuals typically experience a significantly shortened lifespan [1]. Model organisms, such as the yeast *Saccharomyces cerevisiae*, have been instrumental in dissecting the underlying mechanisms of the disease and in the search for effective treatments [10]. Ncr1 and Npc2 are the yeast orthologues of NPC proteins, with Ncr1 localizing at the membrane and Npc2 at the lumen of yeast lysosome-like vacuoles [11,12]. Remarkably, the absence of Ncr1 in yeast mirrors several phenotypic traits seen in NPC1 mammalian cells. Cells lacking Ncr1 exhibit alterations in the homeostasis of ergosterol, the yeast equivalent of cholesterol, as well as in sphingolipid metabolism [13,14]. The accumulation of sphingolipid long chain bases has been shown to activate the Pkh1 protein kinase and its downstream target Sch9, leading to heightened sensitivity to oxidative stress, mitochondrial dysfunction, and a shortened chronological lifespan (CLS) [13]. Furthermore, the accumulation of phytoceramide and activation of Sit4 phosphatase also contribute to these phenotypes [14]. Deletion of *PKH1*, *SCH9*, or *SIT4* alleviates oxidative stress sensitivity, mitochondrial dysfunction, and premature CLS in *ncr1* Δ cells [13,14].

Recently, we conducted a phosphoproteomic analysis of vacuolar membranes from Ncr1-deficient cells, revealing alterations in proteins primarily involved in the organization, transport, and fusion of vesicles, Golgi organization, as well as in processes such as autophagy and the regulation of ion homeostasis and polyphosphate metabolism [15]. Furthermore, our findings revealed that autophagy is disrupted in cells lacking Ncr1, despite the downregulation of TORC1, a negative regulator of autophagy [15]. Instead, iron overload was implicated in the observed autophagic defects, since iron deprivation conditions restored autophagy in *ncr1* Δ cells and improved their CLS [15].

Here, we conducted an in-silico analysis to predict potential transcription factors (TFs) responsible for the observed alterations in the abundance of *ncr1Δ* vacuolar proteins. Additionally, we investigated the impact of H₂S production on the CLS of *ncr1Δ* cells and explored the involvement of Snf1/AMPK, which is negatively regulated by Sit4, in the autophagy impairment observed in *ncr1Δ* cells.

3.2. Materials and methods

3.2.1 Yeast strains and growth conditions

The yeast strains used in this study are listed in Table 1. Cells were cultivated aerobically at 26 °C with a ratio of flask volume:medium volume of 5:1 in an orbital shaker at 140 rpm. The growth medium utilized was synthetic complete (SC) medium, comprising drop-out, 2% (w/v) glucose (ThermoFisher Scientific, Waltham, MA, USA), 0.67% (w/v) yeast nitrogen base without amino acids (BD BioSciences, San Jose, CA, USA), and the necessary amino acids or nucleotides [0.008% (w/v) histidine, 0.038% (w/v) methionine, 0.04% (w/v) leucine, and 0.008% (w/v) uracil; all from Sigma Aldrich, St. Louis, MO, USA].

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

Strain	Genotype	Source
BY4741 ^{a,b,c}	Mata, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i>	EUROSCARF
<i>ncr1Δ</i> ^{a,b,c}	BY4741 <i>ncr1::KanMX4</i>	[13]

Cells harboring ^apRS416-*GFP-ATG8*, ^bpSP-GM2-*ACC1^{wt}*, and ^cpSP-GM2- *ACC1^{Ser659Ala,Ser1157Ala}* are indicated.

3.2.2. Transcription factor enrichment analysis

TF enrichment analysis was conducted on genes encoding proteins differentially regulated in *ncr1Δ* vacuolar membranes (chapter II), using the YEASTRACT repository [16]. The gene list was uploaded, and the TFs were searched based on DNA binding or expression evidence.

3.2.3. H₂S Measurement

To detect H₂S production in live yeast cultures, lead acetate paper strips (Cytiva, Marlborough, MA, USA) were placed on the top of yeast flasks at the time of inoculation. H₂S reacts with lead ions to form black lead sulfide PbS, functioning as a qualitative indicator of H₂S [17]. Images were acquired after growth for 18h.

3.2.4. Chronological lifespan

The CLS was evaluated following a previously established protocol [18]. To assess the impact of exogenous H₂S supply on CLS, cells were treated with 100 μ M GYY4137 dichloromethane complex (dissolved in DMSO; Sigma Aldrich, St. Louis, MO, USA) upon inoculation at OD_{600nm}=0.2. Subsequently, 5 μ M NaHS hydrate (Sigma Aldrich, St. Louis, MO, USA) was added at 6 h, 24 h, and 48 h post-inoculation, as described previously [17]. Control cells were treated with DMSO. Cultures were maintained in the culture medium at 26 °C, and cellular viability was assessed at specified time points using standard dilution plate counts on YPD medium containing 1.5% (w/v) agar. Colony-forming units (CFUs) were counted following 2-3 days of incubation at 26 °C. Cellular viability was expressed as the percentage of CFUs relative to the initial time point (t₀, 24 h after inoculation at OD_{600nm}=0.2).

3.2.5. Western blotting

For the analysis of Snf1 phosphorylation, TCA 100 % (w/v) was added to yeast cultures to a final concentration of 20% and samples were incubated 15 min on ice. After centrifugation at 5,000 rpm (4 °C) during 3 min, the pellet was washed twice with acetone and then suspended in 0.1 M NaOH. After incubation on ice during 10 min, samples were recovered by centrifugation at 13,000 (4 °C) during 15 min and the pellet was resuspended in modified Laemmli buffer [62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] and incubated 5 min at 95 °C. After centrifugation at 13,000 rpm (4 °C) for 3 min, the supernatant was collected. For the analysis of autophagy, cell pellet was incubated in 0.1M NaOH during 5 min at room temperature and harvested by centrifugation at 13,000 rpm (4 °C) during 15 min. The pellet was suspended in modified Laemmli and incubated 5 min at 95 °C. After centrifugation at 13,000 rpm (4 °C) for 3 min, the supernatant was collected.

Protein content was quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA), using bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) as a standard. Protein samples were prepared by adding 5% (v/v) 2-mercaptoethanol (Merck, Darmstadt, Germany), separated by SDS-PAGE, and transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). Immunodetection was performed using anti-Phospho-AMPK α (Thr172) (1:1,000; Cell Signaling, Danvers, MA, USA) or anti-GFP (1:5,000; Roche, Basel, Switzerland) as primary antibodies, and anti-rabbit IgG-peroxidase (1:5,000; Sigma Aldrich, St. Louis, MO, USA) or anti-mouse IgG-peroxidase (1:5,000; Molecular Probes, Eugene, OR, USA) as secondary antibodies. As loading control, Pgk1 levels were analyzed using anti-Pgk1 (1:30,000; Invitrogen, Waltham, MA, USA) as primary antibody and anti-mouse IgG-peroxidase (1:10,000; Molecular

Probes, Eugene, OR, USA) as secondary antibody. Immunoblot was revealed by chemiluminescence (ECL, Advansta, San Jose, CA, USA).

3.2.6. Statistical analysis

Data were analyzed in GraphPad Prism Software v10.0.0 (GraphPad Software, Boston, MA, USA). Values were compared using Student's t-test.

3.3. Results

3.3.1 Production of H₂S is decreased in cells lacking Ncr1

In the work presented in the chapter II, we demonstrated alterations in the levels and phosphorylation of vacuolar membrane proteins of cells lacking Ncr1 [15]. These alterations were mainly found in proteins associated with vesicle trafficking, ion homeostasis (specifically iron, manganese, and calcium), polyphosphate metabolism, Golgi organization, and autophagy. To predict potential TFs that may be responsible for the observed changes in the levels of *ncr1Δ* vacuolar proteins (if regulation occurs at the transcriptional level), we performed an in-silico analysis using the repository YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking) [16]. Among the TFs enriched in this analysis were Cbf1, Met28, Met31 and Met32 (Table 2), which regulate the sulfur metabolism, including the assimilation of inorganic sulphate used for the synthesis of the sulfur-containing amino acids methionine and cysteine [19–21]. A key product of this pathway is H₂S that results from the desulfhydration of cysteine or homocysteine and has important physiological functions [22]. Decreased H₂S production is associated with neurodegenerative diseases [23] whereas the exposure to low concentrations of H₂S extends lifespan of worms and yeast model organisms [17,24,25]. Moreover, increased H₂S production from cysteine during dietary restriction was identified as a mediator of the beneficial effects of these condition on lifespan and stress resistance in yeast and mammals [17]. Vacuoles have a central role in degradation of cysteine to release H₂S, as impairment of vacuolar H⁺-ATPase (V-ATPase) activity, responsible for vacuolar acidification, negatively affects H₂S production [26]. This led us to evaluate if H₂S production is altered in *ncr1Δ* cells, using lead acetate paper strips [17]. The results show that cells lacking Ncr1 exhibited a decreased H₂S production (Figure 1A).

Table 2. Identification of transcription factors (top 10) that may regulate the changes in vacuolar proteins observed in *ncr1Δ* cells.

Transcription factor	Gcn4	Yap1	Pdr3	Rpn4	Pdr1	Met28	Met31	Cbf1	Met32	Bas1
Number of genes	45	45	44	44	41	39	39	37	37	26
List of genes	VPS73	VPS73	VPS73	VPS73	VPS73	VPS73	VPS73	YNL058C	VPS73	VPS73
	YNL058C	YNL058C	YNL058C	YNL058C	OPY2	OPY2	YNL058C	OPY2	YNL058C	YNL058C
	OPY2	OPY2	OPY2	OPY2	EMP70	EMP70	OPY2	EMP70	OPY2	PEP12
	EMP70	EMP70	EMP70	EMP70	MNT2	PEP12	EMP70	MNT2	EMP70	YCK3
	MNT2	MNT2	MNT2	MNT2	PEP12	VAM3	MNT2	PEP12	MNT2	NYV1
	PEP12	PEP12	PEP12	PEP12	VAM3	YCK3	VAM3	VAM3	PEP12	VTC5
	VAM3	VAM3	VAM3	VAM3	YCK3	NYV1	YCK3	YCK3	VAM3	SCP160
	YCK3	YCK3	YCK3	YCK3	NYV1	VTC5	NYV1	NYV1	VTC5	YDL180W
	NYV1	NYV1	NYV1	NYV1	VTC5	MYO2	VTC5	VTC5	MYO2	MCD4
	VTC5	VTC5	VTC5	VTC5	MYO2	IML1	SCP160	MYO2	SCP160	MCH1
	MYO2	MYO2	MYO2	MYO2	SCP160	MCD4	IML1	SCP160	IML1	PEP8
	SCP160	SCP160	SCP160	SCP160	YDL180W	MCH1	YDL180W	IML1	YDL180W	ZRC1
	IML1	IML1	IML1	IML1	MCD4	PEP8	MCD4	MCD4	MCD4	YPT52
	YDL180W	YDL180W	YDL180W	YDL180W	MCH1	ZRC1	MCH1	MCH1	MCH1	PMC1
	MCD4	MCD4	MCD4	MCD4	ZRC1	YPT52	PEP8	PEP8	PEP8	ATG22
	MCH1	MCH1	PEP8	PEP8	YPT52	FUN26	ZRC1	ZRC1	ZRC1	LSB6
	PEP8	PEP8	ZRC1	ZRC1	FUN26	YBT1	YPT52	YPT52	YPT52	YGL114W
	ZRC1	ZRC1	YPT52	YPT52	YBT1	PMC1	FUN26	FUN26	YBT1	FRE8
	YPT52	YPT52	FUN26	FUN26	PMC1	YPT10	YBT1	YBT1	ATG22	PFF1
	FUN26	FUN26	YBT1	YBT1	ATG22	LSB6	PMC1	PMC1	YPT10	YNL115C
	YBT1	YBT1	PMC1	PMC1	YPT10	YGL114W	ATG22	YPT10	LSB6	FTH1
	PMC1	PMC1	ATG22	ATG22	LSB6	VPS30	YPT10	LSB6	YGL114W	SSA1
	ATG22	ATG22	YPT10	YPT10	YGL114W	FRE8	LSB6	YGL114W	FET5	VVS1
	YPT10	YPT10	LSB6	LSB6	FET5	PFF1	YGL114W	FET5	VPS30	ENB1

LSB6	LSB6	YGL114W	YGL114W	VPS30	SEC17	FRE8	VPS30	FRE8	IVY1
YGL114W	YGL114W	FET5	FET5	FRE8	YAR028W	PFF1	FRE8	SEC17	AMS1
FET5	FET5	VPS30	VPS30	PFF1	YNL115C	SEC17	SEC17	YNL115C	
VPS30	VPS30	FRE8	FRE8	SEC17	FTH1	YAR028W	YAR028W	FTH1	
FRE8	FRE8	PFF1	PFF1	YAR028W	VMA2	YNL115C	FTH1	VMA2	
PFF1	PFF1	SEC17	SEC17	FTH1	SMF3	FTH1	SMF3	SMF3	
SEC17	SEC17	YAR028W	YAR028W	VMA2	SLM4	VMA2	SLM4	VHC1	
YAR028W	YAR028W	YNL115C	YNL115C	SMF3	VHC1	SMF3	VHC1	SSA1	
YNL115C	YNL115C	FTH1	FTH1	SLM4	SSA1	SLM4	SSA1	VPS33	
FTH1	FTH1	VMA2	VMA2	VHC1	FAT1	VHC1	FAT1	VVS1	
VMA2	VMA2	SMF3	SMF3	SSA1	VPS33	SSA1	VPS33	ENB1	
SMF3	SMF3	SLM4	SLM4	FAT1	VVS1	FAT1	VVS1	IVY1	
SLM4	SLM4	VHC1	VHC1	VPS33	ENB1	ENB1	AMS1	AMS1	
VHC1	VHC1	SSA1	SSA1	VVS1	IVY1	IVY1			
SSA1	SSA1	FAT1	FAT1	ENB1	AMS1	AMS1			
FAT1	FAT1	VPS33	VPS33	IVY1					
VPS33	VPS33	VVS1	VVS1	AMS1					
VVS1	VVS1	ENB1	ENB1						
ENB1	ENB1	IVY1	IVY1						
IVY1	IVY1	AMS1	AMS1						
AMS1	AMS1								

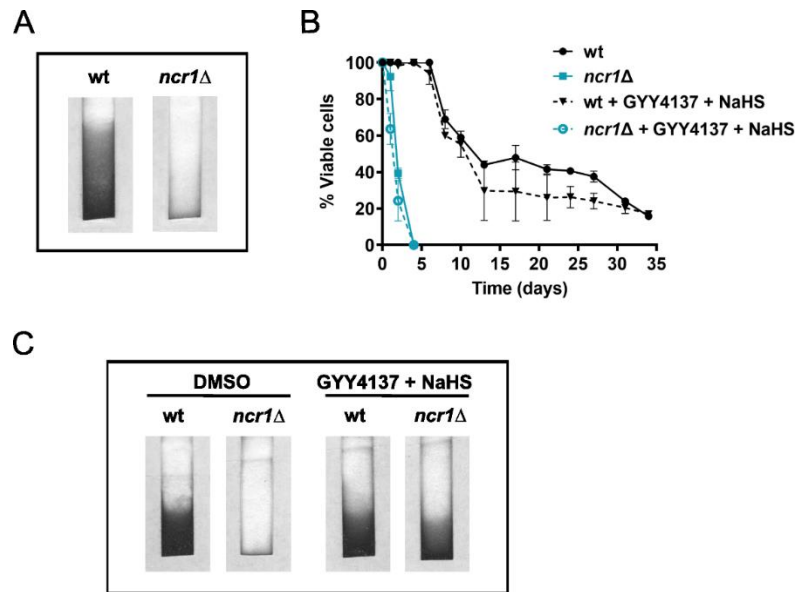


Figure 1. *NCR1* deletion negatively affects H₂S production. (A) H₂S production was detected by black lead sulfide accumulation on lead acetate strips placed on the top of the culture flasks. A representative result is shown (n=2). (B) Cells were inoculated at OD_{600nm}=0.2 in SC medium supplemented with 100 μ M GYY4137 (dissolved in DMSO) and 5 μ M NaHS was added at 6 h, 24 h and 48 h after inoculation (wt + GYY4137 + NaHS, *ncr1* Δ + GYY4137 + NaHS). As control, cells were incubated with DMSO (wt, *ncr1* Δ). Cellular viability was expressed as the percentage of the CFUs in relation to day 0. Data are the mean $\pm$ SEM (n=2). (C) H₂S production was detected as in (A) in cells grown as in (B). A representative result is shown (n=2).

To test if decreased H₂S production is contributing to the shortened CLS of *ncr1* Δ cells, we treated cells with H₂S-donors and its effect on CLS was analyzed (Figure 1B). By using a combination of a slow H₂S-releasing donor, GYY4137, and fast H₂S releasing donor, NaHS, we were able to increase H₂S levels in *ncr1* Δ cells (Figure 1C), but it was not sufficient to increase its CLS (Figure 1B). Treatment with H₂S-donors also did not extend the CLS of wild type cells, contrary to previous reports [17,25]. This may reflect differences in growth conditions. However, these results suggest that decreased H₂S production is not associated with the premature aging in cells lacking Ncr1.

3.3.2 Autophagy impairment in *ncr1* Δ cells is not linked to Snf1 inhibition

The Snf1/AMPK protein kinase is involved in the regulation of multiple cellular processes, including the induction of autophagy, by inhibiting TORC1 through phosphorylation of one of its subunits, Kog1, and by directly phosphorylating Atg1 [27,28]. As the activity of Sit4, a negative regulator of Snf1 [29], is increased in *ncr1* Δ cells [14], we postulated that Snf1 activity may be decreased in *ncr1* Δ mutant, contributing to autophagy impairment. To test this hypothesis, Snf1 phosphorylation at T210 was assessed by immunoblotting. The results shown that the levels of phospho-Snf1 increased in wild type

cells grown for the PDS phase comparing to the exponential phase, as expected (Figure 2A). However, deletion of *NCR1* increased the levels of phospho-Snf1 at PDS phase (Figure 2A,B), suggesting that Snf1 is in fact more active and that the autophagic flux is impaired in a Snf1-independent manner.

Despite Snf1 activity being required for the induction of autophagy, high Snf1 activity may also have a negative impact on autophagy by inhibiting the activity of acetyl-CoA carboxylase (Acc1), which is required for fatty acid synthesis [30] and for autophagy [31]. This led us to hypothesize that Snf1 activation in *ncr1* Δ cells may decrease Acc1 activity, consequently compromising the autophagy flux. To investigate this, we assessed the CLS and autophagy induction in cells expressing a constitutively active form of Acc1 with two site mutations at Ser-659 and Ser-1157 (Acc1-S659A,S1157A), which prevent Acc1 phosphorylation by Snf1 [32]. Our results show that Acc1 activation was not sufficient to restore the autophagic flux in cells lacking Ncr1 (Figure 2C) or increase its CLS (Figure 2D). These results suggest that autophagy impairment in *ncr1* Δ cells did not result from Snf1-inhibition of Acc1.

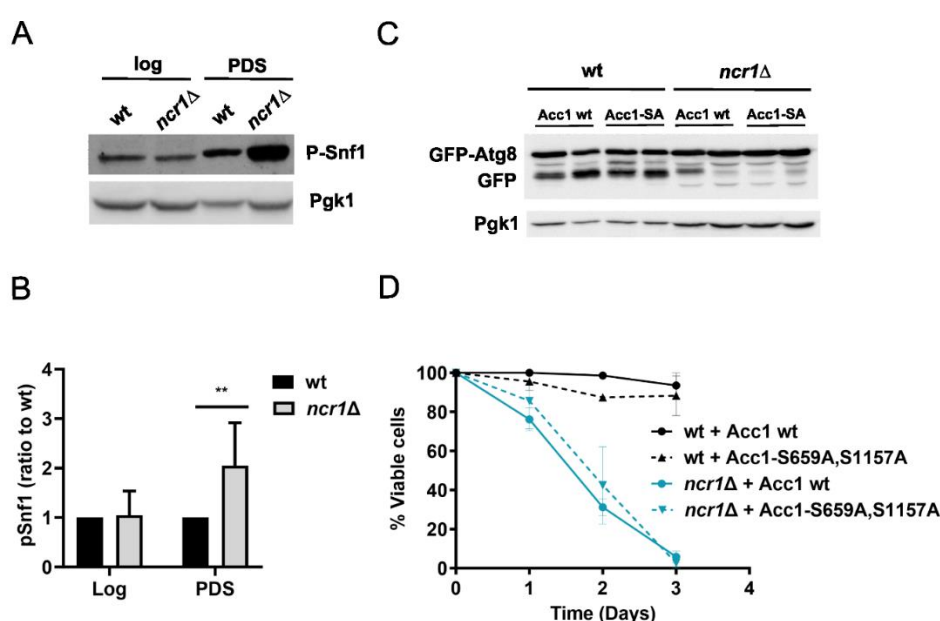


Figure 2. Snf1 activity increases in *ncr1* Δ cells at post-diauxic (PDS) phase. (A) Cells were grown to the exponential (log) and PDS (24 h after log) phases, and Snf1 phosphorylation at T210 analyzed by immunoblotting. Pgk1 was used as loading control. A representative image of at least three independent experiments is shown. (B) Phosphorylation of Snf1-T210 normalized to wt levels is shown (mean $\pm$ SD; t-test: **, $p \leq 0.01$). (C) Cells carrying pSP-GM2-ACC1^{wt} (Acc1 wt) or pSP-GM2-ACC1^{Ser659Ala,Ser1157Ala} (Acc1-SA) were grown to PDS phase (24 h after log) and vacuolar delivery of GFP-Atg8 analyzed by immunoblotting using anti-GFP antibody. Pgk1 was used as loading control. The image shows the results from 2 independent samples. (D) Cells carrying pSP-GM2-ACC1^{wt} or pSP-GM2-ACC1^{Ser659Ala,Ser1157Ala} were grown to the PDS (24 h after log) phase and maintained overtime in the growth medium. Cellular viability was expressed as the percentage of the CFUs in relation to day 0. Data are the mean $\pm$ SEM (n=3).

3.4. Discussion

Recently we identified alterations in the levels of several vacuolar membrane proteins in Ncr1 deficient cells [15]. Here, we conducted an in-silico analysis to predict potential TFs responsible for these alterations. These included a group of TFs (Cbf1, Met28, Met31 and Met32) associated with sulfur metabolism regulation [19–21]. A key product of the pathway regulated by these TFs is H₂S, which plays important physiological functions [22]. Poor production of H₂S is associated with neurodegenerative diseases, whereas increased H₂S production is associated with extended lifespan in several organisms [17,23–25]. Accordingly, our findings revealed that cells lacking Ncr1 exhibited decreased H₂S production. This could potentially be attributed to alterations in vacuolar function of *ncr1Δ* cells, as vacuoles play a crucial role in H₂S production through the degradation of cysteine [26]. Despite the known influence of H₂S on lifespan, our results suggest that the observed decrease in H₂S production in *ncr1Δ* cells is not associated with their shortened lifespan. Indeed, H₂S donors did not increase *ncr1Δ* CLS.

Our previous studies also demonstrated that the disruption of autophagy due to iron overload contributes to the shortened CLS of cells lacking Ncr1 [15]. Furthermore, we found that this occurs independently of TORC1, a negative regulator of autophagy. In this study we show that autophagy impairment is also not linked to the inhibition of Snf1/AMPK, a positive regulator of autophagy [27,28], as Snf1 activity even increased in *ncr1Δ* cells at the PDS phase. Our results also provide evidence that the defects in the autophagic flux do not result from inhibition of Acc1, which catalyzes the limiting step in the biosynthesis of lipids required for autophagy [30,31]. In sum, our results suggest that Snf1 does not contribute to autophagy impairment in *ncr1Δ* cells. Further studies will be required to elucidate the molecular mechanism leading to iron-associated autophagy inhibition in the yeast model of NPC1.

3.5. Acknowledgments

We are grateful to Paula Ludovico (ICVS, Universidade do Minho, Portugal) for generously providing the pRS416-*GFP-ATG8* plasmid and Jens Nielsen (BioInnovation Institute, Technical University of Denmark) for kindly providing the plasmids pSP-GM2-*ACC1^{wt}* and *c*pSP-GM2-*ACC1^{Ser659Ala}*.

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CHAPTER IV

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

This chapter is based on the following published article:

Telma S. Martins, Miguel Correia, Denise Pinheiro, Carolina Lemos, Marta V. Mendes, Clara Pereira and Vítor Costa. Cells. 2024 Apr 9;13(8):655. doi: 10.3390/cells13080655. PMID: 38667270; PMCID: PMC11049076.

Abstract

The Sit4 protein phosphatase plays a key role in orchestrating various cellular processes essential for maintaining cell viability during aging. We have previously shown that *SIT4* deletion promotes vacuolar acidification, mitochondrial derepression, and oxidative stress resistance, increasing yeast chronological lifespan. In this study, we performed a proteomic analysis of isolated vacuoles and yeast genetic interaction analysis to unravel how Sit4 influences vacuolar and mitochondrial function. By employing high-resolution mass spectrometry, we show that *sit4Δ* vacuolar membranes were enriched in Vps27 and Hse1, two proteins that are part of the endosomal sorting complex required for transport-0. In addition, *SIT4* exhibited a negative genetic interaction with *VPS27*, as *sit4Δvps27Δ* double mutants had a shortened lifespan compared to *sit4Δ* and *vps27Δ* single mutants. Our results also show that Vps27 did not increase *sit4Δ* lifespan by improving protein trafficking or vacuolar sorting pathways. However, Vps27 was critical for iron homeostasis and mitochondrial function in *sit4Δ* cells, as *sit4Δvps27Δ* double mutants exhibited high iron levels and impaired mitochondrial respiration. These findings show, for the first time, crosstalk between Sit4 and Vps27, providing new insights into the mechanisms governing chronological lifespan.

Keywords: Sit4; Vps27; vacuolar trafficking; mitochondria; iron; chronological aging

4.1 Introduction

Lysosomes and lysosome-like vacuoles in yeast serve as pivotal hubs for maintaining cellular homeostasis, metabolism, and lifespan. These organelles are enriched in carrier proteins and hydrolases, such as proteases, glycosidases, lipases, nucleases, phosphatases, and sulfatases. The concentration of these hydrolases and the vacuolar H⁺-ATPase-dependent maintenance of an acidic pH render lysosomes a major catabolic center [1–5]. Notably, lysosomal dysfunction is a hallmark of aging common to several age-related diseases, including cancer and neurodegenerative and metabolic disorders [1,6]. The decline in lysosomal function during aging has been linked to the dysregulation of nutrient signaling pathways, an increase in lysosomal pH, and a decrease in autophagy [1,7–11]. Notably, lifespan extension by dietary restriction involves the modulation of nutrient signaling pathways, which in turn depend at least in part on lysosome function in several organisms [7,12–18].

Lysosomes degrade both intracellular and exogenous components, from macromolecules to organelles, such as mitochondria, peroxisomes, or lipid droplets, and even surface receptors and pathogens. The major pathways of substrate delivery to lysosomes are autophagy and the endocytic pathway [2,19]. For example, during macroautophagy (hereafter named autophagy), a double membrane is formed around cytoplasmic material targeted for degradation, leading to the generation of autophagosomes that ultimately merge with lysosomes [20,21]. Conversely, mitophagy allows the selective clearance of mitochondria [22]. Autophagy is highly conserved during evolution and serves critical roles in ensuring cell survival [23,24]. It maintains protein and organelle quality control by removing damaged material that is generated under stress conditions and during aging or diseases. In addition, it allows cells to survive under nutrient deprivation by recycling macromolecules, to provide amino acids, sugars, fatty acids, nucleotides, and other nutrients. The endocytic pathway plays a vital role in regulating cellular functions and immune responses by delivering extracellular and cell surface cargos to lysosomes via endocytosis [25,26]. Internalized proteins localized at early endosomes undergo sorting, being either targeted for degradation in lysosomes or recycled to other compartments (e.g., the Golgi apparatus and the plasma membrane). Proteins destined for degradation are ubiquitinated, allowing their recognition by the endosomal sorting complex required for transport (ESCRT), which facilitates its sorting via the multivesicular body (MVB) pathway to the lysosome. Conversely, proteins that are not ubiquitinated follow the recycling pathway [27,28].

Trafficking pathways play a vital role in the function and biogenesis of vacuoles. In eukaryotic cells, organellar proteins are produced in the endoplasmic reticulum (ER), moved

to the Golgi apparatus, and subsequently sorted into their cellular compartments. In yeast, proteins destined for the vacuole can follow three routes: the carboxypeptidase Y (CPY) pathway (indirectly, through late endosomes/MVB), the alkaline phosphatase (ALP/AP-3) pathway [26,29], or the cytoplasm to vacuole targeting (Cvt) pathway, which uses much of the autophagic machinery to transport hydrolases, such as α -mannosidase (Ams1) and aminopeptidase I (Ape1), from the cytoplasm to the vacuole [30].

Apart from their essential function in degradation, lysosomes also serve as storage site for metabolites derived from the diet (e.g., amino acids, sugars, lipids, nucleotides, and ions) and play a central role in regulating nutrient sensing and signaling pathways [4,5]. For instance, vacuolar iron storage is particularly important to prevent oxidative stress, as it is a redox-active metal [31].

Targeting lysosomes to optimize their function is emerging as a means to promote a healthier lifespan [1,3,6,7,11]. This promising strategy requires a deeper comprehension of the molecular cues driving the decline in lysosomal function with age and its improvement under lifespan extension conditions. We have previously shown that *SIT4* deletion increases yeast chronological lifespan (CLS) by promoting vacuolar acidification as well as mitochondrial derepression and oxidative stress resistance [32–35]. Sit4 is a PP2A-like Ser/Thr protein phosphatase, a homologue of the mammalian protein phosphatase 6 (PP6) [36], which is also involved in the regulation of the cell cycle and budding [37,38], cell wall integrity, actin cytoskeleton organization and ribosomal gene expression mediated by Pkc1 function [39], nutrient signaling [40,41], carbohydrate and lipid metabolism [42,43], pH and monovalent ion homeostasis [44], and ER-to-Golgi trafficking [45]. Sit4 acts as the catalytic subunit of several protein complexes, formed by its association with distinct regulatory subunits, explaining its role across a broad array of cellular functions. For instance, Sit4-associated proteins (SAPs), namely Sap4, Sap155, Sap185, or Sap190, bind Sit4 independently to positively regulate cell cycle progression at the G1 phase [46]. Sit4 can also form a ceramide-activated protein phosphatase complex by interacting with regulatory subunits Tpd3 and Cdc55 [47]. In addition, Sit4 interacts with and is inhibited by Tap42 in response to nutrient signals through TORC1 [40]. The dissociation of the Tap42-Sit4 complex results in Sit4 activation and dephosphorylation of the GATA-binding transcription factor Gln3, which is then translocated into the nucleus where it activates the nitrogen catabolite repression pathway [48].

In this study, we assessed how Sit4 deficiency impacts vacuoles by performing a proteomic analysis of vacuolar membranes from wild-type and *sit4* Δ cells and investigated the role of Vps27 in regulating *sit4* Δ CLS. Our findings reveal that Vps27 is enriched in the vacuolar membranes of *sit4* Δ cells and is crucial for lifespan extension by modulating mitochondrial function.

4.2. Materials and methods

4.2.1 Yeast strains and growth conditions

The *S. cerevisiae* strains utilized in this study are detailed in Table 1. Yeast cells were cultured aerobically at 26 °C on an orbital shaker set at 140 rpm, maintaining a flask volume to medium volume of 5:1. Synthetic complete (SC) medium was used as a growth medium, comprising drop-out, 2% (w/v) glucose (Thermo Fisher Scientific, Waltham, MA, USA), and 0.67% (w/v) yeast nitrogen base without amino acids (BD BioSciences, San Jose, CA, USA), supplemented with the required amino acids and nucleotides. For BY4741 strains, SC medium was supplemented with histidine (0.008% (w/v), Sigma Aldrich, St. Louis, MO, USA), methionine (0.038% (w/v), Sigma Aldrich, St. Louis, MO, USA), leucine (0.04% (w/v), Sigma Aldrich, St. Louis, MO, USA), and uracil (0.008% (w/v), Sigma Aldrich, St. Louis, MO, USA). For BY4742 strains, SC medium was supplemented with histidine (0.008% (w/v), Sigma Aldrich, St. Louis, MO, USA), lysine (0.008% (w/v), Sigma Aldrich, St. Louis, MO, USA), leucine (0.04% (w/v), Sigma Aldrich, St. Louis, MO, USA), and uracil (0.008% (w/v), Sigma Aldrich, St. Louis, MO, USA). To assess Mup1-GFP levels in response to methionine, BY4742 cells were cultured in minimal medium (MM), containing 2% (w/v) glucose (Thermo Fisher Scientific, Waltham, MA, USA) and 0.67% (w/v) yeast nitrogen base without amino acids (BD BioSciences, San Jose, CA, USA), supplemented with amino acids or nucleotides [0.008% (w/v) histidine (Sigma Aldrich, St. Louis, MO, USA), 0.008% (w/v) lysine (Sigma Aldrich, St. Louis, MO, USA), 0.04% (w/v) leucine (Sigma Aldrich, St. Louis, MO, USA), and 0.008% (w/v) uracil (Sigma Aldrich, St. Louis, MO, USA)]. Furthermore, 38 mg L⁻¹ of L-methionine (Thermo Fisher Scientific, Waltham, MA, USA) was added to cell cultures.

To assess the growth in respiratory medium, cells were grown in YPD [1% (w/v) yeast extract (Liofilchem, Roseto degli Abruzzi, Italy), 2% (w/v) peptone (Liofilchem, Roseto degli Abruzzi, Italy), and 2% (w/v) glucose (Thermo Fisher Scientific, Waltham, MA, USA)] or YPG [1% (w/v) yeast extract (Liofilchem, Roseto degli Abruzzi, Italy), 2% (w/v) peptone (Liofilchem, Roseto degli Abruzzi, Italy), and 3% (v/v) glycerol (Thermo Fisher Scientific, Waltham, MA, USA)] supplemented with 1.5% (w/v) agar (Liofilchem, Roseto degli Abruzzi, Italy).

The deletion of *VPS27* was carried out using a deletion fragment encompassing *KanMx4* or *MXHIS3* and *VPS27* flanking regions. Yeast cells were transformed by the lithium acetate/single-stranded carrier DNA/PEG method [51], and gene deletion was validated using standard PCR procedures.

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

Strain	Genotype	Source
BY4741 ^{b,e,f}	Mata, <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>met15Δ0</i> , <i>ura3Δ0</i>	EUROSCARF
<i>sit4Δ</i> ^{b,e,f}	BY4741 <i>sit4::MXHIS3</i>	[34]
<i>vps27Δ</i> ^{a,b,e,f}	BY4741 <i>vps27::KanMX4</i>	This study
<i>sit4Δvps27Δ</i> ^{a,b,e,f}	BY4741 <i>sit4::MXHIS3 vps27::KanMX4</i>	This study
<i>pep4Δ</i>	BY4741 <i>pep4::KanMX4</i>	EUROSCARF
<i>pho8Δ</i> ^c	BY4741 <i>pho8::HPH</i>	[49]
<i>sit4Δpho8Δ</i> ^c	BY4741 <i>sit4::KanMX4 pho8::HPH</i>	[49]
<i>vps27Δpho8Δ</i> ^c	BY4741 <i>vps27::MXHIS3 pho8::HPH</i>	This study
<i>sit4Δvps27Δpho8Δ</i> ^c	BY4741 <i>sit4::KanMX4 vps27::MXHIS3 pho8::HPH</i>	This study
BY4742 ^d	Mata <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>lys2Δ0</i> , <i>ura3Δ0</i>	EUROSCARF
<i>sit4Δ</i> ^d	BY4742 <i>sit4::KanMX4</i>	EUROSCARF
<i>vps27Δ</i> ^d	BY4742 <i>vps27::MXHIS3</i>	This study
<i>sit4Δvps27Δ</i> ^d	BY4742 <i>sit4::KanMX4 vps27::MXHIS3</i>	This study
BY4741 Rho0	BY4741 Rho0	[50]

Cells harboring ^aYCpHAC33-*Prom-VPS27-3xHA*, ^bpRS416-*GFP-ATG8*, ^cpYX242-*mtPho*, ^dpRS416-*MUP1-GFP*, ^epRS426-*GFP-PHO8*, and ^fpVT100U-*preSU9-GFP* are indicated.

4.2.2 Isolation of vacuolar membranes and proteomic analysis by HPLC-MS/MS

Cells were cultured to the late exponential (Log) phase ($OD_{600nm} \approx 2$) and vacuolar membranes were isolated as described [52,53]. Samples were processed for proteomics analysis and proteins were identified and quantified by mass spectrometry (nanoLC-MS/MS), as described [54,55]. Proteomic datasets were analyzed using Proteome Discoverer 2.5.0.400 software (Thermo Fisher Scientific, Waltham, MA, USA), the UniProt database for the *S. cerevisiae* Proteome 2020_03, and a contaminant database from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry, Munich, Germany). The levels of peptides were normalized based on total peptide levels of annotated vacuolar membrane proteins.

To assess group differences in protein levels, the homogeneity of variances was evaluated using the Levene test, and pairwise comparisons were conducted using Student's t-test. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS), version 26 (IBM Corp., Armonk, NY, USA), with the significance levels set at $\alpha = 0.05$. For the statistical analysis of the overrepresentation of functional groups, the Gene Ontology (GO) database (6 March 2023 and 10.5281/zenodo.7709866) was utilized.

4.2.3 Chronological lifespan

CLS was evaluated as described [56]. To study the effect of iron chelation on CLS, the growth medium was supplemented with 80 μ M bathophenanthrolinedisulfonate (BPS; disodium salt hydrate; Sigma Aldrich, St. Louis, MO, USA). Cells were grown overnight in SC medium, supplemented or not with BPS, diluted to an $OD_{600nm} = 0.2$ in SC medium and further grown for 24 h (considered time zero, t_0 , in the lifespan assay). These cultures were kept at 26 °C, and cell viability was assessed over time using standard dilution plate counts on YPD medium supplemented with 1.5% (w/v) agar (Liofilchem, Roseto degli Abruzzi, Italy). Following incubation at 26 °C for 2–3 days, colonies were counted and the percentage of the colony-forming units (CFUs) relative to t_0 was determined.

4.2.4 Glucose and ethanol determination

Cells were grown in SC medium, and samples from the extracellular medium were taken at 12, 15, 24, and 48 h of growth after the Log phase. Glucose levels were quantified using the D-Glucose GOD-POD kit (Nzytech, Lisbon, Portugal) following the method described in the manufacturer's instructions. For ethanol determination, samples were diluted 100-fold with H_2O , and ethanol levels were quantified using the Enzytec Liquid Ethanol kit (r-biopharm, Darmstadt, Germany) following the method described in the manufacturer's instructions.

4.2.5 Glycogen measurement

Cells were grown for 3 days in YPD plates and glycogen accumulation was qualitatively measured by exposing yeast colonies to iodine crystals. Yeast colonies develop a brown coloration that reflects their glycogen content [57].

4.2.6 Western blotting

Cells were collected at the Log ($OD_{600nm} \approx 0.6$), late Log ($OD_{600nm} \approx 2$), and post-diauxic shift (PDS; 24 h after Log) phases, as indicated in figure legends. For the analysis of microautophagy induction, Log phase cells treated for 4 h with 200 ng mL^{-1} of rapamycin were used as positive control. Proteins were extracted using 0.1 M NaOH (Merck, Darmstadt, Germany), dissolved in Laemmli buffer, and quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), as described [53]. Protein samples were prepared by adding 5% (v/v) 2-mercaptoethanol (Merck, Darmstadt, Germany), separated by SDS-PAGE, and transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). For Mup1-GFP detection, cells were vortexed in Laemmli buffer in the presence of zirconium beads and then incubated at 60 °C for 10 min. To assess

Mup1-GFP levels in response to methionine, proteins were extracted as previously reported [58]. Cell lysis was performed by incubating the cell pellets in 0.5 mL of 0.2 M NaOH (Merck, Darmstadt, Germany), 0.2% (v/v) 2-mercaptoethanol (Merck, Darmstadt, Germany) for 10 min on ice and adding 5% (w/v) trichloroacetic acid (Thermo Fisher Scientific, Waltham, MA, USA), followed by a 10 min incubation on ice. The pellet was collected by centrifugation, washed twice with acetone (Vidrolab 2, Gandra, Portugal), suspended in Laemmli buffer plus zirconium beads, vortexed, and then incubated for 10 min at 37 °C.

Immunodetection was performed using mouse anti-HA (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA, sc-7392), mouse anti-GFP (1:5000; Roche, Basel, Switzerland, 11814460001), mouse anti-CPY (1:2000; Invitrogen, Waltham, MA, USA, A-6428), goat anti-Apel (1:200; Santa Cruz Biotechnology, Dallas, TX, USA, sc-26740), mouse anti-Por1 (1:5000; Invitrogen, Waltham, MA, USA, 459500), rabbit anti-Atp2 (1:2000; Abcam, Cambridge, UK, ab128743), or mouse anti-Cox2 (1:7000; Invitrogen, Waltham, MA, USA, 4B12A5) as primary antibodies, and anti-rabbit IgG-peroxidase (1:5000; Sigma Aldrich, St. Louis, MO, USA), anti-mouse IgG-peroxidase (1:5000; Molecular Probes, Eugene, OR, USA), or anti-goat IgG-peroxidase (1:5000; Sigma Aldrich, St. Louis, MO, USA) as secondary antibodies. The primary antibodies used to probe the loading controls were mouse anti-Hxk2 (1:100,000, Rockland, Philadelphia, PA, USA, 200-4159) or mouse anti-GAPDH (1:30,000, Proteintech, Sankt Leon-Rot, Germany, 60004-1). The secondary antibody was anti-mouse IgG-peroxidase (1:5000, Molecular Probes, Eugene, OR, USA). Immunodetection was performed by chemiluminescence (ECL, Advansta, San Jose, CA, USA).

4.2.7 Colony immunoblot assay

Yeast cultures were diluted to OD_{600nm} of 0.15 using phosphate-buffered saline (PBS, 137 mM NaCl (Thermo Fisher Scientific, Waltham, MA, USA), 2.7 mM KCl (Merck, Darmstadt, Germany), 10 mM Na₂HPO₄ (Panreac, Barcelona, Spain), 1.8 mM KH₂PO₄ (Panreac, Barcelona, Spain), pH 7.4), and 13.3 µL of culture was spotted onto a SC glucose plate. Upon 72 h of incubation at 26 °C, colonies were covered with a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). After 24 h at 26 °C, membranes were removed and washed with TBS-0.05% (v/v) Tween (Sigma Aldrich, St. Louis, MO, USA) solution to clear away all cells that remained attached. Detection of CPY secretion was carried out by immunoblotting, with mouse anti-CPY primary antibody (1:2000, Invitrogen, Waltham, MA, USA) and anti-mouse IgG-peroxidase secondary antibody (1:5000, Molecular Probes, Eugene, OR, USA).

4.2.8 Iron levels

Total iron levels were quantified in yeast cells (6×10^8 cells mL⁻¹) grown in SC glucose medium to late Log (OD_{600nm} $\approx$ 2) and PDS phases, using a colorimetric assay, as previously described [59].

4.2.9 Oxidative stress resistance

For the analysis of oxidative stress resistance, cells were grown to Log phase in SC medium supplemented or not with 80 μ M BPS, and then treated with 1.5 mM hydrogen peroxide (H₂O₂; Merck, Darmstadt, Germany) for 1 h. Cell viability was determined as described above.

4.2.10 Growth analysis

Respiratory capacity was assessed by evaluating cell growth in glycerol medium. Yeast cultures were diluted to OD_{600nm} = 0.1, and four ten-fold serial dilutions were prepared in PBS. Then cells were spotted onto YPD (glucose) or YPG (glycerol) containing 1.5% (w/v) agar (Liofilchem, Roseto degli Abruzzi, Italy), and the plates were incubated at 26 °C for 2–3 days. To evaluate iron sensitivity, four ten-fold serial dilutions of each culture were spotted onto SC plates supplemented or not with iron(II) sulfate heptahydrate (10 mM, 20 mM or 25 mM; Sigma Aldrich, St. Louis, MO, USA), previously dissolved in citrate buffer [81.9 mM sodium citrate (Sigma Aldrich, St. Louis, MO, USA), 18.1 mM Citric acid (Merck, Darmstadt, Germany)], and the plates were incubated for 3 days at 26 °C.

4.2.11 Oxygen consumption

Oxygen consumption rate (OCR) was measured using a Clark-type oxygen electrode coupled to an Oxygraph plus system (Hansatech, Norfolk, UK), as described [53].

4.2.12 Alkaline phosphatase assay

For the analysis of alkaline phosphatase activity, protein extracts were prepared and incubated with nitrophenylphosphate (Sigma Fast, Sigma Aldrich, St. Louis, MO, USA), as described [49].

4.2.13 Mitochondrial mass

The mitochondrial mass was evaluated in cells expressing GFP fused to the mitochondrial pre-sequence preSU9 (pVT100U-*preSU9-GFP*), which has low sensitivity to the mitochondrial membrane potential [60,61]. Cells were grown to Log phase, and GFP fluorescence was assessed by flow cytometry using a BD Accuri C6 (20,000 events on the

FL1 channel) and the FlowJo v10.8.1 software version (BD Life Sciences, Franklin Lakes, NJ, USA).

4.2.14 Analysis of mitochondrial DNA by qPCR

Cells were grown to Log phase in SC medium, and total DNA isolated using the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, Waltham, MA, USA), following the method described in the manufacturer's instructions. The concentration and purity of all DNA stock solutions were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and DNA integrity checked on agarose gels. For each sample, the total DNA concentration was normalized to 5 ng/μL. For qPCR amplifications, 2 μL of template DNA (dilution 1/250) was used with the primer pairs (0.25 μM of each primer) listed in Table 2 and 10 μL of iTaq Universal SYBR Green supermix (Bio-Rad, Hercules, CA, USA). qPCR tests were performed using an iCycler iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with the following settings: 95 °C for 3 min; 45 cycles of 95 °C for 15 s, 55 °C for 30 s and 60 °C for 30 s. The relative efficiency and quality of each primer pair was assessed using a 5-fold serial dilution series of the DNA (1/10, 1/50, 1/250, 1/1250, and 1/6250). Negative controls (non-template controls) were included in all qPCR tests. To exclude the formation of nonspecific products, a melting curve analysis was performed at the end of each qPCR. qPCR analysis included two independent biological replicates and four technical replicates for each DNA sample. The data obtained were analyzed using the method described by Pfaffl [62] and the CFX Maestro 2.0 (v 5.0.021.0616) software (Bio-Rad, Hercules, CA, USA), to obtain the total levels of mitochondrial DNA (mtDNA) relative to nuclear DNA in the tested strains. *ACT1* was used as a reference for nuclear DNA, and *COX1* and *COX3* were used as references for mtDNA.

Table 2. Sequences of primers used in qPCR.

Primer	Sequence
COX1 fw	CTACAGATACAGCATTTCCAAGA
COX1 rv	GTGCCTGAATAGATGATAATGGT
COX3 fw	TTGAAGCTGTACAACCTACC
COX3 rv	CCTGCGATTAAGGCATGATG
ACT1 fw	GTATGTGTAAAGCCGGTTTTG
ACT1 rv	CATGATACCTTGGTGTCTTGG

4.2.15 Statistical analysis

Data were analyzed using GraphPad Prism Software v10.0.0 (GraphPad Software, Boston, MA, USA). Statistical comparisons were performed using one-way ANOVA, two-way ANOVA, or Student's t-test as appropriate. Significance levels were represented as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

4.3. Results

4.3.1 Vps27 is enriched in the vacuolar membranes and mediates the lifespan extension of cells lacking the phosphatase Sit4

To investigate the impact of Sit4 deficiency on yeast lysosome-like vacuoles, we conducted a proteomic analysis of vacuolar membranes, isolated as described [52,53] from both wild-type and *sit4Δ* cells grown in SC medium to $OD_{600nm} \approx 2$. We identified a set of 230 vacuolar membrane proteins, which represent approximately 80% of the entire vacuolar membrane proteome of *S. cerevisiae*. Proteins whose levels were altered in the *sit4Δ* vacuolar membranes are presented in Table S1. A volcano plot (Figure 1A) was used to represent the differences in protein levels. The results reveal that 22 proteins exhibited reduced abundance whereas 27 proteins showed increased abundance (Figure 1B) in the vacuolar membranes of yeast lacking Sit4. GO analysis on biological processes revealed an enrichment in proteins related to vacuole fusion and organization, late endosome to vacuole transport, autophagy, microautophagy, polyphosphate metabolism, and metal ion homeostasis (Figure 1C and Table S2). Among the proteins that increased the most in *sit4Δ* vacuolar membranes were Vps27 (4.1-fold) and Hse1 (2.2-fold), which form the endosomal sorting complex required for transport-0 (ESCRT-0).

The localization and levels of Vps27 are regulated by Snf1/AMPK, which promotes Vps27 translocation from endosomes to the vacuolar membranes at the PDS phase due to glucose depletion [63,64]. Notably, Snf1 is activated in *sit4Δ* cells and is critical for lifespan extension in this mutant strain [35]. To assess how *SIT4* deletion affects total Vps27 levels, *vps27Δ* and *sit4Δvps27Δ* cells expressing *VPS27* fused to HA, controlled by its endogenous promoter, were grown to late Log and PDS phases, and Vps27 levels on total protein extracts were assessed by Western blotting. In agreement with the published data [63], Vps27 levels decreased in wild-type cells during growth from the Log to the PDS phase. At the Log phase, total Vps27 levels were lower in the *sit4Δ* mutant in comparison to wild-type cells (Figure S1A,B). Overall, our results suggest that Vps27 is specifically enriched in the *sit4Δ* vacuolar membranes.

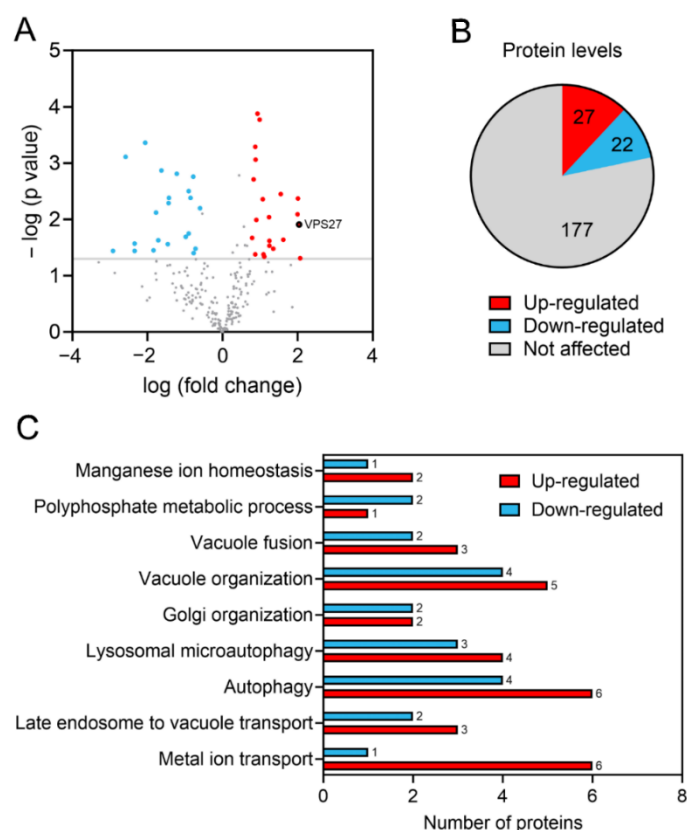


Figure 1. Proteomic analysis of *sit4Δ* vacuolar membranes. (A) Volcano plot for differentially expressed proteins in *sit4Δ* vs. wild-type samples. The log-transformed p-values (Student's t-test) are plotted in the vertical axis against the log-transformed fold change on the horizontal axis. The horizontal dashed line indicates a p-value of 0.05. Red dots represent proteins that are upregulated with a 1.5-fold change threshold, whereas blue dots represent proteins that are downregulated (log2 fold change of ± 0.58). Grey dots represent the unchanged proteins. Vps27 protein is identified with a red dot with a black outline. (B) Number of proteins significantly up- or downregulated in the *sit4Δ* vacuolar membranes. (C) GO term enrichment analysis on biological processes was conducted for proteins exhibiting statistical alterations in *sit4Δ* samples.

ESCRT-0 plays a key role in the late endosome to vacuole transport through the MVB sorting of ubiquitinated cargo [65,66]. In recent years, it was also implicated in the microautophagy of vacuolar membrane proteins [64]. Furthermore, a large-scale study suggests that *VPS27* deletion leads to a decrease in cell survival [67]. This led us to assess the impact of *VPS27* deletion on yeast CLS. Our results show that the loss of Vps27 had a mild negative impact on lifespan, but it abolished the lifespan extension phenotype of *sit4Δ* cells (Figure 2A,B). In fact, the longevity of *sit4Δvps27Δ* cells was even lower compared to that observed in *vps27Δ* cells, indicating a strong negative genetic interaction between *SIT4* and *VPS27*. Although *sit4Δ* and *sit4Δvps27Δ* cells have a slow growth phenotype (Figure S2A), it is unlikely that the differences between the CLS of the different strains are related to changes in cell growth or glucose metabolism. Indeed, 24 h after Log (considered t0 in the CLS assay due to the rapid loss of viability observed in *sit4Δvps27Δ* cells), all strains clearly entered the PDS phase, as shown by the growth curve, extracellular glucose (very

low or absent), and ethanol (produced at similar levels) (Figure S2A–C). Moreover, *sit4Δvps27Δ* cells did not exhibit defects in the accumulation of glycogen, a storage carbohydrate that increases in *sit4Δ* cells and is critical for CLS [68].

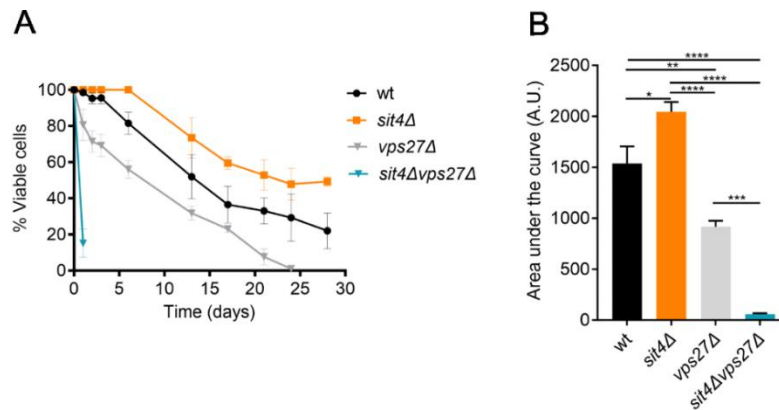


Figure 2. *SIT4* deletion extends lifespan in a *Vps27*-dependent manner. (A) Cells were grown to PDS phase (24 h after Log; t₀) and maintained over time in SC medium. Cellular viability was expressed as the percentage of CFUs (aged vs. t₀). Data are the mean ± SEM (n ≥ 3). (B) The area under each lifespan curve was calculated using GraphPad and expressed in arbitrary units (A.U.). Data are the mean ± SEM (n ≥ 3); * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; ****, p ≤ 0.0001; one-way ANOVA.

4.3.2 The pathways for vacuolar degradation are downregulated in *sit4Δ* cells in a *Vps27*-independent manner

Vps27 is involved in ubiquitinated cargo recognition and subsequently in the recruitment of ESCRT-I, facilitating the sorting of ubiquitinated MVB cargo [65,66]. During nutrient depletion, e.g., during cell growth to PDS and stationary phases, the MVB pathway promotes intracellular amino acid homeostasis and the upregulation of vacuolar hydrolases [69]. In fact, the MVB pathway synergistically cooperates with autophagy to promote cell longevity following nutrient depletion [69,70]. This led us to assess if the enrichment of Vps27 in the *sit4Δ* vacuolar membranes results in MVB pathway induction.

To assess the MVB pathway, yeast cells were transformed with *MUP1-GFP*, to express a plasma membrane methionine permease that is transported for degradation inside vacuoles in response to starvation or to high levels of methionine [71]. When Mup1-GFP is degraded inside vacuoles, free GFP accumulates due to its resistance to the lytic environment of vacuoles. Therefore, the induction of the MVB pathway can be assessed by Western blotting, i.e., by measuring the percentage of Mup1-GFP degradation (free GFP/(Mup1-GFP + GFP) ratio). As expected, during the transition from the Log to PDS phase, the MVB pathway was induced in wild-type but not in *vps27Δ* cells (Figure 3A,B), as it is dependent on the ESCRT machinery [69,72]. In *sit4Δ* cells, Mup1-GFP degradation was significantly reduced at the PDS phase, indicating that MVB pathway induction was

decreased. However, the addition of methionine increased Mup1-GFP degradation in *sit4Δ* cells to wild-type levels, in a Vps27-dependent manner (Figure S3), suggesting that Sit4 mediates MVB induction during cell growth but not during methionine supplementation. Overall, our data suggest that the MVB pathway does not contribute to lifespan extension in the *sit4Δ* mutant.

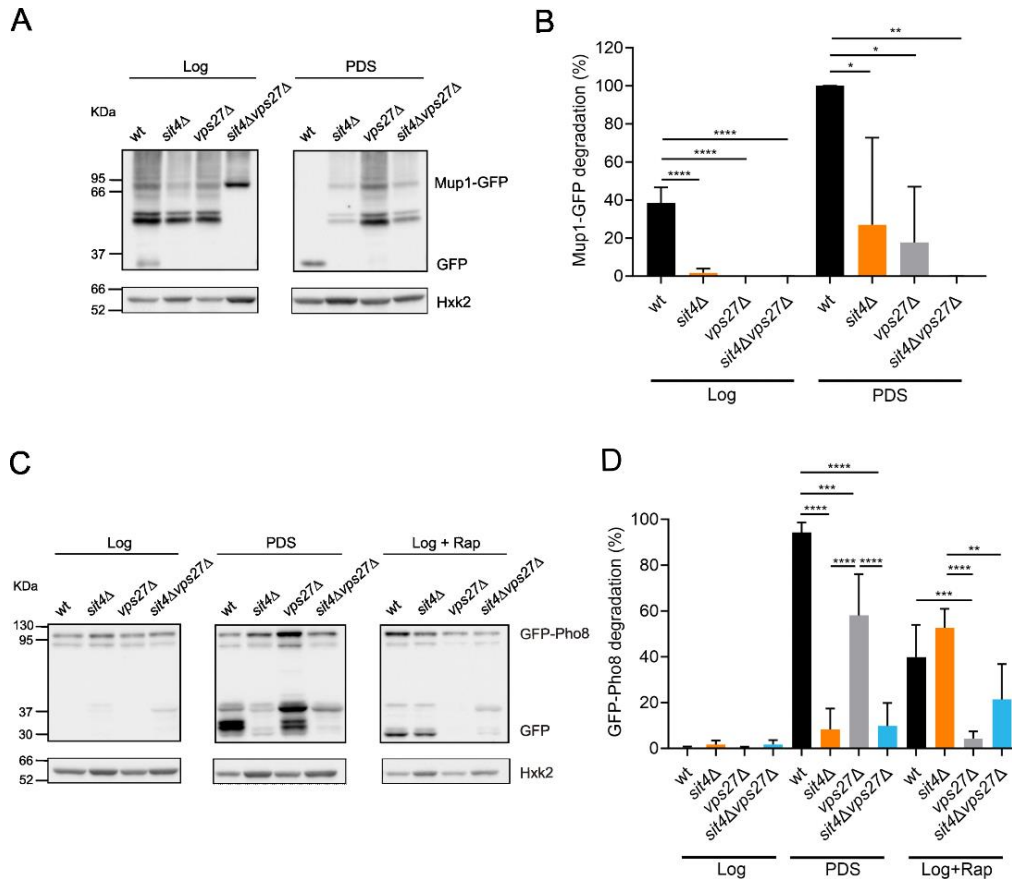


Figure 3. The induction of the MVB pathway and microautophagy at the PDS phase is Sit4-dependent. (A) Immunodetection of Mup1-GFP and GFP in protein extracts from cells expressing pRS416-*MUP1-GFP* grown in SC medium. Hxk2 was used as a loading control. A representative blot is shown. (B) The induction of the MVB pathway (free GFP/(Mup1-GFP + GFP) ratio) was evaluated at the Log and PDS (24 h after Log) phases. Values are the mean $\pm$ SD ($n = 3$); * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; one-way ANOVA. (C) Immunodetection of GFP-Pho8 and GFP in protein extracts from cells expressing pRS426-*GFP-PHO8*. Hxk2 was used as a loading control. A representative blot is shown. (D) The induction of microautophagy (free GFP/(GFP + GFP-Pho8) ratio) was evaluated at the Log and PDS (24 h after Log) phases. Cells treated with rapamycin were used as a positive control. Values are the mean $\pm$ SD ($n = 3$); ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA.

Nutrient depletion also induces the sorting of vacuolar membrane proteins into the lumen followed by its degradation by microautophagy, in a Vps27-dependent manner [64,73]. This process is regulated by TORC1, as it phosphorylates Vps27 to inhibit microautophagy [74], and by Snf1, as Vps27 is incapable of reaching the vacuole in cells lacking Snf1 [63]. Microautophagy can be evaluated by following the processing of GFP-

tagged Pho8 or other vacuolar membrane proteins [73,74]. To monitor microautophagy, cells were transformed with *GFP-PHO8*, and the levels of GFP-Pho8 and GFP were assessed by immunoblotting. Consistent with published findings, microautophagy was induced in wild-type cells at the PDS phase or upon TORC1 inhibition with rapamycin (Figure 3C,D) in a Vps27-dependent manner. *SIT4* deletion significantly reduced GFP-Pho8 processing at the PDS phase, indicating that microautophagy induction was decreased in these mutant cells. However, rapamycin was capable of inducing Pho8 degradation in *sit4Δ* cells, suggesting that inhibition of microautophagy in this mutant may be caused by impaired signaling rather than defects in the machinery assisting this process. Overall, these results suggest that microautophagy does not contribute to lifespan extension in the *sit4Δ* mutant.

In the CPY pathway, the transport of proteins to vacuoles depends on multivesicular endosomes and Vps27 [75,76]. To assess if Vps27 enrichment at vacuolar membranes reflects an altered capacity of the CPY pathway in the *sit4Δ* background, CPY processing was analyzed by immunoblotting. The results showed complete processing of CPY in both growth phases in all strains (Figure 4A). However, compared with the parental strain, *vps27Δ* and *sit4Δvps27Δ* cells had a tendency to display lower levels of mature CPY (mCPY) in the Log phase (Figure 4A,B). This decrease is probably due to an aberrant secretion of the precursor form of CPY (prCPY) to the extracellular space, characteristic of class E vps mutants [77]. At the PDS phase, mCPY levels in *vps27Δ* mutants were restored to wild-type levels, suggesting that other trafficking pathways may be rescuing prCPY to be processed in the vacuole. We also assessed whether deletion of *VPS27* in *sit4Δ* cells results in a CPY secretion phenotype by performing a colony immunoblot assay. As expected, *vps27Δ* cells exhibited a strong CPY secretion phenotype (Figure 4C). Notably, CPY secretion increased in the *sit4Δvps27Δ* double mutant vs. wild-type and *sit4Δ* cells. These findings suggest that the CPY pathway is not altered in *sit4Δ* cells but that *VPS27* deletion impairs this pathway, which may underlie the premature aging of *sit4Δvps27Δ* cells.

In addition to CPY, yeast uses the Cvt pathway, another biosynthetic trafficking pathway, to transport hydrolases, such as the Ape1, into the vacuole. For this, Cvt relies on the molecular machinery involved in autophagy [30]. To determine whether Cvt is altered in *sit4Δ* cells, we monitored Ape1 processing to its mature form. In the PDS phase, all strains exhibited near complete processing of Ape1 (Figure 5A,B). However, in the Log phase, *sit4Δ* cells had a 1.7-fold increase in Ape1 maturation compared to the parental strain. Moreover, Ape1 processing increased in the *sit4Δvps27Δ* mutant vs. *vps27Δ* cells, suggesting that the deletion of *SIT4* increased the Cvt pathway in a Vps27-independent manner.

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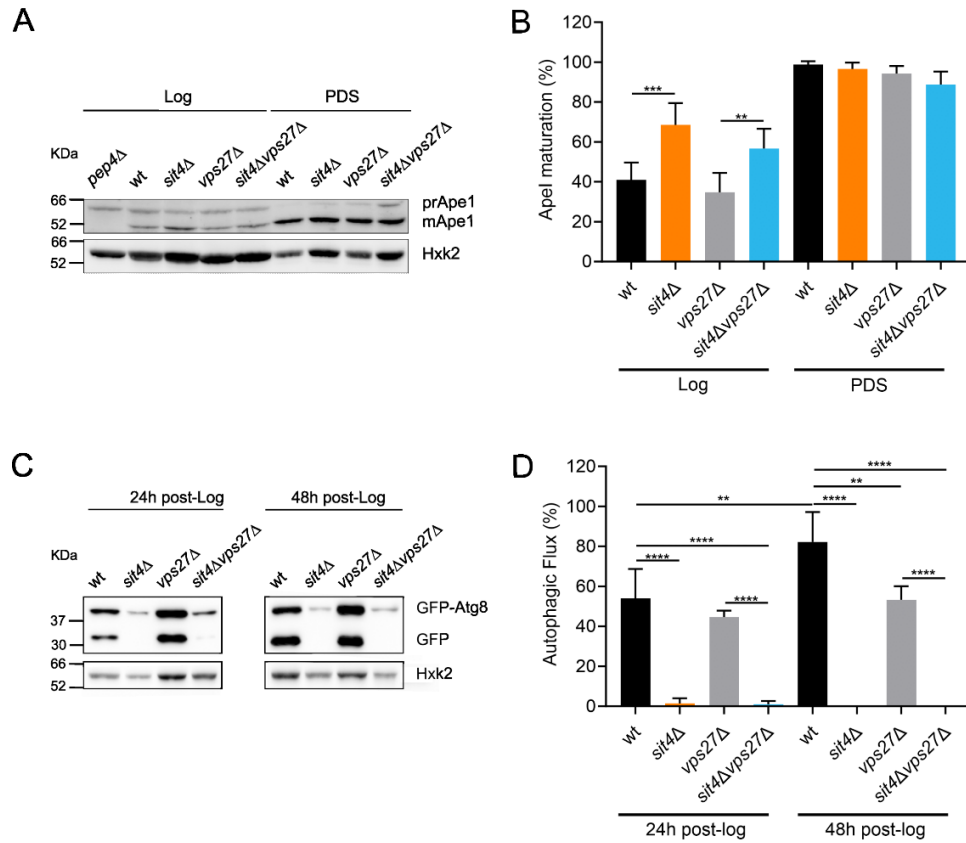


Figure 5. *SIT4* deletion compromises autophagy and induces the Cvt pathway at the Log phase in a *Vps27*-independent manner. (A) Processing of the proenzyme Ape1 (prApe1) to the mature enzyme (mApe1) was analyzed at the Log and PDS (24 h after Log) phases. Hxk2 was used as a loading control. The *pep4Δ* cells (unable to process Ape1) were used as control. A representative blot is shown. (B) Quantification of Ape1 maturation. Values are the mean $\pm$ SD ($n = 4$); ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA. (C) Immunodetection of GFP-Atg8 and GFP in cells expressing pRS416-GFP-ATG8. Hxk2 was used as a loading control. A representative blot is shown. (D) The autophagic flux (free GFP/(GFP + GFP-Atg8) ratio) was evaluated at 24 h and 48 h after the Log phase. Values are the mean $\pm$ SD ($n = 3$); ** $p < 0.01$, **** $p < 0.0001$; one-way ANOVA.

4.3.3 Loss of iron homeostasis in *sit4Δvps27Δ* cells does not contribute to its shortened lifespan

Our proteomic analysis from *sit4Δ* vacuolar membranes also identified alterations in proteins involved in iron trafficking, namely in Smf3, Ccc1, and Fth1 (Tables S1 and S2). It is known that Vps27 impacts iron homeostasis by controlling the degradation of iron transporters. Under high iron conditions, iron transporters are sorted into vesicles by ESCRT-0 and the MVB pathway, to be degraded in vacuoles [83]. This prompted us to investigate whether *VPS27* deletion affects iron homeostasis, contributing to the shortened CLS of *sit4Δvps27Δ* cells. The results showed that iron levels were similar in all strains at the Log phase (Figure 6A). However, at the PDS phase, the *sit4Δvps27Δ* mutant exhibited higher levels of iron. This result led us to evaluate the sensitivity of cells to grow in medium supplemented with iron. We found that *sit4Δ* cells exhibited a higher sensitivity to iron supplementation that was aggravated by *VPS27* deletion (Figure 6B). *VPS27* deletion in *sit4Δ* cells may decrease the degradation of iron transporters, leading to the observed high iron levels and iron sensitivity. We hypothesized that iron accumulation leads to iron toxicity, contributing to the premature aging of *sit4Δvps27Δ* cells. To examine this, we evaluated the impact of iron chelation with BPS, known to cause intracellular iron deprivation, on oxidative stress resistance and CLS. To assess oxidative stress resistance, cells grown in SC medium with or without BPS were treated with 1.5 mM H₂O₂. Our data show that *sit4Δvps27Δ* cells did not exhibit increased sensitivity to H₂O₂ (Figure 6C). Moreover, BPS decreased the H₂O₂ sensitivity in all strains, but this protective effect was lower in *sit4Δvps27Δ* mutant cells, suggesting that iron accumulation does not aggravate H₂O₂ sensitivity. Moreover, BPS had a minor effect on the CLS of *sit4Δvps27Δ* cells as it only increased cell survival from 1 to 2 days (Figure 6D). Overall, these findings suggest that iron accumulation does not seem to be a primary contributing factor to the drastic viability loss of *sit4Δvps27Δ* cells.

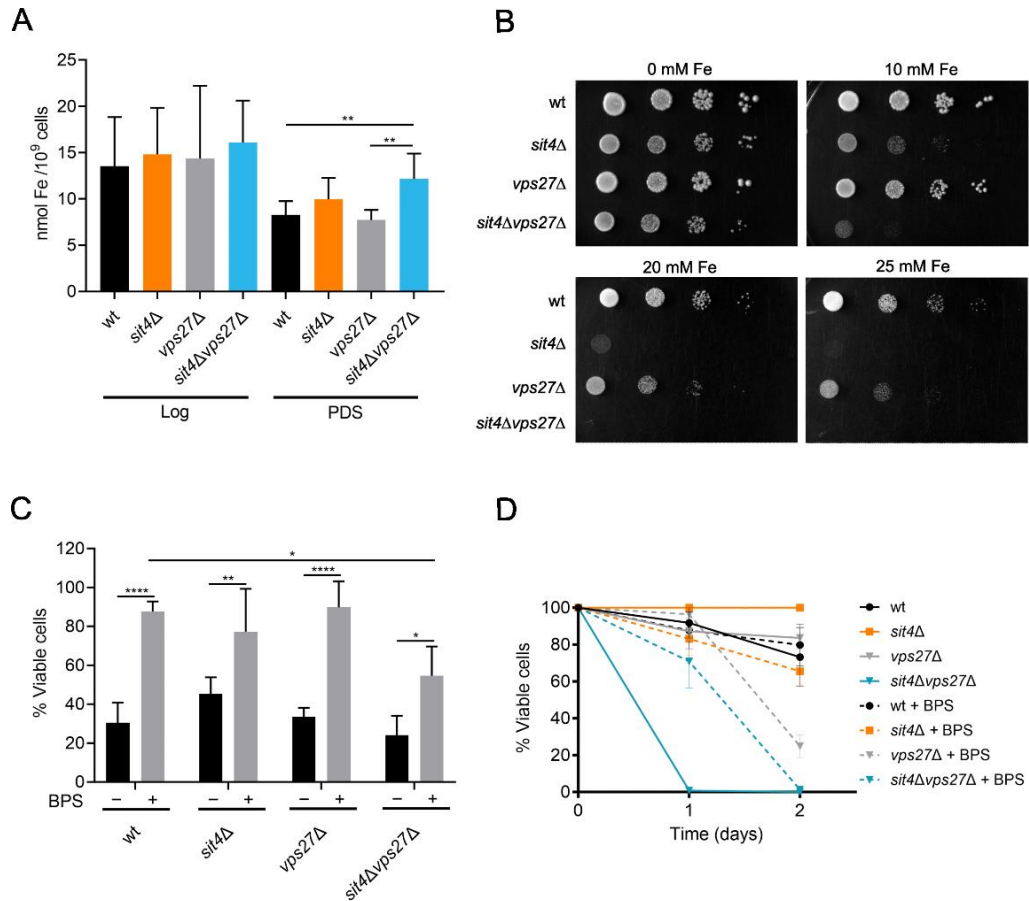


Figure 6. Iron levels and the effect of iron chelation on H₂O₂ resistance and CLS in *sit4Δvps27Δ* cells. (A) Total iron levels were quantified in cells grown to late Log and PDS (24 h after Log) phases. Values are the mean $\pm$ SD ($n \geq 6$); ** $p \leq 0.01$; one-way ANOVA. (B) Cells were grown to the Log phase and four ten-fold serial dilutions were spotted onto SC plates with or without supplementation of iron (II) sulphate heptahydrate. A representative image is shown ($n = 3$). (C) Cells were grown to Log phase in medium with or without supplementation of 80 μ M of BPS and treated with 1.5 mM H₂O₂ for 1 h. Cell viability was expressed as the percentage of the CFUs (treated cells vs. non-stressed cells). Values are the mean $\pm$ SD ($n \geq 3$); * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$; one-way ANOVA. (D) Cells were grown to the PDS phase (t₀) in SC medium with or without supplementation of 80 μ M BPS and maintained over time in the same medium. Cellular viability was quantified as the percentage of the CFUs (aged vs. t₀). Data are the mean $\pm$ SEM ($n \geq 3$).

4.3.4 Vps27 is crucial for mitochondrial function in *sit4Δ* cells

Sit4 regulates mitochondrial metabolism by repressing respiratory genes in high glucose conditions [42], and mitochondrial derepression is crucial for the increased CLS of *sit4Δ* cells [32,34,35]. Sit4 modulates the catabolite repression through regulation of Mig1 and Hxk2. During cellular growth in a glucose medium, the unphosphorylated Mig1-Hxk2 complex accumulates in the nucleus, where it represses the expression of genes required for cellular growth on non-fermentable carbon sources [84,85]. In the absence of Sit4, Hxk2 is hyperphosphorylated [86] and Mig1 levels decrease [87]. Moreover, Snf1 is activated [88,89], leading to Mig1 phosphorylation and inhibition [90]. Thus, cells lacking Sit4 exhibit a high oxygen consumption rate (OCR) already at the Log phase even when grown in

fermentable media [32]. A higher respiratory capacity early in growth has been related to an improved tolerance to reactive oxygen species during the stationary phase and also to an increase in lifespan [91,92]. Moreover, *sit4Δ* cells exhibit a higher dependency on the respiratory metabolism than wild-type cells [42]. These data and the lack of alterations in vacuolar trafficking pathways regulated by Vps27 that may explain its role in *sit4Δ* lifespan led us to analyze the function of Vps27 in the modulation of *sit4Δ* mitochondrial function.

To assess if Vps27 is required for the increased mitochondrial respiration displayed by *sit4Δ* cells, the OCR was measured in cells at both Log and PDS phases. Notably, *VPS27* deletion abolished mitochondrial derepression at the Log phase in cells lacking Sit4 (Figure 7A). In fact, *sit4Δvps27Δ* cells had lower OCR in both growth phases, compared to wild-type and *sit4Δ* cells, indicating that the double mutant has severe deficiencies in mitochondrial respiration. Furthermore, the *vps27Δ* mutant failed to fully induce mitochondrial respiration during growth from the Log to the PDS phase and exhibited a lower OCR, compared to the parental strain. Thus, Vps27 seems to potentiate optimal mitochondrial activity, and its decrease may contribute to the shortened CLS exhibited by *vps27Δ* mutants (Figure 2). We also evaluated the ability of *sit4Δvps27Δ* cells to grow in a medium containing glycerol (YPG), a respiratory carbon source, instead of glucose (YPD). The results demonstrated that *sit4Δvps27Δ* cells were incapable of growing in YPG (Figure 7B), further suggesting that mitochondrial function is impaired. Overall, these results suggest that Vps27 is essential to ensure respiratory fitness and extended longevity in *sit4Δ* cells.

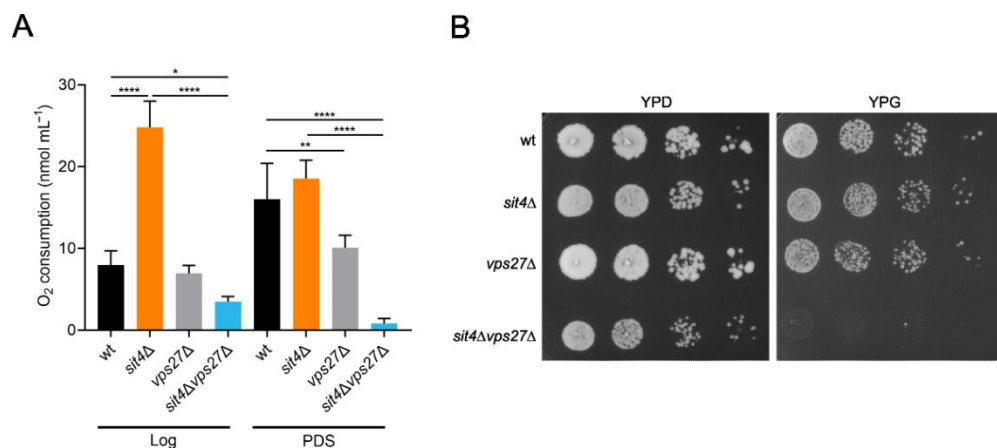


Figure 7. The *sit4Δvps27Δ* mutant has impaired mitochondrial respiration. (A) Cells were grown to Log and PDS phases and mitochondrial respiration was determined by measuring oxygen consumption rate. Values are the mean $\pm$ SD ($n \geq 4$); * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; one-way ANOVA. (B) Cells were grown to Log phase and four ten-fold serial dilutions were spotted onto YPD or YPG plates. A representative image is shown ($n = 4$).

Numerous studies support a key role of vacuolar trafficking pathways, such as autophagy and mitophagy, in mitochondrial fitness. Mitophagy is a selective type of autophagy that targets damaged mitochondria for degradation in vacuoles. Along with mitochondrial dynamics, mitophagy is of central importance to the sustenance of mitochondrial integrity and activity [93–96]. This led us to investigate whether Vps27 is involved in the modulation of mitophagy and mitochondrial biogenesis in cells lacking Sit4. Mitophagy was assessed in cells expressing the vacuolar alkaline phosphatase Pho8 targeted to mitochondria (mtPho8) [97]. Since maturation of the Pho8 pro-enzyme requires vacuolar Pep4, the alkaline phosphatase activity is an indicator of mitochondrial degradation in vacuoles. Our results show that the alkaline phosphatase activity was higher in cells lacking Sit4 at the Log and PDS phases, although in a Vps27-independent manner (Figure 8A). In fact, we even observed higher activity in *sit4Δvps27Δ* cells at the Log phase compared to *sit4Δ* cells. This indicates that the mitochondrial respiration defects in the double mutant *sit4Δvps27Δ* are not associated with impaired mitophagy. Moreover, these findings suggest that enhanced mitophagy activity in *sit4Δ* cells is not associated with Vps27-dependent CLS extension.

Mitochondrial integrity depends on the coordination of both mitochondrial degradation and mitochondrial biogenesis [93,94,98]. To evaluate if *VPS27* deletion affects mitochondrial biogenesis, the levels of mitochondrial proteins (Por1, Atp2, and Cox2) were assessed in cells at the Log and PDS phases. As expected, the levels of the porin Por1 increased in wild-type cells from the Log to the PDS phase (Figure 8B,C), reflecting the higher mitochondrial mass characteristic of the respiratory phase. Accordingly with the early catabolite derepression in cells lacking Sit4, the levels of Por1 increased at the Log phase. The increase in mitochondrial biogenesis in the *sit4Δ* mutant was Vps27 independent, as *sit4Δvps27Δ* also exhibited higher levels of Por1 at the Log phase. Similar changes were observed when mitochondrial mass was assessed by flow cytometry, using cells expressing preSU9-GFP (Figure S4). Notably, deletion of *VPS27* in *sit4Δ* cells abolished the higher levels of the oxidative phosphorylation proteins Atp2 and Cox2 (Figure 8B,D,E). In fact, Cox2 was not detected in *sit4Δvps27Δ* cells, even at the PDS phase. As Cox2 is encoded by the mitochondrial genome, we speculated that *sit4Δvps27Δ* exhibits defects in the maintenance of mtDNA. However, analysis by qPCR revealed that mtDNA levels were not affected in *sit4Δvps27Δ* compared to wild-type cells (Figure 8F). These observations suggest that the defects in *sit4Δvps27Δ* mitochondrial function did not result from defects in mitochondrial biogenesis or the loss of mtDNA.

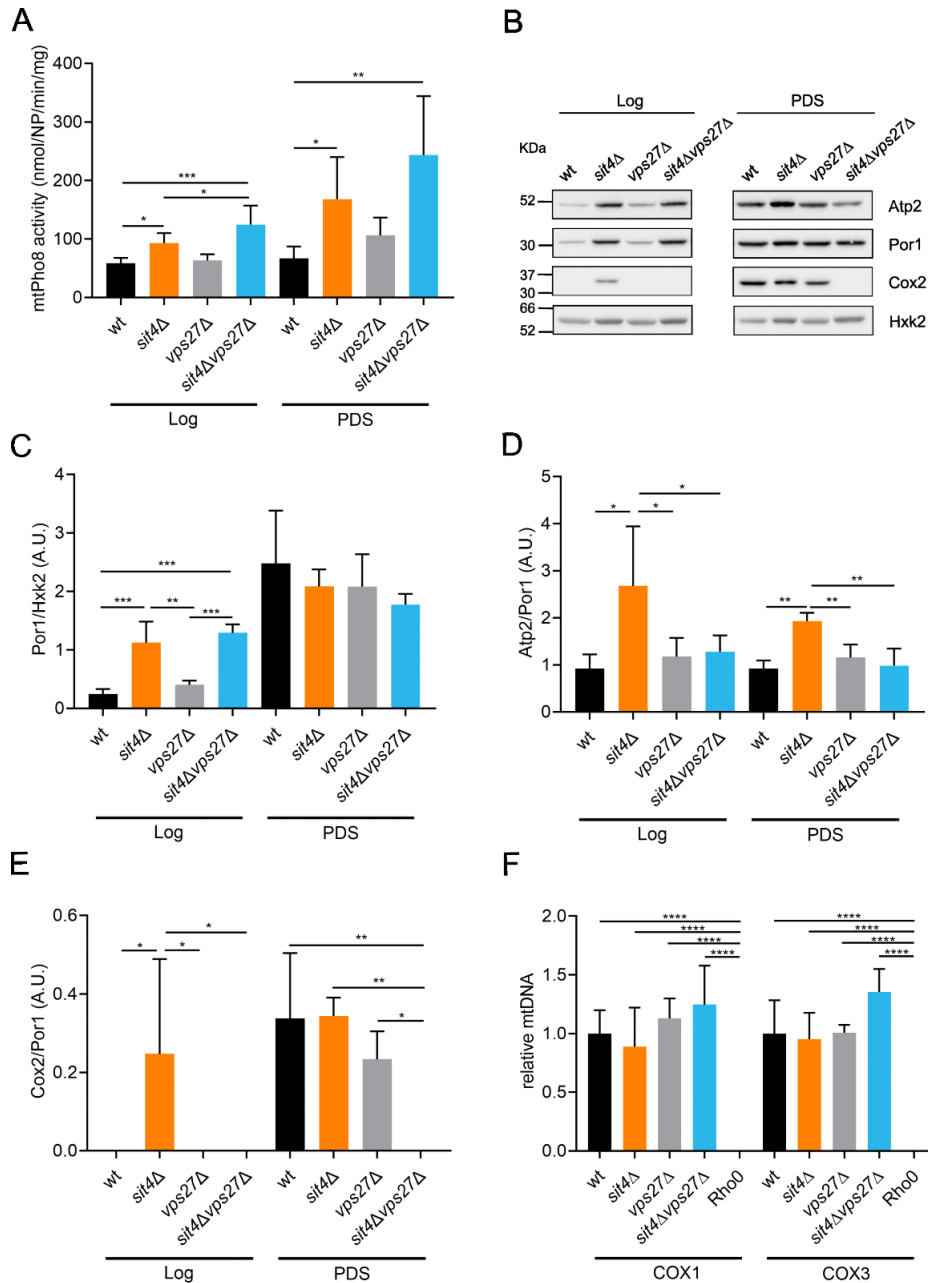


Figure 8. VPS27 deletion in *sit4Δ* cells leads to a decrease in Atp2 and Cox2 levels, but it does not affect mitophagy or mitochondrial biogenesis. (A) Mitophagy is induced in *sit4Δ* cells in a Vps27-independent manner. Cells expressing pYX242-*mtPHO8* were grown to Log and PDS (24 h after Log) phases and mitophagy was determined by measuring the alkaline phosphatase activity. Values are the mean $\pm$ SD ($n = 4$); * $p \leq 0.05$, ** $p \leq 0.01$, *** $p < 0.001$; one-way ANOVA. (B) Cells were grown to Log and PDS phases and the levels of mitochondrial proteins (Por1, Atp2, and Cox2) were assessed by immunoblotting. Hxk2 was used as a loading control. A representative blot is shown ($n = 3$). (C) Relative amount of Por1 (a mitochondrial outer membrane protein) to Hxk2 is shown as an indicator of mitochondrial mass. Values are the mean $\pm$ SD; ** $p \leq 0.01$, *** $p < 0.001$; one-way ANOVA. (D,E) Quantification of the ratio Atp2/Por1 (D) and Cox2/Por1 (E). Values are the mean $\pm$ SD; * $p \leq 0.05$, ** $p \leq 0.01$; one-way ANOVA. (F) Analysis of mtDNA/nuclear DNA by qPCR. COX1 and COX3 were used as reference for mtDNA and *ACT1* for nuclear DNA. Rho0 cells were used as control. Values are the mean $\pm$ SD ($n = 4$); **** $p < 0.0001$; one-way ANOVA.

4.4. Discussion

The decline in the function of lysosomes (vacuoles in yeast) and mitochondria has been linked to the aging process and the development of age-related diseases [1,6]. Furthermore, numerous interventions that improve the function of these organelles also increase lifespan [7,12–18]. Our previous studies showed that *SIT4* deletion increases mitochondrial respiration and yeast CLS, in part mediated by increased phosphorylation levels of hexokinase 2 [86] and ATP synthase catalytic beta subunit Atp2 [34], as well as Snf1/AMPK activation [35]. The deletion of *SIT4* also suppresses the mitochondrial dysfunction exhibited by *Isc1*-deficient cells that accumulate ceramide during aging [32], as well as the vacuolar fragmentation and dysfunctions exhibited by this mutant [33]. However, the mechanism by which Sit4 regulates the vacuolar–mitochondrial axis is not fully understood, and its characterization may help to define new strategies to improve lifespan.

In this study, we identified 49 vacuolar membrane proteins whose levels change in cells lacking Sit4. These proteins were mainly associated with cellular processes, such as vacuole fusion and organization, late endosome to vacuole transport, autophagy, microautophagy, polyphosphate metabolism, and metal ion homeostasis. Proteins that increased significantly in *sit4Δ* vacuolar membranes include Vps27 (4.1-fold) and Hse1 (2.2-fold), components of the ESCRT-0 [65]. The enrichment of Vps27 at *sit4Δ* vacuolar membranes was not due to upregulation of Vps27 since its total protein levels were not increased. The translocation of Vps27 from endosomes to vacuolar membranes depends on Snf1 activity at the PDS phase [63] and Sit4 deficiency results in Snf1 activation early during growth in glucose medium [88,89]. Whether Snf1 activation promotes Vps27 vacuolar localization in *sit4Δ* cells remains to be clarified.

Our results suggest that Vps27 is crucial for lifespan extension in *sit4Δ* cells. Indeed, the CLS of *sit4Δvps27Δ* cells was much shorter than that of each single mutants, indicating a negative genetic interaction between *SIT4* and *VPS27*. The analysis of vacuolar trafficking pathways that are known to be dependent on Vps27 showed that the MVB pathway and microautophagy were impaired in both *sit4Δ* and *sit4Δvps27Δ* cells. However, *VPS27* deletion in *sit4Δ* cells resulted in aberrant CPY secretion. Thus, defects in the transport of cargo proteins through the CPY pathway may contribute to vacuolar dysfunction and a short lifespan in *sit4Δvps27Δ* cells. We also showed that the Cvt pathway was induced in both *sit4Δ* and *sit4Δvps27Δ* cells at the Log phase, suggesting that Vps27 does not promote *sit4Δ* CLS through regulation of Cvt. Intriguingly, the induction of autophagy at the PDS phase was impaired in *sit4Δ* and *sit4Δvps27Δ* cells. Autophagy induction has been largely associated with increased longevity and described as a requirement for lifespan extension by pharmacological and nutritional interventions [24,99].

However, several *atg* mutants with defective autophagy still exhibit lifespan extension under caloric restriction conditions [100]. Furthermore, several pieces of evidence suggest that autophagy and the ubiquitin proteasome system are coordinated for proper control of protein homeostasis and housekeeping functions, and that the inhibition of autophagy can be compensated by the induction of the proteasome, and vice versa [101–104]. Although Sit4 does not regulate proteasome activity, Sit4 and proteasome are functionally linked as they act in concert in osmoregulation and nutrient sensing [105]. More studies are required to assess if the ubiquitin proteasome system contributes to *sit4Δ* lifespan extension.

Our studies also suggest that Vps27 contributes to iron homeostasis, with *sit4Δvps27Δ* cells accumulating high iron levels and exhibiting increased sensitivity to iron supplementation. However, iron accumulation does not seem to be involved in the *sit4Δvps27Δ* premature aging, as BPS treatment only promoted a minor increase in the mutant CLS. In addition, we found that Vps27 affects the levels of proteins critical for oxidative phosphorylation in *sit4Δ* cells, namely of Atp2 and Cox2. How Vps27 regulates these proteins in cells lacking Sit4 to increase mitochondrial fitness, which is critical for the *sit4Δ* mutant extended longevity, requires further investigation.

4.5. Conclusions

In summary, this study suggests that Vps27, a component of the ESCRT-0 complex, is enriched at the vacuolar membrane of *sit4Δ* cells, playing a crucial role in the CLS extension observed in this mutant strain. Interestingly, the deletion of *VPS27* in *sit4Δ* cells did not affect protein trafficking or vacuolar sorting pathways. However, it impaired mitochondrial respiration, associated with reduced levels of Atp2 and Cox2. Together, these findings unveil a novel link between Sit4, Vps27, and mitochondrial respiration, providing valuable insights into the intricate mechanisms governing cellular longevity.

4.6. Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [106] partner repository with the dataset identifier PXD048450.

4.7. Acknowledgments

We are grateful to Paula Ludovico (ICVS, Universidade do Minho, Portugal), Helder Maiato (i3S, Universidade do Porto, Portugal), Sylvie Friant (Université de Strasbourg and Centre National de la Recherche Scientifique, Strasbourg, France), David Teis (Division of Cell Biology, Biocenter, Medical University of Innsbruck, and Austrian Drug Screening

Institute, Innsbruck, Austria), Claudio de Virgilio (Department of Biology, University of Fribourg, Fribourg, Switzerland), and Benedikt Westermann (Institute for Physiological Chemistry, Ludwig Maximilian University of Munich, Munich, Germany) for providing, respectively, the pRS416-*GFP-ATG8* plasmid and mouse anti-CPY antibody, the mouse anti-GAPDH antibody, the YCpHAC33-*Prom-Vps27-3xHA* plasmid, the pRS416-*MUP1-GFP* plasmid, the pRS426-*GFP-PHO8* plasmid, and the plasmid pVT100U-*preSU9-GFP*. We also thank Hugo Osório (i3S Proteomics Scientific Platform) for his support in the proteomic analysis.

4.8. Supplementary material

4.8.1 Supplementary figures

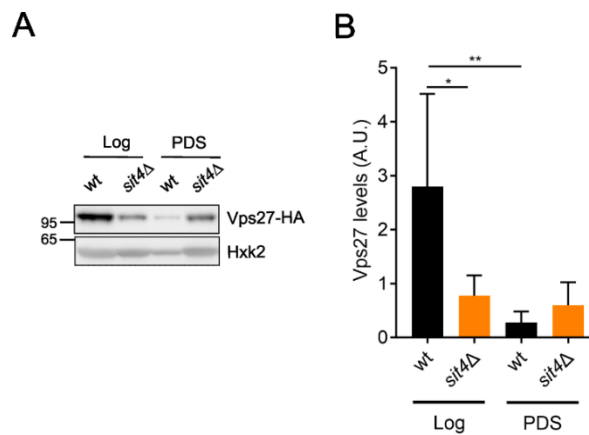


Figure S1. *SIT4* deletion decreases Vps27 levels at the Log phase. (A) Cells harboring the plasmid YCpHAC33-*Prom-Vps27-3xHA* were grown to late exponential (Log) and post-diauxic shift (PDS) phases and Vps27 levels on total protein extracts were assessed by immunodetection. Hxk2 was used as a loading control. A representative blot of at least three independent experiments is shown. (B) Quantification of Vps27 (normalized to Hxk2). Values are the mean $\pm$ SD; * $p \leq 0.05$; ** $p \leq 0.01$; one-way ANOVA.

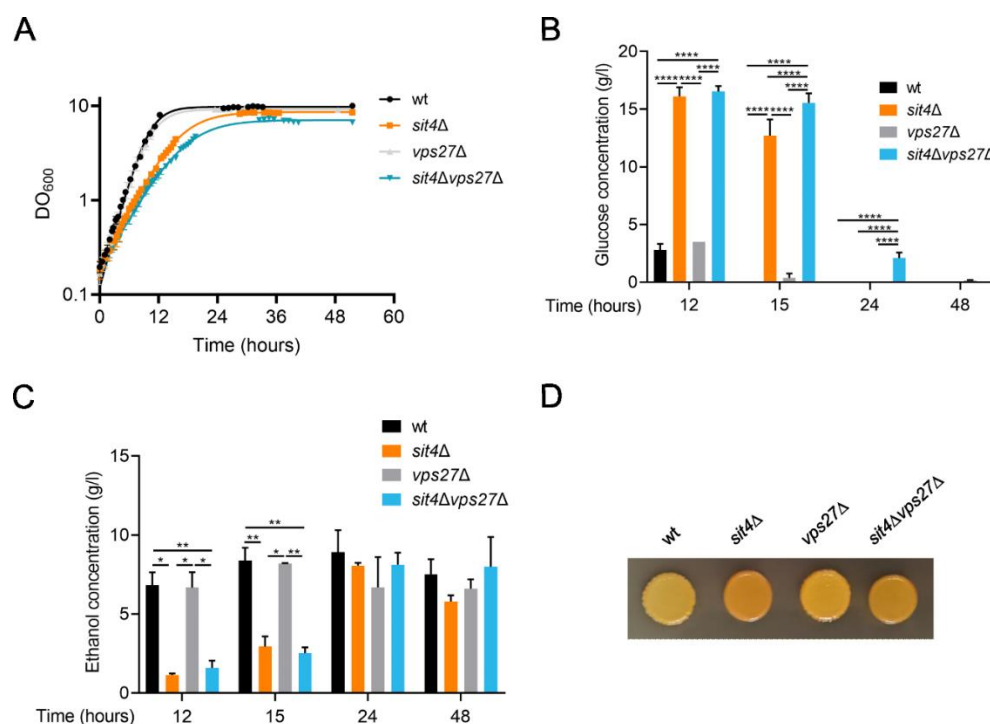


Figure S2. Growth and metabolic profile of indicated strains. (A) Cells were grown until OD_{600nm}=0.2 (time 0) in SC media and the OD_{600nm} was monitored over time. Data are the mean $\pm$ SD ($n = 2$). (B) glucose and (C) ethanol levels were quantified at 12, 15, 24, and 48 h. Data are the mean $\pm$ SD ($n \geq 3$); * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$; one-way ANOVA. (D) Yeast colonies were exposed to iodine vapor to qualitatively evaluate the glycogen content. A representative figure is shown ($n = 2$).

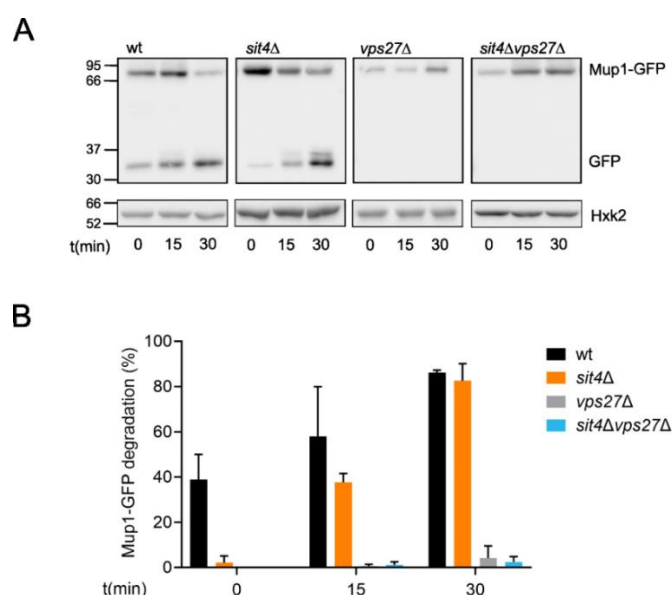


Figure S3. Methionine increases Mup1-GFP degradation in *sit4Δ* cells. (A) Cells expressing pRS416-MUP1-GFP were grown in MM medium to the Log phase (0 min) and 38 mg L⁻¹ of methionine was added as indicated. Mup1-GFP and GFP were detected by immunoblotting. Hxk2 was used as a loading control. (B) The induction of the MVB pathway (ratio between free GFP and the sum of GFP and Mup1-GFP) in response to methionine was evaluated. Values are the mean $\pm$ SD ($n = 2$).

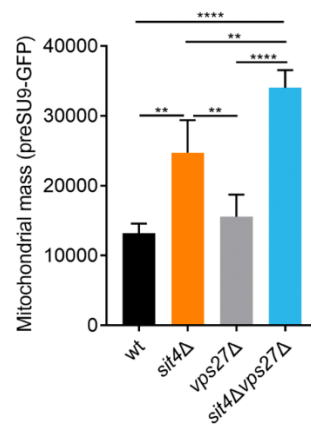


Figure S4. Mitochondrial mass increases in *sit4Δ* and *sit4Δvps27Δ* cells. Cells expressing a fusion protein containing GFP with a mitochondrial presequence (pVT100U-*preSU9-GFP*), were grown in SC medium to Log phase. GFP intensity was quantified by flow cytometry and used as an indicator of mitochondrial mass. Values are the mean $\pm$ SD ($n = 4$); ** $p \leq 0.01$; **** $p \leq 0.0001$; one-way ANOVA.

4.8.2 Supplementary tables

Table S1 - Levels of vacuolar membrane proteins in wild-type and *sit4*Δ samples.

Levels of vacuolar membrane proteins in wild type and <i>sit4</i> Δ samples												
Accession	Gene Symbol	Gene ID	Abundance (normalized) wild type				Abundance (normalized) <i>sit4</i> Δ				Ratio <i>sit4</i> Δ/wt	p-value
			Sample 1	Sample 2	Sample 3	Mean	Sample 1	Sample 2	Sample 3	Mean		
Q12078	SMF3	D6VY36; Q12078; sce:YLR034C	1,07E+09	1,09E+09	1,16E+09	1,11E+09	2,24E+09	2,03E+09	2,08E+09	2,11E+09	1,91	0,00
P25351	YCR023C	D6VR32; P25351; Q8NKJ7; sce:YCR023C	1,65E+08	1,94E+08	1,87E+08	1,82E+08	3,42E+08	3,71E+08	3,72E+08	3,61E+08	1,99	0,00
P53262	VOA1	D6VUN8; P53262; sce:YGR106C	6,81E+07	7,12E+07	8,27E+07	7,40E+07	1,20E+07	2,08E+07	2,03E+07	1,77E+07	0,24	0,00
P47818	CCC1	D6VYM0; P47818; sce:YLR220W	1,46E+09	1,70E+09	1,46E+09	1,54E+09	2,88E+09	2,94E+09	2,63E+09	2,82E+09	1,83	0,00
P53059	MNT2	D6VV78; P53059; sce:YGL257C	6,86E+06	8,13E+06	7,29E+06	7,43E+06	3,00E+05	1,18E+06	2,24E+06	1,24E+06	0,17	0,00
P38310	FTH1	D6VQK4; P38310; Q9URQ1; sce:YBR207W	2,41E+08	2,60E+08	2,51E+08	2,51E+08	4,74E+08	4,16E+08	4,93E+08	4,61E+08	1,84	0,00
P36051	MCD4	D6VX34; P36051; sce:YKL165C	2,02E+08	2,53E+08	2,58E+08	2,37E+08	5,80E+07	8,56E+07	8,69E+07	7,68E+07	0,32	0,00
P33894	STE13	D6W2S4; P33894; Q9URF1; sce:YOR219C	2,29E+07	2,41E+07	2,11E+07	2,27E+07	6,89E+06	1,09E+07	1,15E+07	9,76E+06	0,43	0,00
Q04301	VBA1	D6VZR1; Q04301; sce:YMR088C	2,33E+08	2,64E+08	2,48E+08	2,49E+08	3,52E+08	3,26E+08	3,34E+08	3,37E+08	1,36	0,00
P38146	YPT10	D6VQR1; P38146; sce:YBR264C	1,28E+08	1,27E+08	1,35E+08	1,30E+08	8,90E+07	6,64E+07	7,23E+07	7,59E+07	0,58	0,00
P53925	YNL115C	B0KZS0; D6W167; P53925; Q6B2V3; sce:YNL115C	1,18E+09	1,42E+09	1,29E+09	1,30E+09	2,29E+09	2,54E+09	2,12E+09	2,32E+09	1,78	0,00
Q02896	YDC1	D6W3T0; Q02896; sce:YPL087W	2,61E+07	3,05E+07	2,46E+07	2,71E+07	1,62E+07	1,31E+07	1,41E+07	1,45E+07	0,53	0,00

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

P38247	SLM4	D6VQ76; P38247; sce:YBR077C	9,16E+06	9,48E+06	8,20E+06	8,95E+06	2,71E+07	3,06E+07	2,11E+07	2,63E+07	2,94	0,00
P43585	VTC2	D6VTM5; P43585; sce:YFL004W	2,19E+09	1,94E+09	1,91E+09	2,01E+09	1,33E+09	9,10E+08	1,10E+09	1,11E+09	0,55	0,00
P47170	IML1	D6VWV7; P47170; sce:YJR138W	2,96E+07	2,74E+07	3,45E+07	3,05E+07	1,52E+07	6,60E+06	1,22E+07	1,13E+07	0,37	0,00
P22855	AMS1	D6VTZ5; P22855; sce:YGL156W	1,64E+08	2,79E+08	8,89E+07	1,78E+08	5,73E+08	8,18E+08	7,52E+08	7,14E+08	4,02	0,00
P32602	SEC17	D6VPU9; P32602; sce:YBL050W	1,05E+08	6,88E+07	7,09E+07	8,16E+07	1,79E+08	1,84E+08	1,51E+08	1,71E+08	2,10	0,00
Q12697	YPK9	D6W2Y9; Q12697; sce:YOR291W	1,18E+09	1,43E+09	1,75E+09	1,45E+09	5,47E+08	4,82E+08	5,84E+08	5,38E+08	0,37	0,01
P32386	YBT1	D6VXW0; P32386; Q07882; sce:YLL048C	9,73E+09	1,03E+10	1,16E+10	1,05E+10	7,65E+09	7,00E+09	6,18E+09	6,94E+09	0,66	0,01
P19524	MYO2	D6W323; P19524; sce:YOR326W	1,90E+08	1,50E+08	1,17E+08	1,52E+08	5,14E+07	3,50E+07	4,79E+07	4,48E+07	0,29	0,01
Q06538	CSC1	D6VYN9; Q06538; sce:YLR241W	5,62E+08	5,80E+08	6,53E+08	5,98E+08	3,62E+08	4,38E+08	4,37E+08	4,12E+08	0,69	0,01
P39548	YAR028W	D6VPN0; P39548; sce:YAR028W	4,53E+07	3,74E+07	2,21E+07	3,49E+07	1,80E+08	1,13E+08	1,27E+08	1,40E+08	4,01	0,01
P25591	VAC17	D6VQV5; P25590; P25591; sce:YCL063W	2,15E+06	8,64E+05	1,29E+06	1,44E+06	3,32E+06	3,13E+06	3,69E+06	3,38E+06	2,35	0,01
P53134	YGL114W	D6VU33; P53134; sce:YGL114W	2,25E+08	2,31E+08	2,27E+08	2,28E+08	4,37E+08	4,96E+08	3,46E+08	4,26E+08	1,87	0,01
P40343	VPS27	D6W111; P40343; sce:YNR006W	4,73E+05	8,33E+05	1,58E+05	4,88E+05	2,44E+06	1,46E+06	2,11E+06	2,00E+06	4,10	0,01
P25568	ATG22	D6VQX7; P25568; sce:YCL038C	2,04E+08	2,24E+08	2,00E+08	2,09E+08	3,22E+08	2,67E+08	3,45E+08	3,11E+08	1,49	0,01
Q03212	EAR1	D6VZZ3; Q03212; Q03830; sce:YMR171C	2,42E+07	2,43E+07	2,19E+07	2,34E+07	8,96E+06	1,78E+07	1,10E+07	1,26E+07	0,54	0,02
Q03897	MTC5	D6VSB4; Q03897; sce:YDR128W	3,70E+07	2,74E+07	2,42E+07	2,95E+07	1,50E+07	1,37E+07	1,63E+07	1,50E+07	0,51	0,02
Q04991	FMP42	D6W046; Q04991; sce:YMR221C	8,64E+08	1,01E+09	8,82E+08	9,20E+08	1,87E+09	1,62E+09	1,27E+09	1,59E+09	1,72	0,02
Q04934	IVY1	D6VSL1; Q04934; sce:YDR229W	9,67E+06	1,18E+07	2,43E+07	1,53E+07	6,19E+07	4,15E+07	3,76E+07	4,70E+07	3,08	0,02
P39925	AFG3	D3DLR5; P39925; sce:YER017C	2,23E+08	1,86E+08	1,11E+08	1,73E+08	4,81E+07	4,54E+07	6,61E+07	5,32E+07	0,31	0,02
P34240	ZRT3	D6VX25; P34240; sce:YKL175W	2,73E+08	2,71E+08	3,94E+08	3,13E+08	5,32E+08	7,78E+08	9,28E+08	7,46E+08	2,39	0,02

P38270	ATG14	D6VQC5; P38270; sce:YBR128C	8,07E+06	7,96E+06	1,49E+07	1,03E+07	1,02E+06	3,58E+06	1,52E+06	2,04E+06	0,20	0,03
P27809	KRE2	D6VTA6; P27809; sce:YDR483W	2,43E+08	2,20E+08	1,27E+08	1,97E+08	6,14E+07	6,08E+07	9,21E+07	7,14E+07	0,36	0,03
P42839	VNX1	D6W0M5; P42839; sce:YNL321W	6,59E+08	6,54E+08	5,01E+08	6,05E+08	1,06E+09	7,85E+08	9,14E+08	9,18E+08	1,52	0,03
P42951	LSB6	D6VW84; P42951; sce:YJL100W	7,44E+07	6,84E+07	6,95E+07	7,08E+07	1,05E+08	8,35E+07	8,96E+07	9,26E+07	1,31	0,03
Q05902	ECM38	D6VYU3; Q05902; sce:YLR299W	2,46E+07	3,54E+07	1,09E+07	2,37E+07	4,57E+07	5,38E+07	6,89E+07	5,62E+07	2,37	0,03
P40570	VLD1	D6VVU4; P40570; sce:YIR014W	4,33E+07	5,00E+07	5,76E+07	5,03E+07	8,13E+07	9,12E+07	6,49E+07	7,91E+07	1,57	0,03
P38966	YDR089W	D6VS76; P38966; Q03192; sce:YDR089W	1,65E+09	1,33E+09	1,08E+09	1,35E+09	7,71E+08	8,76E+08	8,16E+08	8,21E+08	0,61	0,03
P43601	ATG18	D6VTQ1; P43601; sce:YFR021W	3,49E+05	3,73E+05	1,11E+05	2,78E+05	6,82E+05	7,28E+05	7,12E+05	7,08E+05	2,55	0,03
P32802	EMP70	D6VY83; P32802; Q12101; sce:YLR083C	1,10E+08	1,13E+08	6,95E+07	9,72E+07	2,41E+07	2,80E+07	2,93E+07	2,71E+07	0,28	0,04
P53947	YNL058C	D6W1C1; P53947; sce:YNL058C	1,11E+07	7,00E+06	4,53E+06	7,55E+06	1,02E+06	2,07E+06	1,37E+06	1,49E+06	0,20	0,04
P25625	PER1	D6VR53; P25625; sce:YCR044C	8,62E+06	2,99E+06	6,20E+06	5,93E+06	1,42E+06	6,50E+05	2,93E+05	7,87E+05	0,13	0,04
P39109	YCF1	D6VSB9; P39109; Q03905; sce:YDR135C	6,10E+09	6,99E+09	7,64E+09	6,91E+09	8,27E+09	8,57E+09	8,12E+09	8,32E+09	1,20	0,04
P06105	SCP160	D6VWA3; P06105; sce:YJL080C	1,06E+08	6,76E+07	8,04E+07	8,45E+07	5,55E+07	4,34E+07	4,93E+07	4,94E+07	0,58	0,04
P40152	YNL217W	D6W0X3; P40152; sce:YNL217W	1,54E+08	1,60E+08	1,50E+08	1,55E+08	3,61E+08	2,55E+08	3,71E+08	3,29E+08	2,13	0,04
P50944	AVT4	D6W178; P50944; sce:YNL101W	5,55E+07	4,59E+07	3,45E+07	4,53E+07	1,00E+08	6,20E+07	8,61E+07	8,27E+07	1,83	0,04
P38753	HSE1	D3DKR2; P38753; sce:YHL002W		3,89E+05		3,89E+05	9,17E+05	7,47E+05	8,57E+05	8,40E+05	2,16	0,05
P53191	PIB2	D6VUB4; E9P8Z2; P53191; sce:YGL023C	2,05E+07	2,01E+07	2,11E+07	2,06E+07	1,30E+08	4,91E+07	8,01E+07	8,64E+07	4,19	0,05
P36018	YPT52	D6VX79; P36018; sce:YKR014C	1,83E+08	2,00E+08	1,67E+08	1,83E+08	7,64E+07	1,02E+08	1,58E+08	1,12E+08	0,61	0,05
P40074	AVT6	D3DM25; P40074; sce:YER119C	1,37E+08	1,16E+08	1,04E+08	1,19E+08	2,46E+08	1,80E+08	1,60E+08	1,95E+08	1,64	0,05
Q12013	AKR2	D6W2A0; Q12013; sce:YOR034C	1,55E+08	9,13E+07	1,68E+08	1,38E+08	3,95E+07	4,15E+07	4,11E+07	4,07E+07	0,29	0,05

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

P53838	BOR1	D6W0R9; P53838; sce:YNL275W	7,68E+06	9,45E+06	3,45E+06	6,86E+06	1,45E+06	2,06E+06	2,58E+06	2,03E+06	0,30	0,06
Q12116	TMS1	D6VS91; Q12116; sce:YDR105C	3,33E+07	3,19E+07	2,61E+07	3,04E+07	7,89E+07	4,95E+07	8,33E+07	7,06E+07	2,32	0,06
P53283	TPO2	D6VUR9; P53283; sce:YGR138C	8,84E+07	1,28E+08	3,13E+07	8,26E+07	5,68E+06	6,48E+06	1,30E+07	8,38E+06	0,10	0,06
P38959	VPS41	D6VS67; P38959; P87334; Q12011; sce:YDR080W	6,27E+07	5,83E+07	8,12E+07	6,74E+07	8,99E+07	8,32E+07	1,06E+08	9,30E+07	1,38	0,06
Q05521	DPP1	D6VSR4; Q05521; sce:YDR284C	5,75E+08	5,87E+08	7,58E+08	6,40E+08	4,34E+08	4,08E+08	5,31E+08	4,58E+08	0,72	0,06
Q08921	TCO89	D6W3I8; Q08921; sce:YPL180W	1,10E+07	7,98E+06	1,53E+07	1,14E+07	2,48E+07	6,21E+07	3,40E+07	4,03E+07	3,53	0,06
P38287	CSH1	D6VQF6; P38287; sce:YBR161W	1,86E+06	2,18E+06	1,10E+06	1,71E+06	4,98E+05	2,65E+05	1,18E+06	6,49E+05	0,38	0,07
P53879	RHO5	D6W106; P53879; sce:YNL180C	6,07E+07	5,11E+07	2,54E+07	4,57E+07	2,40E+07	7,00E+06	1,85E+07	1,65E+07	0,36	0,07
Q12255	NYV1	D6VY93; Q12255; sce:YLR093C	4,50E+08	3,46E+08	4,07E+08	4,01E+08	2,78E+08	2,83E+08	3,53E+08	3,04E+08	0,76	0,07
P47159	YJR124C	D6VWU3; P47159; Q6Q5M7; sce:YJR124C	6,41E+07	6,43E+07	9,70E+07	7,51E+07	1,00E+08	1,15E+08	9,88E+07	1,05E+08	1,39	0,07
Q03308	VPS16	D6W3W9; Q03078; Q03308; sce:YPL045W	5,26E+07	4,05E+07	7,25E+07	5,52E+07	3,29E+07	1,72E+07	3,59E+07	2,87E+07	0,52	0,07
P06780	RHO1	D6W4G6; P06780; sce:YPR165W	1,34E+09	1,06E+09	9,60E+08	1,12E+09	8,64E+08	6,43E+08	8,78E+08	7,95E+08	0,71	0,08
P38142	YBR241C	D6VQN7; P38142; Q6B2Y5; sce:YBR241C	1,23E+08	1,44E+08	8,98E+07	1,19E+08	2,54E+08	1,50E+08	1,89E+08	1,97E+08	1,66	0,08
P27810	KTR1	D6W2G0; P27810; sce:YOR099W	2,99E+08	2,56E+08	1,40E+08	2,32E+08	8,44E+07	1,21E+08	1,44E+08	1,16E+08	0,50	0,08
P41318	LST8	D6W1H1; P41318; sce:YNL006W	2,47E+07	2,16E+07	2,36E+07	2,33E+07	5,68E+07	6,46E+07	3,45E+07	5,20E+07	2,23	0,08
P48836	VMA10	D3DKY7; P48836; sce:YHR039C-A	2,01E+07	4,50E+07	1,02E+07	2,51E+07	3,70E+07	7,36E+07	7,74E+07	6,27E+07	2,50	0,09
P38130	KTR3	D6VQK3; E9P931; P38130; sce:YBR205W	2,24E+08	2,06E+08	9,50E+07	1,75E+08	7,24E+07	6,02E+07	1,05E+08	7,91E+07	0,45	0,09
P32798	COT1	D6W314; P32798; sce:YOR316C	6,42E+08	5,31E+08	6,10E+08	5,94E+08	8,72E+08	6,30E+08	8,34E+08	7,79E+08	1,31	0,09
Q08234	YOL075C	D6W1Z3; Q08233; Q08234; sce:YOL075C	1,00E+07	9,76E+06	3,34E+06	7,71E+06	1,61E+06	3,33E+05	1,21E+06	1,05E+06	0,14	0,09
Q12407	YDL199C	D6VRF5; Q12407; sce:YDL199C	6,49E+07	8,23E+07	6,27E+07	7,00E+07	7,92E+07	8,75E+07	9,74E+07	8,81E+07	1,26	0,09

Q00582	GTR1	D6W0G3; Q00582; sce:YML121W	9,23E+06	9,50E+06	1,86E+07	1,24E+07	7,56E+06	4,41E+06	3,14E+06	5,04E+06	0,40	0,09
P39962	YCK3	D3DM29; P39962; sce:YER123W	6,36E+08	5,22E+08	4,82E+08	5,47E+08	4,99E+08	2,94E+08	3,30E+08	3,74E+08	0,68	0,09
P53388	DIP5	D6W3A5; P53388; sce:YPL265W	8,10E+07	7,15E+07	3,38E+07	6,21E+07	1,94E+07	1,77E+07	1,72E+07	1,81E+07	0,29	0,09
P42837	FIG4	D6W0M2; P42837; sce:YNL325C	9,78E+07	2,05E+08	1,00E+08	1,34E+08	2,00E+08	2,08E+08	2,58E+08	2,22E+08	1,65	0,09
P40528	SYG1	D6VVN4; P40528; P40964; sce:YIL047C	6,47E+08	5,15E+08	3,74E+08	5,12E+08	1,01E+09	6,18E+08	8,10E+08	8,13E+08	1,59	0,09
P35724	MNR2	D6VXM3; P35724; sce:YKL064W	1,66E+08	1,57E+08	1,36E+08	1,53E+08	1,75E+08	1,93E+08	1,68E+08	1,78E+08	1,17	0,10
P38329	VHC1	D6VQN1; P38329; sce:YBR235W	3,29E+08	3,12E+08	2,59E+08	3,00E+08	4,40E+08	3,26E+08	3,85E+08	3,84E+08	1,28	0,10
P34756	FAB1	D6VTQ0; P34756; sce:YFR019W	1,72E+07	2,30E+07	3,58E+07	2,54E+07	1,23E+07	1,20E+07	1,57E+07	1,33E+07	0,53	0,10
Q08227	INP54	D6W202; Q08227; sce:YOL065C	2,29E+08	2,12E+08	1,61E+08	2,00E+08	3,35E+08	2,22E+08	4,10E+08	3,22E+08	1,61	0,11
Q99385	VCX1	D6VRM2; O13580; O13581; Q99385; sce:YDL128W	8,52E+08	9,21E+08	1,03E+09	9,36E+08	7,22E+08	8,82E+08	7,54E+08	7,86E+08	0,84	0,11
P46989	ATG27	D6VW11; P46989; Q86ZR9; sce:YJL178C	1,51E+09	1,48E+09	1,95E+09	1,65E+09	1,89E+09	2,87E+09	2,22E+09	2,33E+09	1,41	0,11
Q12010	YPQ1	D6W1X6; Q12010; sce:YOL092W	4,92E+08	7,11E+08	5,54E+08	5,86E+08	8,22E+08	7,79E+08	6,60E+08	7,53E+08	1,29	0,11
P13856	RSR1	D6VUT2; P13856; sce:YGR152C	2,80E+07	2,21E+07	1,85E+07	2,29E+07	1,60E+07	1,84E+07	1,67E+07	1,71E+07	0,75	0,11
P53142	VPS73	D6VU42; P53142; sce:YGL104C	2,85E+06	2,09E+06	1,85E+06	2,27E+06	6,68E+05			6,68E+05	0,29	0,12
P43561	FET5	D6VTI9; P43561; sce:YFL041W	1,23E+09	1,57E+09	9,16E+08	1,24E+09	1,47E+09	1,67E+09	1,85E+09	1,66E+09	1,34	0,13
P10591	SSA1	D6VPL2; P10591; sce:YAL005C	2,52E+07	2,18E+07	1,01E+07	1,90E+07	2,93E+07	2,64E+07	4,66E+07	3,41E+07	1,79	0,13
Q07895	YLR001C	D6VY03; Q07895; sce:YLR001C	2,82E+08	3,34E+08	4,90E+08	3,68E+08	2,80E+08	2,31E+08	2,32E+08	2,48E+08	0,67	0,13
P40514	YIL067C	D6VVL7; P40514; Q6B2R2; sce:YIL067C	4,34E+06	3,48E+06	1,21E+06	3,01E+06	1,78E+06	9,49E+05	8,52E+05	1,19E+06	0,40	0,14
P47075	VTC4	D6VWG5; P47075; Q7Z872; Q86ZR4; sce:YJL012C	2,67E+09	1,68E+09	1,33E+09	1,89E+09	2,69E+09	2,40E+09	3,69E+09	2,93E+09	1,54	0,14

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

P38225	FAT1	D6VQ41; O60021; P38225; Q86ZS3; sce:YBR041W	2,33E+08	3,22E+08	7,43E+08	4,33E+08	8,34E+08	6,08E+08	7,97E+08	7,46E+08	1,73	0,14
P35169	TOR1	D6VWN7; P35169; sce:YJR066W	6,11E+08	6,28E+08	9,66E+08	7,35E+08	5,99E+08	3,58E+08	5,14E+08	4,90E+08	0,67	0,15
Q03823	YMR160W	D6VZY2; Q03823; sce:YMR160W	1,01E+08	9,47E+07	1,19E+08	1,05E+08	2,14E+08	1,07E+08	1,66E+08	1,62E+08	1,55	0,15
Q06810	OPY2	D6W479; Q06810; Q6B149; sce:YPR075C	3,31E+07	1,54E+07	2,09E+07	2,31E+07	9,21E+06	1,65E+07	1,32E+07	1,30E+07	0,56	0,15
P53290	GTR2	D6VUU7; P53290; sce:YGR163W	5,22E+06	7,21E+06	1,35E+07	8,65E+06	5,41E+06	3,11E+06	3,58E+06	4,03E+06	0,47	0,15
Q03441	RMD1	D6VRY7; Q03441; Q05210; sce:YDL001W	2,26E+08	2,25E+08	2,55E+08	2,35E+08	3,41E+08	2,37E+08	2,94E+08	2,91E+08	1,24	0,15
P32867	SSO1	D6W3D8; P32867; sce:YPL232W	1,32E+08	5,91E+07	2,60E+07	7,22E+07	2,17E+07	2,53E+06	2,50E+07	1,64E+07	0,23	0,16
Q12016	VPS68	D6W1T9; Q12016; sce:YOL129W	3,07E+07	9,23E+07	8,19E+07	6,83E+07	1,55E+07	3,49E+07	4,57E+07	3,20E+07	0,47	0,16
P34110	VPS35	D6VW33; P34110; sce:YJL154C	8,70E+07	6,01E+07	2,53E+07	5,75E+07	3,89E+07	1,56E+07	1,86E+07	2,44E+07	0,42	0,16
P17064	FCY2	D3DLV9; P17064; sce:YER056C	1,26E+08	1,29E+08	5,15E+07	1,02E+08	5,55E+07	3,81E+07	5,30E+07	4,89E+07	0,48	0,17
P32343	SSH4	D6VX71; P32343; sce:YKL124W	8,87E+07	9,58E+07	1,00E+08	9,49E+07	9,66E+07	1,51E+08	1,19E+08	1,22E+08	1,29	0,17
P38742	NPR3	D3DKU5; P38742; sce:YHL023C	7,05E+06	5,28E+06	1,09E+07	7,73E+06	4,61E+06		2,75E+06	3,68E+06	0,48	0,17
P12868	PEP5	D6W057; P12868; sce:YMR231W	1,41E+08	1,20E+08	2,17E+08	1,59E+08	1,05E+08	8,92E+07	9,83E+07	9,77E+07	0,61	0,17
Q06247	YLR173W	D6VYH7; Q06247; sce:YLR173W	1,13E+08	1,24E+08	1,35E+08	1,24E+08	1,22E+08	1,78E+08	1,59E+08	1,53E+08	1,23	0,17
P20795	VPS33	D6VZ31; P20795; sce:YLR396C	7,75E+07	9,92E+07	4,22E+07	7,29E+07	1,04E+08	9,62E+07	1,01E+08	1,01E+08	1,38	0,18
Q12096	GNT1	D6W318; Q12096; sce:YOR320C	4,59E+07	5,08E+07	3,12E+07	4,26E+07	1,11E+07	3,21E+07	3,63E+07	2,65E+07	0,62	0,18
Q3E7B6	VMA9	D6VR05; Q3E7B6; sce:YCL005W-A	1,31E+09	9,54E+08	1,34E+09	1,20E+09	1,21E+09	6,91E+08	5,64E+08	8,22E+08	0,68	0,18
P21576	VPS1	D6VXT7; P21576; sce:YKR001C	2,89E+08	2,36E+08	3,99E+08	3,08E+08	1,83E+08	2,45E+08	2,46E+08	2,25E+08	0,73	0,19
Q04491	SEC13	D6VYK9; Q04491; sce:YLR208W	6,65E+05	2,16E+06	8,28E+04	9,68E+05	3,19E+06	9,88E+05	3,40E+06	2,53E+06	2,61	0,19
P32610	VMA8	D3DLJ9; P32610; sce:YEL051W	1,32E+08	1,10E+08	7,58E+07	1,06E+08	1,74E+08	1,04E+08	1,70E+08	1,49E+08	1,41	0,20

P30605	ITR1	D6VTB9; P30605; sce:YDR497C	1,31E+08	7,26E+07	5,01E+07	8,45E+07	6,22E+07	3,91E+07	3,40E+07	4,51E+07	0,53	0,20
P39980	SIT1	D3DLI5; E9P951; P39980; sce:YEL065W	1,20E+06	1,41E+06	2,44E+05	9,53E+05	2,61E+06	7,81E+05	3,29E+06	2,23E+06	2,34	0,20
Q03516	RSN1	D6W092; Q03516; sce:YMR266W	4,58E+08	4,07E+08	1,23E+08	3,29E+08	1,79E+08	1,01E+08	1,30E+08	1,37E+08	0,41	0,20
Q12241	VAM3	D6W2G5; Q12241; sce:YOR106W	1,82E+08	9,47E+07	1,25E+08	1,34E+08	1,24E+08	6,54E+07	6,62E+07	8,52E+07	0,64	0,20
P47008	SFH5	D6VW40; P47008; Q6Q535; sce:YJL145W	7,14E+06	5,44E+06	3,85E+06	5,47E+06	8,02E+06	5,81E+06	1,24E+07	8,74E+06	1,60	0,20
P31412	VMA5	D6VXK7; P31412; sce:YKL080W	4,98E+07	1,36E+08	4,88E+07	7,81E+07	9,01E+07	1,48E+08	2,91E+08	1,76E+08	2,26	0,21
P38182	ATG8	D6VPS5; P38182; sce:YBL078C	2,33E+07	2,79E+07	3,70E+07	2,94E+07	3,06E+07	5,44E+07	3,89E+07	4,13E+07	1,40	0,22
Q04121	NHX1	D6VT80; Q04121; sce:YDR456W	4,08E+06	2,73E+06	1,22E+06	2,68E+06	1,53E+06	1,87E+06	7,47E+05	1,38E+06	0,52	0,22
Q04562	TMN2	D6VS93; Q04562; sce:YDR107C	4,63E+06	4,34E+06	1,84E+06	3,60E+06	1,96E+06	2,40E+06	1,86E+06	2,08E+06	0,58	0,22
P14772	BPT1	D6VXY7; P14772; sce:YLL015W	1,20E+09	1,26E+09	1,80E+09	1,42E+09	1,70E+09	1,79E+09	1,73E+09	1,74E+09	1,23	0,23
P53011	SEH1	D6VU45; P53011; sce:YGL100W	1,44E+08	1,47E+08	7,75E+07	1,23E+08	1,16E+08	4,51E+07	8,18E+07	8,09E+07	0,66	0,24
P18962	DAP2	D3DKX5; E9P957; P18962; sce:YHR028C	2,10E+09	2,18E+09	2,39E+09	2,22E+09	2,18E+09	2,56E+09	2,59E+09	2,44E+09	1,10	0,24
P53918	ESBP6	D6W158; P53918; sce:YNL125C	4,08E+06	3,45E+06	2,77E+05	2,60E+06	7,64E+05	4,28E+05	8,50E+05	6,81E+05	0,26	0,24
P50945	MIC27	D6W179; P50945; Q45U03; sce:YNL100W	3,57E+08	2,82E+08	1,04E+08	2,48E+08	2,10E+08	9,41E+07	9,44E+07	1,33E+08	0,54	0,24
P25356	BPH1	D6VR42; P25356; Q02396; Q07348; sce:YCR032W	3,45E+07	3,12E+07	4,16E+07	3,57E+07	4,11E+07	9,80E+06	1,48E+07	2,19E+07	0,61	0,25
P22543	VPS34	D6VYN8; P22543; sce:YLR240W	7,49E+07	7,35E+07	9,46E+07	8,10E+07	7,44E+07	6,26E+07	7,41E+07	7,04E+07	0,87	0,25
P47040	YHC3	D6VWC3; O60004; P47040; Q6B1E1; sce:YJL059W	1,64E+07	1,73E+07	1,48E+07	1,62E+07	2,84E+07	1,75E+07	1,89E+07	2,16E+07	1,34	0,25
Q06451	TPO3	D6W4F2; Q06451; sce:YPR156C	1,82E+08	2,19E+08	3,98E+07	1,47E+08	8,31E+07	4,47E+07	9,05E+07	7,28E+07	0,49	0,26
P32600	TOR2	D6VX00; P32600; sce:YKL203C	1,65E+08	1,71E+08	4,04E+08	2,47E+08	1,32E+08	1,12E+08	1,30E+08	1,24E+08	0,50	0,26

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

P38301	GDT1	D6VQI2; E9PAD4; P38301; Q6Q545; sce:YBR187W	4,75E+07	3,66E+07	1,28E+07	3,23E+07	2,33E+07	1,28E+07	1,97E+07	1,86E+07	0,58	0,27
Q12057	PIN2	D6W2G4; Q12057; sce:YOR104W	3,03E+06	1,30E+06	6,47E+05	1,66E+06	1,96E+05		6,28E+05	4,12E+05	0,25	0,27
P38356	BSD2	D6VQT5; P38356; sce:YBR290W	1,09E+06	1,35E+06	2,22E+05	8,88E+05	4,77E+05	3,23E+05	3,44E+05	3,81E+05	0,43	0,27
Q02205	MEH1	D6VX73; Q02205; sce:YKR007W	9,67E+07	4,78E+07	1,24E+08	8,96E+07	9,40E+07	3,45E+07	2,53E+07	5,13E+07	0,57	0,29
P21801	SDH2	D6VXW5; P21801; sce:YLL041C	5,90E+07	6,39E+07	1,83E+07	4,71E+07	5,03E+07	6,69E+07	9,88E+07	7,20E+07	1,53	0,29
P27514	PHO91	D6W1I8; P27514; sce:YNR013C	1,68E+09	1,35E+09	1,11E+09	1,38E+09	1,36E+09	1,09E+09	9,33E+08	1,13E+09	0,82	0,29
P47041	BIT61	D6VWC4; P47041; sce:YJL058C	4,80E+05	5,19E+05	1,03E+06	6,76E+05		4,66E+05	2,72E+05	3,69E+05	0,55	0,29
Q12301	YDL180W	D6VRH2; Q12301; sce:YDL180W	9,81E+07	8,03E+07	8,21E+07	8,68E+07	1,28E+08	8,55E+07	9,74E+07	1,04E+08	1,19	0,29
P39968	VAC8	D3DLN6; P39968; Q549T0; sce:YEL013W	5,62E+09	4,10E+09	6,47E+09	5,40E+09	4,49E+09	4,27E+09	4,88E+09	4,54E+09	0,84	0,30
Q04338	VTI1	D6W022; Q04338; sce:YMR197C	6,90E+08	5,50E+08	5,27E+08	5,89E+08	7,17E+08	7,96E+08	5,60E+08	6,91E+08	1,17	0,30
P38244	PFF1	D6VQ74; P38244; P38245; Q86ZS2; sce:YBR074W	1,70E+09	1,78E+09	2,05E+09	1,85E+09	1,77E+09	1,79E+09	1,44E+09	1,66E+09	0,90	0,30
Q04162	YDR387C	D6VT21; Q04162; sce:YDR387C	1,66E+07	1,89E+07	2,56E+07	2,04E+07	2,44E+07	2,83E+07	2,06E+07	2,45E+07	1,20	0,31
P27801	PEP3	D6VYE3; P27801; sce:YLR148W	9,32E+07	1,08E+08	1,89E+08	1,30E+08	5,08E+07	7,91E+07	1,29E+08	8,64E+07	0,66	0,31
Q06328	YPQ2	D6VSY2; Q06328; sce:YDR352W	2,00E+06	3,04E+06	8,91E+05	1,97E+06	1,80E+06	1,15E+06	3,82E+05	1,11E+06	0,56	0,31
Q08299	ENB1	D6W1R2; Q08299; sce:YOL158C	2,95E+07	2,17E+07	5,92E+06	1,91E+07	3,97E+07	1,69E+07	3,36E+07	3,01E+07	1,58	0,32
Q06651	PIB1	D6VSU2; Q06651; Q6Q5S5; sce:YDR313C	3,11E+06		1,78E+06	2,44E+06	1,11E+06	1,27E+06	1,38E+06	1,25E+06	0,51	0,32
Q04602	VBA4	D6VSA4; Q04602; sce:YDR119W	4,85E+08	3,59E+08	3,09E+08	3,84E+08	6,39E+08	3,63E+08	4,72E+08	4,91E+08	1,28	0,33
P22219	VPS15	D6VQ98; P22219; sce:YBR097W	4,01E+09	4,15E+09	1,49E+09	3,21E+09	2,16E+09	1,67E+09	2,73E+09	2,19E+09	0,68	0,33
P32366	VMA6	D6VZ81; P32366; sce:YLR447C	1,28E+09	1,99E+09	7,39E+08	1,34E+09	7,48E+08	9,06E+08	1,10E+09	9,17E+08	0,69	0,33

P11491	PHO8	D6VTA4; E9P949; P11491; Q03374; sce:YDR481C	2,91E+09	2,75E+09	2,43E+09	2,70E+09	3,52E+09	2,90E+09	2,64E+09	3,02E+09	1,12	0,34
Q03730	YML018C	D6VZF6; Q03730; Q6B2K7; sce:YML018C	1,64E+08	1,03E+08	1,05E+08	1,24E+08	2,84E+08	1,21E+08	1,39E+08	1,81E+08	1,46	0,36
P38731	ARN1	D3DKS9; P38731; sce:YHL040C	3,93E+05	4,58E+05	2,18E+05	3,56E+05	3,29E+05	6,82E+05	2,87E+06	1,29E+06	3,63	0,36
P43555	EMP47	D6VTI2; P43555; sce:YFL048C	2,57E+08	2,95E+08	1,73E+08	2,42E+08	2,20E+08	3,86E+08	3,03E+08	3,03E+08	1,26	0,36
P31381	FUN26	D6VPJ6; P31381; sce:YAL022C	1,31E+08	1,04E+08	1,16E+08	1,17E+08	1,93E+08	1,23E+08	1,15E+08	1,44E+08	1,22	0,37
P38090	AGP2	D6VQC9; P38090; sce:YBR132C	4,23E+07	3,48E+07	2,01E+07	3,24E+07	2,49E+07	2,19E+07	2,97E+07	2,55E+07	0,79	0,37
Q08157	YOL019W	D6W247; Q08157; sce:YOL019W	3,04E+07	2,40E+07	8,84E+06	2,11E+07	3,13E+07	2,41E+07	2,81E+07	2,78E+07	1,32	0,37
Q05919	VPS38	D6VYZ7; Q05919; Q6B1J7; sce:YLR360W	1,39E+07	1,35E+07	3,46E+07	2,07E+07	1,01E+07	1,27E+07	1,56E+07	1,28E+07	0,62	0,38
Q12209	FRE8	D6VY49; Q12209; sce:YLR047C	1,36E+08	1,27E+08	1,10E+08	1,24E+08	1,31E+08	1,22E+08	1,65E+08	1,39E+08	1,12	0,38
P39923	NPR2	D3DLI8; P39923; Q6B264; sce:YEL062W	9,35E+06	3,77E+06	2,76E+07	1,36E+07	1,18E+07	3,78E+06	4,19E+06	6,58E+06	0,48	0,41
Q12473	FRE6	D6VXV7; Q12473; sce:YLL051C	1,33E+08	1,17E+08	1,63E+08	1,37E+08	1,79E+08	1,17E+08	1,86E+08	1,60E+08	1,17	0,42
P40501	AVT7	D6VVJ9; P40501; sce:YIL088C	2,96E+08	4,12E+08	4,66E+08	3,91E+08	4,09E+08	4,57E+08	4,46E+08	4,37E+08	1,12	0,43
P39111	VMA7	D6VUF5; P39111; sce:YGR020C	2,47E+05	8,35E+05		5,41E+05	2,77E+06	9,96E+05	3,90E+05	1,39E+06	2,56	0,44
P23643	VPS3	D6VTB7; P23643; sce:YDR495C	3,98E+06	5,00E+06	5,78E+06	4,92E+06	6,81E+06	3,90E+06	6,93E+06	5,88E+06	1,20	0,44
P40857	PHS1	D6VW87; P40857; sce:YJL097W	4,06E+07	3,69E+07	2,12E+07	3,29E+07	2,78E+07	2,39E+07	3,09E+07	2,75E+07	0,84	0,44
Q07468	VAM6	D6VRS2; Q07468; sce:YDL077C	1,04E+08	8,77E+07	1,89E+08	1,27E+08	8,93E+07	9,59E+07	1,07E+08	9,74E+07	0,77	0,45
P38778	SMF2	D3DKZ8; P38778; sce:YHR050W	5,54E+06	6,06E+06	1,95E+06	4,52E+06	7,69E+06	4,59E+06	5,26E+06	5,85E+06	1,29	0,45
P32854	PEP12	D6W2A3; P32854; sce:YOR036W	4,24E+07	2,45E+07	2,85E+07	3,18E+07	3,15E+07	2,91E+07	1,85E+07	2,64E+07	0,83	0,46
Q12284	ERV2	D6W448; Q12284; sce:YPR037C	2,83E+07	2,92E+07	1,69E+07	2,48E+07	1,91E+07	2,18E+07	2,34E+07	2,14E+07	0,86	0,46
P32563	VPH1	D6W2X0; P32563; sce:YOR270C	1,57E+10	1,72E+10	1,98E+10	1,76E+10	1,32E+10	1,94E+10	1,48E+10	1,58E+10	0,90	0,47

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

P22203	VMA4	D6W328; P22203; sce:YOR332W	2,77E+08	4,51E+08	1,60E+08	2,96E+08	2,33E+08	5,56E+08	4,02E+08	3,97E+08	1,34	0,47
Q12256	TPO4	D6W2X3; Q12256; sce:YOR273C	2,07E+08	2,60E+08	1,17E+08	1,95E+08	2,35E+08	1,99E+08	2,57E+08	2,31E+08	1,18	0,47
Q02948	VPS30	D6W3P7; Q02948; sce:YPL120W	3,83E+07	3,49E+07	5,48E+07	4,27E+07	3,32E+07	4,11E+07	3,87E+07	3,76E+07	0,88	0,49
P20107	ZRC1	D6W070; P20107; sce:YMR243C	2,88E+09	2,75E+09	2,38E+09	2,67E+09	4,10E+09	2,43E+09	2,73E+09	3,08E+09	1,15	0,49
P53285	VPS62	D6VUS1; P53285; sce:YGR141W	5,31E+08	4,03E+08	4,34E+08	4,56E+08	6,15E+08	4,37E+08	4,69E+08	5,07E+08	1,11	0,49
P33311	MDL2	D6W3A0; P33311; Q08983; sce:YPL270W	1,27E+08	9,17E+07	4,82E+07	8,89E+07	1,02E+08	3,92E+07	5,93E+07	6,70E+07	0,75	0,50
P11792	SCH9	D3DLF4; P11792; sce:YHR205W	1,88E+06	1,38E+06	3,03E+06	2,10E+06	9,86E+05	3,41E+06	4,30E+06	2,90E+06	1,38	0,51
Q07549	SNA4	D6VRM7; Q07549; sce:YDL123W	3,84E+08	1,83E+08	9,64E+07	2,21E+08	4,23E+08	1,91E+08	2,80E+08	2,98E+08	1,35	0,52
Q12003	ENV7	D6W3D4; Q12003; Q7LGU7; sce:YPL236C	6,89E+07	8,05E+07	1,05E+08	8,48E+07	9,51E+07	7,42E+07	1,21E+08	9,66E+07	1,14	0,53
P50079	HSV2	D6VV05; P50079; sce:YGR223C	3,75E+07	1,98E+07	7,26E+07	4,33E+07	6,08E+07	3,54E+07	7,00E+07	5,54E+07	1,28	0,55
Q07824	TPO1	D6VXX6; Q07824; sce:YLL028W	1,33E+08	1,38E+08	3,64E+07	1,02E+08	1,01E+08	5,31E+07	8,31E+07	7,91E+07	0,77	0,55
P17255	VMA1	D6VRG7; O74301; P17255; Q9Y7W5; sce:YDL185W	1,18E+09	1,77E+09	6,35E+08	1,19E+09	9,71E+08	1,81E+09	1,60E+09	1,46E+09	1,22	0,56
P19073	CDC42	D6VYN1; P19073; Q05978; sce:YLR229C	7,89E+08	7,53E+08	3,20E+08	6,21E+08	1,15E+09	5,05E+08	6,71E+08	7,74E+08	1,25	0,56
P53882	TDA7	D6W109; P53882; sce:YNL176C	9,49E+06	1,76E+06	1,33E+06	4,20E+06	3,06E+06	2,11E+06	2,03E+06	2,40E+06	0,57	0,57
P25371	ADP1	D6VR20; P25371; sce:YCR011C	1,51E+09	1,57E+09	2,10E+09	1,73E+09	2,01E+09	2,03E+09	1,58E+09	1,87E+09	1,09	0,57
P40335	PEP8	D6VWC9; E9P910; P40335; sce:YJL053W	1,08E+07	1,13E+07	1,06E+07	1,09E+07	1,36E+07	7,05E+06	8,05E+06	9,56E+06	0,88	0,58
P41807	VMA13	D6W446; P41807; sce:YPR036W	1,14E+08	2,03E+08	1,53E+08	1,57E+08	1,54E+08	1,41E+08	2,54E+08	1,83E+08	1,17	0,58
Q04272	VPS20	D6VZQ1; Q04272; sce:YMR077C	2,73E+06	2,37E+06	1,43E+06	2,18E+06	1,96E+06	9,62E+05	2,53E+06	1,82E+06	0,84	0,59
P10592	SSA2	D6VXY0; P10592; sce:YLL024C	3,46E+08	3,14E+08	2,36E+08	2,99E+08	2,52E+08	3,19E+08	4,36E+08	3,36E+08	1,12	0,59
P38873	KOG1	D3DLD4; P38873; sce:YHR186C	1,10E+08	1,42E+08	2,97E+08	1,83E+08	1,86E+08	2,24E+08	2,42E+08	2,17E+08	1,19	0,60

P37296	STV1	D6VZM9; P37296; sce:YMR054W	2,24E+08	2,65E+08	2,98E+08	2,63E+08	2,01E+08	2,89E+08	2,43E+08	2,44E+08	0,93	0,61
P32939	YPT7	D6VZH4; P32939; sce:YML001W	1,29E+09	1,42E+09	1,83E+09	1,51E+09	1,33E+09	1,82E+09	1,74E+09	1,63E+09	1,08	0,62
P47082	AVT1	D6VWH5; P47082; sce:YJR001W	3,12E+08	3,02E+08	3,40E+08	3,18E+08	3,43E+08	3,08E+08	2,61E+08	3,04E+08	0,96	0,63
Q12296	MAM3	D6W207; Q12296; sce:YOL060C	5,93E+08	3,91E+08	5,95E+08	5,26E+08	6,68E+08	5,17E+08	5,22E+08	5,69E+08	1,08	0,64
P21560	CBP3	D6W3F5; P21560; sce:YPL215W	6,84E+07	6,47E+07	1,39E+07	4,90E+07	2,93E+07	3,70E+07	5,34E+07	3,99E+07	0,81	0,65
P25515	VMA3	D3DLM1; P25515; sce:YEL027W	2,49E+08	2,19E+08	7,63E+08	4,11E+08	1,03E+09	3,24E+08	3,06E+08	5,54E+08	1,35	0,66
Q06708	VAC14	D6VZ21; Q06708; sce:YLR386W	1,11E+08	1,43E+08	2,59E+08	1,71E+08	1,57E+08	1,43E+08	3,15E+08	2,05E+08	1,20	0,66
P32912	VAM7	D6VTU3; P32912; sce:YGL212W	1,61E+08	1,23E+08	1,00E+08	1,28E+08	1,21E+08	1,34E+08	1,02E+08	1,19E+08	0,93	0,69
Q03446	RCR2	D6VRZ0; Q03446; sce:YDR003W	1,48E+07	1,15E+07	4,55E+06	1,03E+07	8,68E+06	7,99E+06	1,04E+07	9,01E+06	0,88	0,70
P53950	VAC7	D6W1C5; P53950; sce:YNL054W	6,39E+07	5,16E+07	3,61E+07	5,05E+07	4,56E+07	4,81E+07	4,81E+07	4,73E+07	0,94	0,71
P02994	TEF1; TEF2	D6VQB6; P02994; Q7Z7U8; Q7Z8Q8; Q7Z8Q9; sce:YBR118W; sce:YPR080W	1,18E+09	9,08E+08	1,25E+09	1,11E+09	8,94E+08	9,25E+08	1,32E+09	1,05E+09	0,94	0,71
P38164	SEA4	D6VPP9; P38164; Q86ZS4; sce:YBL104C	3,44E+07	2,95E+07	7,61E+07	4,67E+07	4,76E+07	3,86E+07	3,48E+07	4,03E+07	0,86	0,71
Q02887	ATG21	D6W3R6; Q02887; sce:YPL100W	1,53E+07	8,59E+06	2,96E+07	1,78E+07	3,28E+07	1,34E+07	1,73E+07	2,11E+07	1,19	0,72
P33300	SUR1	D6W3V7; P33300; sce:YPL057C	9,80E+06	9,47E+06	2,59E+06	7,28E+06	8,26E+06	3,44E+06	6,97E+06	6,22E+06	0,85	0,72
Q12200	NCR1	D6W406; Q12200; sce:YPL006W	1,36E+09	1,60E+09	1,93E+09	1,63E+09	1,42E+09	1,75E+09	1,49E+09	1,55E+09	0,95	0,72
Q08281	RTC1	D6W1T1; Q08281; Q92271; sce:YOL138C	1,53E+07	2,21E+07	2,49E+07	2,08E+07	1,68E+07	2,61E+07	2,35E+07	2,22E+07	1,07	0,74
Q04119	PPN1	D6VT77; Q04119; sce:YDR452W	2,46E+08	3,12E+08	5,26E+07	2,04E+08	1,77E+08	1,60E+08	1,91E+08	1,76E+08	0,86	0,76
P40482	SEC24	D6VVH8; P40482; sce:YIL109C	4,22E+07	5,05E+07	5,72E+07	5,00E+07	5,74E+07	3,40E+07	5,05E+07	4,73E+07	0,95	0,76
P25621	FEN2	D6VR38; P25621; sce:YCR028C	4,16E+06	3,48E+06	5,51E+05	2,73E+06	1,18E+06	4,12E+06	1,55E+06	2,28E+06	0,84	0,77

Sit4 genetically interacts with Vps27 to regulate mitochondrial function and lifespan in *Saccharomyces cerevisiae*

Q12324	YVC1	D6W2E9; Q08500; Q12324; Q7LGN3; sce:YOR087W	7,86E+08	7,80E+08	9,68E+08	8,45E+08	8,06E+08	6,50E+08	9,91E+08	8,16E+08	0,97	0,82
Q07376	MCH1	D6VRU2; Q07376; sce:YDL054C	2,20E+08	1,82E+08	1,97E+08	2,00E+08	2,80E+08	1,42E+08	2,03E+08	2,08E+08	1,04	0,85
P36062	AVT3	D6VX50; P36062; sce:YKL146W	1,51E+08	1,98E+08	2,05E+08	1,85E+08	1,93E+08	1,88E+08	1,63E+08	1,81E+08	0,98	0,85
Q12424	YDL206W	D6VRE8; Q12424; sce:YDL206W	7,10E+06	8,24E+06	7,95E+06	7,76E+06	8,60E+06	3,73E+06	9,80E+06	7,38E+06	0,95	0,86
Q12042	YPL162C	D6W3K6; Q12042; sce:YPL162C	8,25E+07	7,91E+07	8,76E+07	8,31E+07	1,43E+08	5,11E+07	3,60E+07	7,67E+07	0,92	0,87
P32842	VMA11	D6W3D6; P32365; P32842; sce:YPL234C	2,62E+08	2,17E+08	4,42E+08	3,07E+08	5,51E+08	2,57E+08	1,84E+08	3,31E+08	1,08	0,87
P38929	PMC1	D6VUD2; P38929; sce:YGL006W	2,32E+09	2,65E+09	2,76E+09	2,58E+09	2,28E+09	2,56E+09	3,02E+09	2,62E+09	1,02	0,87
Q02725	VTC3	D6W3Z3; Q02725; sce:YPL019C	3,48E+09	1,92E+09	1,45E+09	2,28E+09	2,26E+09	1,23E+09	2,96E+09	2,15E+09	0,94	0,88
P48558	BXI1	D6W0P0; P48558; Q6B2Q1; sce:YNL305C	1,27E+08	1,20E+08	9,88E+07	1,15E+08	9,73E+07	9,34E+07	1,66E+08	1,19E+08	1,03	0,89
P14359	SNA3	D6VW35; P14359; Q6B236; sce:YJL151C	8,22E+06	2,41E+06	3,58E+05	3,66E+06	6,73E+06	2,21E+06	3,08E+06	4,01E+06	1,09	0,91
P42836	PFA3	D6W0M1; P42836; sce:YNL326C	8,15E+07	9,23E+07	6,57E+07	7,98E+07	1,08E+08	5,46E+07	8,32E+07	8,18E+07	1,02	0,91
P27614	CPS1	D6VW16; P27614; sce:YJL172W	9,86E+08	8,77E+08	2,29E+08	6,97E+08	6,26E+08	7,63E+08	6,21E+08	6,70E+08	0,96	0,92
P16140	VMA2	D6VQC4; P16140; P32123; sce:YBR127C	1,09E+09	2,10E+09	7,41E+08	1,31E+09	8,79E+08	1,83E+09	1,39E+09	1,36E+09	1,04	0,92
P53273	YGR125W	D6VUQ7; P53273; sce:YGR125W	5,90E+08	4,26E+08	4,14E+08	4,77E+08	5,29E+08	4,55E+08	4,63E+08	4,83E+08	1,01	0,92
P39547	UIP3	D6VPM9; P39547; sce:YAR027W	4,00E+07	3,04E+07	1,23E+07	2,76E+07	4,19E+07	1,97E+07	1,98E+07	2,71E+07	0,98	0,97
P40046	VTC1	D3DLX7; P40046; sce:YER072W	1,20E+09	7,89E+08	6,80E+08	8,90E+08	1,01E+09	7,23E+08	9,35E+08	8,87E+08	1,00	0,99
P46996	YJL163C	D6VW24; P46996; sce:YJL163C	1,19E+07	1,44E+07	1,39E+07	1,34E+07	2,16E+07	7,31E+06	1,13E+07	1,34E+07	1,00	1,00
P32335	MSS51	D6VYK4; P32335; sce:YLR203C	1,59E+07	1,77E+07	7,90E+06	1,38E+07	1,24E+07	1,12E+07	1,79E+07	1,38E+07	1,00	1,00
P38724	ARN2	D3DKS4; P38724; sce:YHL047C					2,75E+06		3,83E+05	1,56E+06	*	*
P38735	VMR1	D3DKT3; P38735; sce:YHL035C		1,05E+06		1,05E+06					**	**

P38273	CCZ1	D6VQC8; P38273; sce:YBR131W	1,92E+07		1,31E+07	1,62E+07					**	**
P38279	RTC2	D6VQE2; P38279; sce:YBR147W			1,04E+05	1,04E+05			2,19E+05	2,19E+05	2,10	

* - protein only detected in *sit4*Δ samples; ** - proteins only detected in wild type samples

Table S2 - Gene Ontology (GO) enrichment analysis on biological processes.

Gene Ontology (GO) enrichment analysis on biological processes					
GO Biological process	Protein name	Description	Ratio protein abundance (<i>sit4Δ</i> /wt)	Fold enrichment	P value
Manganese ion homeostasis	SMF3	Putative divalent metal ion transporter involved in iron homeostasis (iron mobilization from vacuoles; important for the switch from fermentation to respiration at the DS; regulated by Aft2); transcriptionally regulated by metal ions; member of Nramp family of metal transport proteins; targeted to vacuole via AP-3 pathway; protein abundance increases in response to DNA replication stress.	1.91	36.30	1.40E-04
	CCC1	Vacuolar Fe ²⁺ /Mn ²⁺ transporter; transports Fe into the vacuoles; upregulated by Yap5 (when Fe levels are high), and by Snf1-Msn2/4 (Fe-S independent); suppresses respiratory deficit of yfh1 mutants, which lack ortholog of mammalian frataxin, by preventing mitochondrial iron accumulation; relative distribution to vacuole decreases upon DNA replication stress; targeted to vacuole via AP-3 pathway.	1.83		
	PER1	Protein of the endoplasmic reticulum; required for GPI-phospholipase A2 activity that remodels the GPI anchor as a prerequisite for association of GPI-anchored proteins with lipid rafts.	0.13		
Polyphosphate metabolic process	YDR089W	Novel subunit of the vacuolar transporter chaperone complex; vacuolar transmembrane protein that regulates biosynthesis of polyphosphate; deletion reduces and overexpression increases polyP accumulation; SPX domain (Syg1, Pho81, Xpr1)-containing protein involved in phosphate homeostasis; relocates from vacuole to cytoplasm upon DNA replication stress.	0.61	25.93	3.25E-04
	VTC2	Regulatory subunit of vacuolar transporter chaperone (VTC) complex; involved in membrane trafficking, vacuolar polyphosphate accumulation, microautophagy and non-autophagic vacuolar fusion; important regulator of substrate invagination from the vacuolar membrane; targeted to vacuole via AP-3 pathway.	0.55		
	YNL217W	Zn ²⁺ -dependent endopolyphosphatase; required with PPN1 to mobilize polyphosphate stores in response to phosphate starvation; member of the PPP-superfamily of metalloproteases; localizes to the vacuolar lumen via the MVB pathway; null mutant is highly sensitive to azaserine and resistant to sodium-O-vandate.	2.13		

Vacuole fusion	IVY1	Phospholipid-binding protein that interacts with both Ypt7p and Vps33p; may partially counteract the action of Vps33p and vice versa, localizes to the rim of the vacuole as cells approach stationary phase; contains a putative inverse BAR (I-BAR) domain that can mold lipid bilayer membranes into protrusions.	3.08	16.81	1.81E-05
	YDR089W	Novel subunit of the vacuolar transporter chaperone complex; vacuolar transmembrane protein that regulates biosynthesis of polyphosphate; deletion reduces and overexpression increases polyP accumulation; SPX domain (Syl1, Pho81, Xpr1)-containing protein involved in phosphate homeostasis; relocates from vacuole to cytoplasm upon DNA replication stress.	0.61		
	VTC2	Regulatory subunit of vacuolar transporter chaperone (VTC) complex; involved in membrane trafficking, vacuolar polyphosphate accumulation, microautophagy and non-autophagic vacuolar fusion; important regulator of substrate invagination from the vacuolar membrane; targeted to vacuole via AP-3 pathway.	0.55		
	YCF1	Vacuolar glutathione S-conjugate transporter; ABC-C transporter of the ATP-binding cassette family; required for vacuole fusion; forms stable complexes with vacuole fusion machinery; regulates Vam7p recruitment to vacuoles; role in detoxifying metals (Cd, Hg, As); transports GSSG that is not immediately reduced in cytosol to vacuole; transports unconjugated bilirubin, selenodiglutathione, oxidized glutathione; similar to human cystic fibrosis protein CFTR.	1.20		
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF) during the disassembly of postfusion cis-SNARE complexes; stimulates the ATPase activity of Sec18p; peripheral membrane protein required for vesicular transport between ER and Golgi, the 'priming' step in homotypic vacuole fusion, and autophagy; similar to mammalian alpha-SNAP.	2.10		
Vacuole organization	MYO2	Type V myosin motor involved in actin-based transport of cargos; required for the polarized delivery of secretory vesicles, the vacuole, late Golgi elements, peroxisomes, lipid droplets, and the mitotic spindle; effector of Ypt11p, a rab-type small GTPase.	0.29	12.24	7.15E-08
	IVY1	Phospholipid-binding protein that interacts with both Ypt7p and Vps33p; may partially counteract the action of Vps33p and vice versa, localizes to the rim of the vacuole as cells approach stationary phase; contains a putative inverse BAR (I-BAR) domain that can mold lipid bilayer membranes into protrusions.	3.08		
	YDR089W	Novel subunit of the vacuolar transporter chaperone complex; vacuolar transmembrane protein that regulates biosynthesis of polyphosphate; deletion reduces and overexpression increases polyP accumulation; SPX domain (Syl1, Pho81, Xpr1)-containing protein involved in phosphate homeostasis; relocates from vacuole to cytoplasm upon DNA replication stress.	0.61		
	CSC1	Calcium permeable gated cation channel; functions as an osmosensitive, calcium-permeable cation channel when expressed in a heterologous system; may be involved in detoxification.	0.69		
	VTC2	Regulatory subunit of vacuolar transporter chaperone (VTC) complex; involved in membrane trafficking, vacuolar polyphosphate accumulation, microautophagy and non-autophagic vacuolar fusion; important regulator of substrate invagination from the vacuolar membrane; targeted to vacuole via AP-3 pathway.	0.55		

	YCF1	Vacuolar glutathione S-conjugate transporter; ABC-C transporter of the ATP-binding cassette family; required for vacuole fusion; forms stable complexes with vacuole fusion machinery; regulates Vam7p recruitment to vacuoles; role in detoxifying metals (Cd, Hg, As); transports GSSG that is not immediately reduced in cytosol to vacuole; transports unconjugated bilirubin, selenodiglutathione, oxidized glutathione; similar to human cystic fibrosis protein CFTR.	1.20		
	VAC17	Phosphoprotein involved in vacuole inheritance; degraded in late M phase of the cell cycle; acts as a vacuole-specific receptor for myosin Myo2p; involved in regulation of asymmetric inheritance of aggregated/misfolded proteins and age reset.	2.35		
	ATG18	Phosphoinositide binding protein; required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (CVT) pathway; binds PtdIns(3,5)P ₂ , PI3P and PI4P; WD-40 repeat containing protein and PROPPIN family member; relocalizes from the vacuole to cytoplasm upon DNA replication stress; mammalian homologs include: WIPI1, WIPI2, WIPI3 and WIPI4/WDR45; mutations in human WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood (SENDA).	2.55		
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF) during the disassembly of postfusion cis-SNARE complexes; stimulates the ATPase activity of Sec18p; peripheral membrane protein required for vesicular transport between ER and Golgi, the 'priming' step in homotypic vacuole fusion, and autophagy; similar to mammalian alpha-SNAP.	2.10		
Golgi organization	MYO2	Type V myosin motor involved in actin-based transport of cargos; required for the polarized delivery of secretory vesicles, the vacuole, late Golgi elements, peroxisomes, lipid droplets, and the mitotic spindle; effector of Ypt11p, a rab-type small GTPase.	0.29	15.12	1.94E-04
	YPT10	Rab family GTP-binding protein; contains the PEST signal sequence specific for proteolytic enzymes; may be involved in vesicular transport; overexpression leads to accumulation of Golgi-like cisternae with budding vesicles.	0.58		
	LSB6	Type II phosphatidylinositol 4-kinase; binds Las17p, a homolog of human Wiskott-Aldrich Syndrome protein involved in actin patch assembly and actin polymerization.	1.31		
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF) during the disassembly of postfusion cis-SNARE complexes; stimulates the ATPase activity of Sec18p; peripheral membrane protein required for vesicular transport between ER and Golgi, the 'priming' step in homotypic vacuole fusion, and autophagy; similar to mammalian alpha-SNAP.	2.10		
Lysosomal microautophagy	ATG14	Autophagy-specific subunit of phosphatidylinositol 3-kinase complex I; Atg14p targets complex I to the phagophore assembly site (PAS); required for localizing additional ATG proteins to the PAS; required for overflow degradation of misfolded proteins when ERAD is saturated; homolog of human Barkor; other members are Vps34, Vps15, and Vps30p.	0.20	15.12	6.28E-07
	YDR089W	Novel subunit of the vacuolar transporter chaperone complex; vacuolar transmembrane protein that regulates biosynthesis of polyphosphate; deletion reduces and overexpression increases polyP accumulation; SPX domain (Syg1, Pho81, Xpr1)-containing protein involved in phosphate homeostasis; relocalizes from vacuole to cytoplasm upon DNA replication stress.	0.61		

	HSE1	Subunit of the endosomal Vps27p-Hse1p complex; complex is required for sorting of ubiquitinated membrane proteins into intraluminal vesicles prior to vacuolar degradation, as well as for recycling of Golgi proteins and formation of luminal membranes.	2.16		
	VTC2	Regulatory subunit of vacuolar transporter chaperone (VTC) complex; involved in membrane trafficking, vacuolar polyphosphate accumulation, microautophagy and non-autophagic vacuolar fusion; important regulator of substrate invagination from the vacuolar membrane; targeted to vacuole via AP-3 pathway.	0.55		
	VPS27	Ubiquitin binding protein involved in endosomal protein sorting; K48-, K63-Ub chain binding protein that preferentially binds K63-linked chains in vitro; subunit of the ESCRT-0 complex that binds to ubiquitin via two ubiquitin-interacting motifs (UIMs) and is involved in sorting cargo into multivesicular bodies; required for recycling Golgi proteins, forming luminal membranes and sorting ubiquitinated proteins destined for degradation.	4.10		
	ATG18	Phosphoinositide binding protein; required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (CVT) pathway; binds PtdIns(3,5)P ₂ , PI3P and PI4P; WD-40 repeat containing protein and PROPPIN family member; relocalizes from the vacuole to cytoplasm upon DNA replication stress; mammalian homologs include: WIPI1, WIPI2, WIPI3 and WIPI4/WDR45; mutations in human WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood (SENDA).	2.55		
	SLM4	Subunit of EGO/GSE complex; vacuolar/endosomal membrane-associated EGO/GSE complex regulates exit from rapamycin-induced growth arrest, stimulating microautophagy and sorting of Gap1p from endosome to plasma membrane; essential for integrity and function of EGO; targeted to vacuole via AP-3 pathway; gene exhibits synthetic genetic interaction with MSS4.	2.94		
Autophagy	ATG14	Autophagy-specific subunit of phosphatidylinositol 3-kinase complex I; Atg14p targets complex I to the phagophore assembly site (PAS); required for localizing additional ATG proteins to the PAS; required for overflow degradation of misfolded proteins when ERAD is saturated; homolog of human Barkor; other members are Vps34, Vps15, and Vps30p.	0.20	6.51	3.12E-06
	YDR089W	Novel subunit of the vacuolar transporter chaperone complex; vacuolar transmembrane protein that regulates biosynthesis of polyphosphate; deletion reduces and overexpression increases polyP accumulation; SPX domain (Syg1, Pho81, Xpr1)-containing protein involved in phosphate homeostasis; relocalizes from vacuole to cytoplasm upon DNA replication stress.	0.61		
	ATG22	Vacuolar membrane protein; required for efflux of amino acids during autophagic body breakdown in vacuole; targeted to vacuole via AP-3 pathway; null mutation causes gradual loss of viability during starvation.	1.49		
	HSE1	Subunit of the endosomal Vps27p-Hse1p complex; complex is required for sorting of ubiquitinated membrane proteins into intraluminal vesicles prior to vacuolar degradation, as well as for recycling of Golgi proteins and formation of luminal membranes.	2.16		
	CSC1	Calcium permeable gated cation channel; functions as an osmosensitive, calcium-permeable cation channel when expressed in a heterologous system; may be involved in detoxification.	0.69		

	VTC2	Regulatory subunit of vacuolar transporter chaperone (VTC) complex; involved in membrane trafficking, vacuolar polyphosphate accumulation, microautophagy and non-autophagic vacuolar fusion; important regulator of substrate invagination from the vacuolar membrane; targeted to vacuole via AP-3 pathway.	0.55		
	VPS27	Ubiquitin binding protein involved in endosomal protein sorting; K48-, K63-Ub chain binding protein that preferentially binds K63-linked chains in vitro; subunit of the ESCRT-0 complex that binds to ubiquitin via two ubiquitin-interacting motifs (UIMs) and is involved in sorting cargo into multivesicular bodies; required for recycling Golgi proteins, forming luminal membranes and sorting ubiquitinated proteins destined for degradation.	4.10		
	ATG18	Phosphoinositide binding protein; required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (CVT) pathway; binds PtdIns(3,5)P2, PI3P and PI4P; WD-40 repeat containing protein and PROPPIN family member; relocalizes from the vacuole to cytoplasm upon DNA replication stress; mammalian homologs include: WIPI1, WIPI2, WIPI3 and WIPI4/WDR45; mutations in human WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood (SENDA).	2.55		
	SLM4	Subunit of EGO/GSE complex; vacuolar/endosomal membrane-associated EGO/GSE complex regulates exit from rapamycin-induced growth arrest, stimulating microautophagy and sorting of Gap1p from endosome to plasma membrane; essential for integrity and function of EGO; targeted to vacuole via AP-3 pathway; gene exhibits synthetic genetic interaction with MSS4.	2.94		
	SEC17	Alpha-SNAP cochaperone; SNARE-complex adaptor for Sec18 (NSF) during the disassembly of postfusion cis-SNARE complexes; stimulates the ATPase activity of Sec18p; peripheral membrane protein required for vesicular transport between ER and Golgi, the 'priming' step in homotypic vacuole fusion, and autophagy; similar to mammalian alpha-SNAP.	2.10		
Late endosome to vacuole transport	EAR1	Specificity factor required for Rsp5p-dependent ubiquitination; also required for sorting of specific cargo proteins at the multivesicular body; mRNA is targeted to the bud via the mRNA transport system involving She2p.	0.54	9.60	2.13E-04
	HSE1	Subunit of the endosomal Vps27p-Hse1p complex; complex is required for sorting of ubiquitinated membrane proteins into intraluminal vesicles prior to vacuolar degradation, as well as for recycling of Golgi proteins and formation of luminal membranes.	2.16		
	CSC1	Calcium permeable gated cation channel; functions as an osmosensitive, calcium-permeable cation channel when expressed in a heterologous system; may be involved in detoxification.	0.69		
	VPS27	Ubiquitin binding protein involved in endosomal protein sorting; K48-, K63-Ub chain binding protein that preferentially binds K63-linked chains in vitro; subunit of the ESCRT-0 complex that binds to ubiquitin via two ubiquitin-interacting motifs (UIMs) and is involved in sorting cargo into multivesicular bodies; required for recycling Golgi proteins, forming luminal membranes and sorting ubiquitinated proteins destined for degradation.	4.10		

	ATG18	Phosphoinositide binding protein; required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (CVT) pathway; binds PtdIns(3,5)P ₂ , PI3P and PI4P; WD-40 repeat containing protein and PROPPIN family member; relocalizes from the vacuole to cytoplasm upon DNA replication stress; mammalian homologs include: WIPI1, WIPI2, WIPI3 and WIPI4/WDR45; mutations in human WDR45 cause static encephalopathy of childhood with neurodegeneration in adulthood (SENDA).	2.55		
Metal ion transport	ZRT3	Vacuolar membrane zinc transporter; transports zinc from storage in vacuole to cytoplasm when needed; transcription induced under conditions of zinc deficiency; targeted to vacuole via AP-3 pathway.	2.39	8.64	1.99E-05
	SMF3	Putative divalent metal ion transporter involved in iron homeostasis; transcriptionally regulated by metal ions; member of Nramp family of metal transport proteins; targeted to vacuole via AP-3 pathway; protein abundance increases in response to DNA replication stress.	1.91		
	CCC1	Vacuolar Fe ²⁺ /Mn ²⁺ transporter; suppresses respiratory deficit of yfh1 mutants, which lack ortholog of mammalian frataxin, by preventing mitochondrial iron accumulation; relative distribution to vacuole decreases upon DNA replication stress; targeted to vacuole via AP-3 pathway.	1.83		
	VNX1	Calcium/H ⁺ antiporter localized to endoplasmic reticulum membrane; member of calcium exchanger (CAX) family; targeted to vacuole via AP-3 pathway.	1.52		
	YBT1	Transporter of ATP-binding cassette (ABC) family; targeted to vacuole via AP-3 pathway; involved in bile acid transport; negative regulator of vacuole fusion; regulates release of luminal Ca ²⁺ stores; similar to mammalian bile transporters; YBT1 has a paralog, VMR1, that arose from the whole genome duplication.	0.66		
	YCF1	Vacuolar glutathione S-conjugate transporter; ABC-C transporter of the ATP-binding cassette family; required for vacuole fusion; forms stable complexes with vacuole fusion machinery; regulates Vam7p recruitment to vacuoles; role in detoxifying metals (Cd, Hg, As); transports GSSG that is not immediately reduced in cytosol to vacuole; transports unconjugated bilirubin, selenodiglutathione, oxidized glutathione; similar to human cystic fibrosis protein CFTR.	1.20		
	FTH1	Putative high affinity iron transporter; involved in transport of intravacuolar stores of iron; forms complex with Fet5p; expression is regulated by iron; proposed to play indirect role in endocytosis; protein abundance increases in response to DNA replication stress.	1.84		

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CHAPTER V

General discussion

5.1. General discussion

Lysosomes and their equivalents in yeast, the vacuoles, play critical roles in storing metabolites and serve as major catabolic centers involved in the degradation of both intracellular and extracellular components, ranging from macromolecules to organelles. Beyond their roles in storage and degradation, vacuoles and lysosomes act as platforms for metabolic signaling, playing a crucial role in the activation of signaling pathways that enable cells to adapt to stress conditions such as nutrient starvation, oxidative stress, and osmotic stress [1–6]. There is also substantial evidence suggesting that lysosomal and vacuolar functions regulate lifespan. As organisms age, lysosomal function declines, which is linked to aging and age-related diseases [5,7]. Notably, many age-related diseases as Parkinson's disease [8] and lysosomal storage disorders (LSDs), including Niemann-Pick type C (NPC) [9], have lysosomal impediments and lysosomal instability. Moreover, several lifespan-extending interventions operate by modulating lysosomal function [7,10–14], indicating that changes in lysosomal proteins influence the aging process. Understanding how lysosomes are regulated in the context of aging and lifespan extension conditions may provide new insights into potential therapeutic targets for promoting healthier and longer lives.

This work aimed to identify vacuolar proteins and pathways altered under conditions of premature aging and increased lifespan, using *Saccharomyces cerevisiae* as a model, thereby contributing to our understanding of how vacuoles regulate lifespan. To achieve this, we performed a proteomic analysis of vacuolar membranes isolated from wild type, *ncr1Δ* (a yeast model of NPC that exhibits premature aging), and *sit4Δ* (a model of extended longevity) cells.

The NPC disease results from the malfunction of NPC1 or NPC2 proteins, which are involved in the export of cholesterol and lipids from lysosomes [15]. This LSD is characterized by compromised intracellular trafficking, leading to the accumulation of cholesterol and other lipids within late endosomes/lysosomes (LE/Lys). Sphingosine accumulation at LE/Lys results in loss of calcium homeostasis, further contributing to trafficking defects and the aberrant accumulation of cholesterol and lipids [16]. Impaired intracellular trafficking of cholesterol leads to autophagy dysfunction, which in turn exacerbates cholesterol accumulation [17–19]. Additionally, changes in cholesterol and calcium homeostasis, along with autophagic defects, contribute to mitochondrial dysfunction in NPC1 cells and premature death [20–26]. In yeast cells lacking *Ncr1*, we observed alterations in the levels or phosphorylation of vacuolar membrane proteins associated with several diverse processes, including vesicle trafficking pathways and ion homeostasis. These alterations are consistent with the previously reported trafficking

defects and calcium/iron dyshomeostasis associated with NPC1 disease [17,19,20,27–40]. Proteomic alterations were also consistent with the impairment of autophagy and iron homeostasis in cells lacking *Ncr1* (chapter 2) and with the defects in autophagy widely reported in NPC1-deficient cells [17,19,20,34]. Some studies showed that TORC1, a major regulator of autophagy, is hyperactivated in NPC1 cells and that its inhibition restores autophagy in these cells [41–43], although other studies suggest that TORC1 activity is not altered or is even decreased in NPC1 cells [17,44]. In the yeast model of NPC1, the analysis of two downstream targets of TORC1, *Rps6* and *Sch9*, indicated that TORC1 signaling is downregulated (chapter 2), suggesting that these cells are unable to induce autophagy at the post-diauxic shift (PDS) phase in a manner independent of TORC1 activation. We also demonstrated that autophagy impairment in *ncr1Δ* cells is not linked to the inhibition of Snf1/AMPK, a protein kinase that activates autophagy by phosphorylating Atg1 [45]. In fact, we found that Snf1 activity is increased in cells lacking *Ncr1* (chapter 3), despite the increased activity of Sit4, a negative regulator of Snf1 [46,47]. Interestingly, our results implicated iron overload in the autophagy impairment and premature lifespan of *ncr1Δ* cells (Figure 1). Indeed, chelating iron with bathophenanthrolinedisulfonic acid (BPS) restores autophagy and increases *ncr1Δ* lifespan. We found that the low-iron sensing transcription factor Aft1 is more active in *ncr1Δ* cells and mediates the accumulation of iron at the PDS phase. Iron accumulation may occur in the cytoplasm, as we observed an increase in the levels of proteins involved in vacuolar iron mobilization. Moreover, we demonstrated that BPS restores the oxidative stress resistance and reduces the levels of reactive oxygen species (ROS) (Figure 1), further indicating that iron is accumulating outside vacuoles, where it promotes intracellular oxidation, rather than inside the vacuoles, which typically protects against iron toxicity [48].

Changes in iron homeostasis have been largely associated with aging and aging-related diseases [49]. High levels of free iron increase the production of ROS, which can damage DNA, proteins, lipids, organelles, and cells, accelerating the aging process. Moreover, iron in excess increases the susceptibility to develop several age-related diseases, such as cancer, diabetes, neurodegenerative disorders, and cardiovascular disorders. Iron chelators have been used as a therapeutic strategy for neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases [49] and limiting iron increases lifespan in mammalian models by promoting autophagy and decreasing oxidative stress [50,51]. Alterations in iron homeostasis have also been associated with NPC disease. However, treatment with deferiprone (DFP), a brain-penetrable iron chelator, does not improve disease phenotypes or the lifespan of NPC1-deficient mice [30]. Identifying the molecular players involved in the regulation of autophagy in response to iron levels will enhance our understanding of the molecular basis of NPC1 disease and aid in the

development of new potential therapeutical strategies against NPC. Studies in yeast have demonstrated that iron limitation induces autophagy and increases lifespan by inhibiting TORC1 [52]. However, our results suggest the existence of an alternative mechanism by which iron levels control autophagy, since TORC1 activity is already downregulated in the yeast model of NPC1, yet BPS is able to induce autophagy and increase the lifespan of *ncr1* Δ cells.

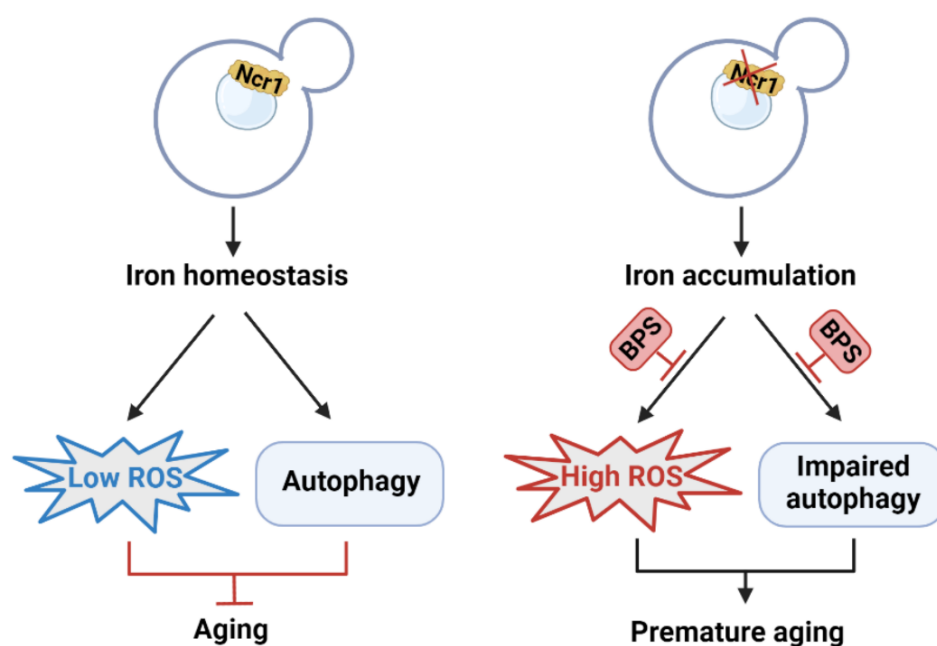


Figure 1 - Ncr1 regulates autophagy and oxidative stress resistance by affecting iron homeostasis. Loss of Ncr1 results in iron accumulation, leading to increased oxidative stress, impaired autophagy, and premature lifespan.

Characterizing changes in lysosomal function in cells with increased lifespan is another strategy that may enhance our understanding of the molecular mechanisms associated with cell longevity. Previous studies showed that downregulation of the yeast Sit4/PP6, a protein phosphatase involved in the modulation of vATPase and vacuolar acidification, increases mitochondrial fitness, oxidative stress resistance, and chronological lifespan (CLS) [53–56]. Sit4 represses respiratory genes when glucose levels are high by promoting the stability of Mig1 and the dephosphorylation of Hxk2, favoring the formation of the repressor complex Mig1-Hxk2 [57–59]. Additionally, Sit4 dephosphorylates and inhibits Snf1, preventing the phosphorylation and inactivation of Mig1 [46,60,61]. The activation of Snf1 in *sit4* Δ cells underlies the increased mitochondrial fitness, resistance to oxidative stress, and extended CLS observed in these cells [56]. Sit4 can be associated with different protein complexes and is involved in the regulation of many biological processes, such as the cell cycle [62,63], cell wall integrity [64], ER-to-Golgi trafficking [65],

ion homeostasis [66], lipid and carbohydrate metabolism [57,67], and nutrient signaling [68,69]. Sit4 interacts with and is inhibited by Tap42 in response to nutrient signals via TORC1 [68]. When TORC1 is inhibited, the Tap42-Sit4 complex dissociates, activating Sit4. Active Sit4 then dephosphorylates the transcription factor Gln3, activating the nitrogen catabolite repression pathway [70]. Sit4 associated with Sit4-associated proteins (SAPs) promote cell progression [71]. Association of Sit4 with Tpd3 and Cdc55 regulates sphingolipid metabolism [72].

In this work, we also performed a proteomic analysis of vacuolar membranes isolated from cells lacking Sit4 (chapter 4). Our results revealed alterations in proteins associated to vacuole fusion and organization, Golgi organization, late endosome to vacuole transport, autophagy, microautophagy, polyphosphate metabolism, and metal ion homeostasis. Several of these biological processes were also altered in *ncr1Δ* cells, further supporting their likely importance in cellular aging. The analysis of trafficking pathways implicated Sit4 in the regulation of autophagy, microautophagy, mitophagy, the MVB pathway, and the Cvt pathway. We found that the Cvt pathway is induced in *sit4Δ* cells grown to the exponential phase whereas mitophagy is increased in this mutant during both exponential and PDS phases. Moreover, deletion of *SIT4* disrupts the induction of autophagy, microautophagy, and the MVB pathway at the PDS phase. This effect is probably associated with cell signaling dysregulation and not with defects in these pathways as they can still be induced under conditions known to activate them. These results suggest that *SIT4* deletion increases CLS independently of these pathways. This was unexpected due to the critical importance of autophagy for cell longevity. However, there are examples of mutants with defective autophagy exhibiting lifespan extension under certain circumstances. For instance, caloric restriction (CR) still increases the lifespan of many atg mutants [73].

Among the top proteins enriched in *sit4Δ* vacuolar membranes were Vps27 and Hse1, components of the endosomal sorting complex required for transport-0 (ESCRT-0). We also found that Vps27 is crucial for the lifespan extension of *sit4Δ* cells and in fact *SIT4* and *VPS27* have a strong negative genetic interaction, as *sit4Δvps27Δ* cells exhibit a short CLS, even when compared to *vps27Δ* cells. The role of Vps27 in *sit4Δ* lifespan extension could not be attributed to alterations in the MVB pathway and microautophagy, which are known to depend on Vps27, as these pathways are already impaired in the *sit4Δ* single mutant. However, the deletion of *VPS27* in *sit4Δ* cells negatively affects the CPY pathway, which may contribute to the shortened lifespan of *sit4Δvps27Δ* double mutant cells. Our results also suggest that Vps27 does not promote *sit4Δ* CLS through regulation of the Cvt pathway or mitophagy, since these pathways are also induced in *sit4Δvps27Δ* cells.

Interestingly, we found that iron homeostasis in *sit4Δ* cells depends on Vps27, as *SIT4* and *VPS27* double deletion results in iron accumulation and aggravates the sensitivity of *sit4Δ* cells to iron supplementation. However, iron accumulation does not seem to be a major factor contributing to the premature aging of *sit4Δvps27Δ* cells, as limiting cellular iron did not significantly impact their CLS.

Notably, our results demonstrated for the first time that Vps27 regulates mitochondrial function and is crucial for mitochondrial derepression in *sit4Δ* cells (Figure 2). Cells lacking Vps27 exhibit a decrease in mitochondrial respiration and the *sit4Δvps27Δ* cells exhibit a severe decrease in oxygen consumption rate and are unable to grow on a nonfermentable carbon source. Mitochondrial function is essential for lifespan as they are critical for the production of energy and biomolecules and act as signaling hubs. Impairment of mitochondrial function has been clearly associated with aging and several pathologies. During aging, mitochondrial function decreases, which affects cellular bioenergetics and increases the production of ROS, thereby increasing oxidative stress and cell death [10]. Modulation of mitochondrial function has been associated with the beneficial effects of several interventions that increase lifespan in various organisms. For instance, CR induces mitochondrial oxidative phosphorylation and antioxidant defenses, while decreasing the production of ROS [74].

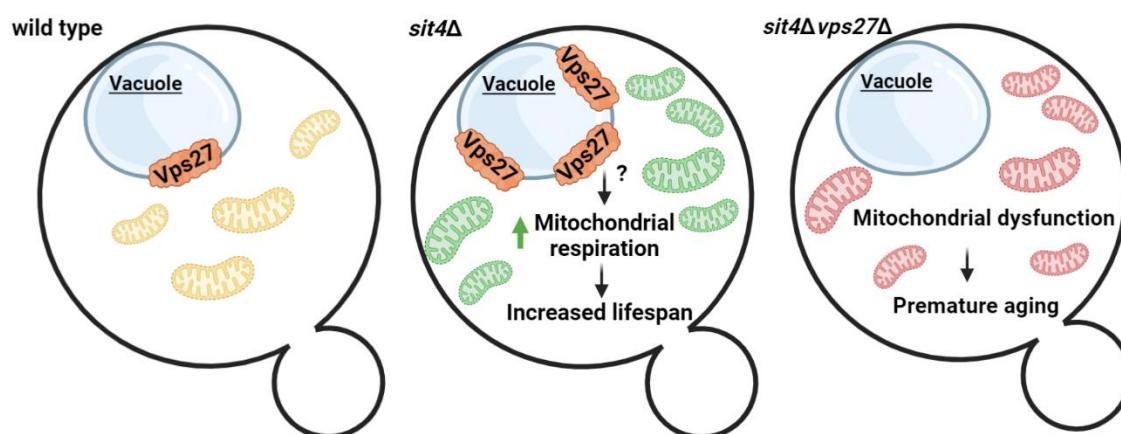


Figure 2 - Vps27 regulate lifespan in *Saccharomyces cerevisiae* by affecting mitochondrial function. Vps27, a component of the ESCRT-0 complex, is enriched at the vacuolar membrane of *sit4Δ* cells and promote mitochondrial respiration, which is critical for *sit4Δ* increased lifespan. Deletion of *VPS27* in *sit4Δ* cells impaired mitochondrial respiration, associated with reduced levels of Atp2 and Cox2, causing premature aging.

It is unlikely that the mitochondrial dysfunction in *sit4Δvps27Δ* cells results from an impaired degradation of damaged mitochondria or to defects in mitochondrial biogenesis, as mitophagy is increased in *sit4Δvps27Δ* cells and the levels of the mitochondrial porin Por1 are not affected by *VPS27* deletion. However, *sit4Δvps27Δ* cells exhibit low levels of

Atp2 and Cox2, two proteins required for oxidative phosphorylation. The downregulation of Cox2 (encoded by the mitochondrial genome) is not associated with defects in the maintenance of mtDNA. It remains to be demonstrated how Vps27 regulates the levels of mitochondrial proteins in *sit4* Δ cells to control mitochondrial respiration, which is critical for the extended longevity of cells lacking Sit4. It is also unknown if the human Vps27 homologue, the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) [75], also plays a role in the regulation of mitochondrial function. Overall, our results provide additional evidence for a cross-talk between vacuolar protein sorting and mitochondria and its importance for lifespan regulation.

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