# Regulation of mitochondrial function by Isc1p and Sch9p in *Saccharomyces cerevisiae*

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Todas as correções determinadas

pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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# Resumo

As mitocôndrias desempenham um importante papel na obtenção de energia através da fosforilação oxidativa. No entanto, são também responsáveis pela regulação de diversos processos biológicos nomeadamente a resposta ao stress e morte celular. Os esfingolipídos, tal como a ceramida, a esfingosina e a esfingosina-1-fosfato são componentes estruturais das membranas celulares e os seus metabolitos desempenham importantes funções reguladoras, que incluem a modelação de vias de sinalização e de uma série de processos celulares, incluindo a apoptose, proliferação, ciclo celular, respostas ao stress e o envelhecimento. Nos últimos anos, temos assistido a um grande interesse por parte da comunidade científica no estudo do papel dos esfingolípidos na função mitocondrial, homeostasia redox e envelhecimento, uma vez que o metabolismo dos esfingolípidos poderá ser terapeuticamente relevante no tratamento de patologias associadas com o envelhecimento.

Na levedura *Saccharomyces cerevisiae*, a degradação de esfingolípidos complexos é catalizada pela enzima Isc1p, ortóloga da esfingomielinase neutra tipo 2 em mamíferos. Células com deficiência na proteína Isc1p apresentam alterações no metabolismo e função mitocondrial, hipersensibilidade ao peróxido de hidrogénio e envelhecimento cronológico prematuro. As disfunções mitocondriais das celulas *isc1*Δ estão associadas com uma sobrecarga de ferro e aumento de morte celular por apoptose induzida pelo peróxido de hidrogénio e envelhecimento cronológico. Estudos anteriores implicam a proteína cinase da família das proteínas AGC Sch9p nos fenótipos das células *isc1*Δ. Apesar da ativação da via ocorrer em resposta a nutrientes e sinais de stress por parte do complexo TORC1, a proteína Sch9p regula também a função mitocondrial e o envelhecimento cronológico integrando sinais de esfingolípidos. Como esta complexa via regula o envelhecimento cronológico, a resistência ao stress oxidativo, a função mitocondrial e a autofagia permanence muito pouco caraterizada.

O trabalho apresentado nesta dissertação aborda o papel da esfingomielinase neutral Isc1p e a proteína cinase Sch9p na regulação da função mitochondrial e autofagia, e a sua revelância no stress oxidativo e envelhecimento cronológico. Os resultados demonstram que a deleção do gene *SCH9* suprime a hipersensibilidade ao peróxido de hidrogénio e as disfunções mitocondriais das células *isc1*Δ*,* tal como a incapacidade de crescer em fontes de carbono não fermentáveis como o glicerol, a baixa atividade da enzima citocromo *c* oxidase (COX) e o reduzido consumo de oxigénio. Por fim, também foi possível demonstrar que o mutante *isc1*Δ exibe um decréscimo no fluxo autofágico e uma hiperativação da

mitofagia, em parte mediado pela proteína cinase Sch9p.
Os resultados obtidos sugerem que a ativação da proteina cinase Sch9p desempenha um importante papel nas disfunções mitocondriais e na desregulação da autofagia e mitofagia em células *isc1*Δ, contribuindo desta forma para a hipersensibilidade ao stress oxidativo e ao envlhecimento cronológico prematuro exibido por este mutante.

**Palavras chave:** esfingolípidos, função mitocondrial, stress oxidativo, longevidade, Isc1p, Sch9p.

# Abstract

Mitochondria play a vital role in energy production through oxidative phosphorylation and have important functions in the regulation of important biological processes, including stress responses and cell death. Sphingolipids such as ceramide, sphingosine and sphingosine-1-phosphate, are structural components of cell membranes and act as key regulators of cell signalling pathways and control a wide range of cellular processes, including apoptosis, proliferation, cell cycle arrest, stress responses and aging. In recent years, interest into the role of sphingolipids in mitochondrial function, redox homeostasis and lifespan has greatly increased, since the regulation of sphingolipid metabolism may be of potential therapeutic relevance in age-associated pathologies.

In the yeast *Saccharomyces cerevisiae,* the turnover of complex sphingolipids is catalyzed by the enzyme Isc1p, the orthologue of the mammalian neutral sphingomyelinase type 2. Cells lacking Isc1p display mitochondrial dysfunctions, hypersensitivity to hydrogen peroxide and a shortened chronological lifespan. The mitochondrial dysfunction of *isc1*Δ cells is associated with iron overload and contributes to increased apoptotic cell death induced by hydrogen peroxide and chronological aging. Previous studies have implicated the activation of the AGC protein kinase Sch9p in *isc1*Δ phenotypes. Apart from sensing nutrient and stress signals from the Target of Rapamycin (TOR) complex 1 (TORC1), this protein also regulates mitochondrial function and chronological lifespan by integrating sphingolipid signalling. How this complex network of interacting pathways regulates chronological lifespan, oxidative stress resistance, mitochondrial function and autophagy remains poorly characterized.

The work reported in this dissertation addressed the role of the Isc1p neutral sphingomyelinase and the Sch9p protein kinase in the regulation of mitochondrial function and autophagy, and its relevance on oxidative stress resistance and chronological lifespan. The results show that *SCH9* deletion suppresses the hydrogen peroxide hypersensitivity and the mitochondrial dysfunctions of *isc1*Δ cells, such as the inability to grow in non-fermentable carbon sources like glycerol, and reduced cytochrome *c* oxidase (COX) activity and oxygen consumption. We have also shown that *isc1*Δ cells exhibit decreased autophagic flux and a hyperactivation of mitophagy, which is in part mediated by Sch9p.
The overall results suggest that the activation of the protein kinase Sch9p leads to mitochondrial dysfunctions and deregulates autophagy and mitophagy in *isc1*Δ cells, leading to enhanced oxidative stress sensitivity and premature aging exhibited by the mutant strain.

**Key-words:** sphingolipids, mitochondrial function, oxidative stress, chronological lifespan, Isc1p and Sch9p.

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# General Abbreviation

| | |
|---|---|
| AKT/PKB | Protein kinase B |
| ATP | Adenosine triphosphate |
| CFU | Colony forming units |
| CLS | Chronological lifespan |
| COX | Cytochrome *c* oxidase |
| $DIOC_6(3)$ | 3,3'-Dihexyloxacarbocyanine Iodide |
| DHE | Dihydroethidium |
| DHS | Dihydrosphingosine |
| DHS-1-P | Dihydrosphingosine-1-phosphate |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| EtBr | Ethidium bromide |
| EDTA | Ehylenediamine tetracetic acid |
| ER | Endoplasmic reticulum |
| FCCP | Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone |
| GFP | Green fluorescent protein |
| $H_2O_2$ | Hydrogen peroxide |
| IPC | Inositol-phosphoceramide |
| LCB | Long chain sphingoid base |
| LCB-P | Long chain base-phosphate |
| MIPC | Mannose-inositol-phosphoceramide |

| | |
|---|---|
| M(IP)2C | Mannose diinositolphosphoryl-ceramide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| nSMase | Neutral sphingomyelinase |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophorese |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PHS | Phytosphingosine |
| PHS-1-P | Phytosphingosine-1-phosphate |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SM | Sphingomyelin |
| SMase | Sphingomyelinase |
| SPT | Serine palmitoyltransferase |
| S1P | Sphingosine-1-phosphate |
| S6K | Ribosomal protein S6 kinase |
| TBS | Tris buffered saline |
| TOR | Target of Rapamycin |
| TORC1 | Target of Rapamycin Complex 1 |
| TTBS | Tris-buffered saline plus Tween |
| UV | Ultraviolet |
| YPD | Yeast peptone dextrose |

| | |
|---|---|
| YPG | Yeast peptone glycerol |

# Chapter I
# Introduction

# I-1. *Saccharomyces cerevisiae* as a biological model to study basic cellular processes

The budding yeast *Saccharomyces cerevisiae* is a unicellular fungus of the *Ascomycete* family with approximately 6000 genes. Due to the high conservation of fundamental biochemical pathways, yeast has been used as a model organism to unravel new aspects of important biological processes in higher eukaryotes (Mager and Winderickx, 2005). The studies of aging (Longo, 2003; Piper, 2006; Barros *et al.,* 2010), cell cycle (Humphrey and Pearce, 2005), stress responses (Costa *et al.,* 2007; Rodrigues-Pousada *et al.,* 2010) or apoptosis (Almeida *et al.,* 2008; Carmona-Gutierrez *et al.,* 2010; Greenwood and Ludovico, 2010) have served in many ways to foster our understanding about these processes.

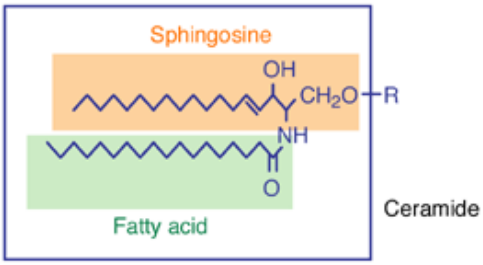
Among all eukaryotic model organisms, *S. cerevisiae* combines several advantages. For instance, it can be cultured in different media, it has a short doubling time, and it has a convenient experimental tractability, due to simple growth conditions and easy genetic manipulations (Mager and Winderickx, 2005). The yeast model has well established genomic and proteomic methodologies and there are well-curated databases that provide overall information about protein-protein interactions, genetic interactions, protein function and predicted orthologues in higher organisms (Mager and Winderickx, 2005; Pena-Castillo and Hughes, 2007; Petranovic and Nielsen, 2008).

The elucidation of sphingolipid metabolism and dynamics in yeast cells and their route of synthesis have been important to uncover new functions of sphingolipids and to understand the mechanisms for sphingolipid homeostasis in both physiological and pathological conditions. The budding yeast has been used to identify nearly all of the genes that encode sphingolipid metabolic enzymes and many of these were critical in identifying mammalian homologs (Dickson and Lester, 2002; Sims *et al.*, 2004.), showing that yeast and mammals share many similarities in sphingolipid metabolism. Therefore, the budding yeast is considered to be a useful model organism to study sphingolipid metabolism and regulation.

# I-2. An overview of sphingolipid structure and bioactivity

## I-2.1. Structure

Sphingolipids are important structural components of cell membranes found in essentially all animals, plants and fungi, as well as some prokaryotic organisms (Merrill *et al*., 2007). They are mostly found on the outer leaflet of the plasma membrane, although they are also present at membranes of different organelles at variable ratio. In addition, they are major constituents of lipoproteins. Several species of sphingolipids have been identified and some of them are bioactive lipids since they have the ability to modulate signalling pathways and the variation of their ratio results in important modifications in cellular functions and fate (Hannun and Obeid, 2008). In fact, sphingolipids such as sphingosine, ceramide and sphingosine-1-phosphate have emerged as core sphingolipids in this metabolism as they regulate a vast number of cellular processes, including cell growth, adhesion, migration, senescence, apoptosis, and autophagy (Hannun and Obeid, 2008; Ryland *et al*., 2011).
From a structural point of view, sphingolipids have an amphipathic nature and are composed by a long chain sphingoid base (LCB), generally 18 carbons long (sphingosine), with the C2-amino group amide-linked to a fatty acid, thereby forming the core unit, to which polar groups are added to form different types of sphingolipids (Malagarie *et al*., 2002, figure 1). The nature of the fatty acid (carbon length, degree of unsaturation and hydroxylation) along with other modifications of the long-chain bases and the polar head group define the vast family of sphingolipids (Merrill *et al*., 2007; Hannun and Obeid, 2008).




| Substituent (R) | Sphingolipid |
|---|---|
| H | Ceramide |
| Phosphocholine | Sphingomyelin |
| Sugar(s) | Glycosphingolipid |

**Figure -1. General sphingolipid structure.** The image was modified from refrence (Malagarie *et al*., 2002).

## I-2.2 Bioactive sphingolipids and regulation of biological processes

The complexity of sphingolipid metabolism arises from the interconnectivity of bioactive lipids (Hannun and Obeid, 2008), which enable cells to orchestrate different cellular responses by regulating sphingolipid interconversions (figure 2).

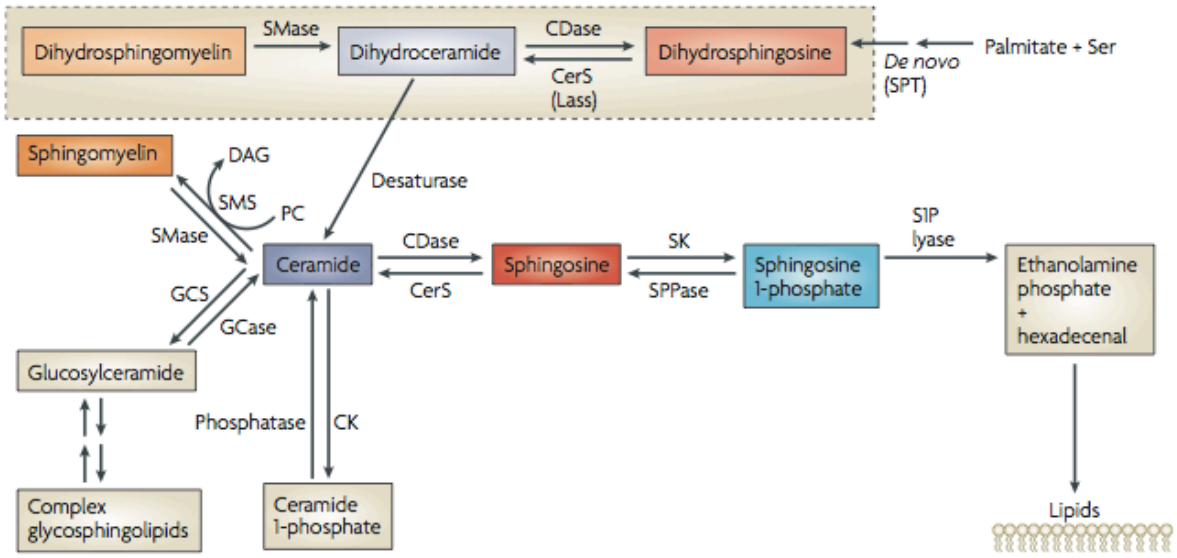



**Figure-2. Overview of sphingolpid metabolism and interconnectivity of bioactive sphingolipids.** The image was obtained from reference (Hannun and Obeid, 2008).

In response to both extracellular stress (e.g., UV, hypoxia, toxins, heat stress) and alterations in cell physiology, the enzymes involved in sphingolipid metabolism act in a coordinate manner to regulate not only the levels of individual bioactive lipids, but also their metabolic interconversion (figure 3).

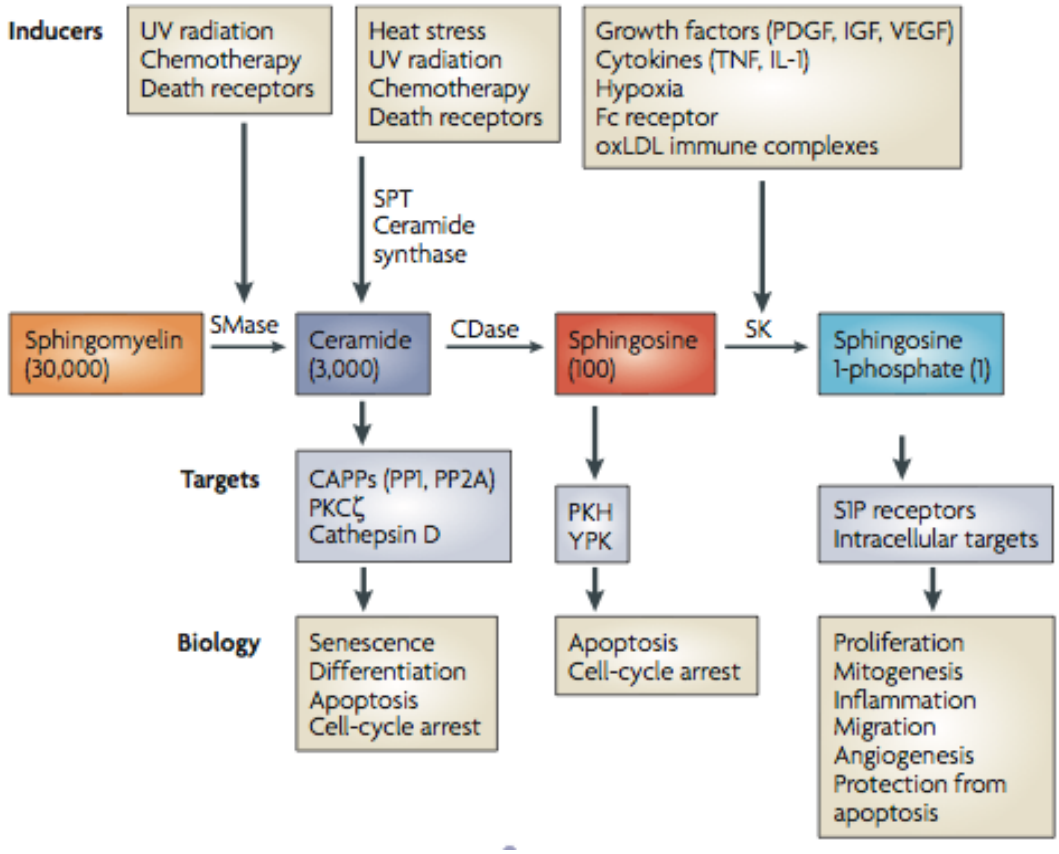



**Figure-3. An overview of the roles of sphingolipids in Biology.** The image was obtained from reference (Hannun and Obeid, 2008).

The sphingolipids ceramide, sphingosine and sphingosine-1-phosphate (S1P) are the main representatives of sphingolipid metabolism and play crucial roles in the regulation of many cellular processes (Hannun and Obeid, 2008). The first sphingolipid to be identified was sphingosine and it exerts pleiotropic effects on protein kinases and other targets (Hannun *et al.,* 1986). Sphingosine and its related sphingoid bases have roles in regulating the actin cytoskeleton, endocytosis, cell cycle and apoptosis (Smith *et al.,* 2000). Ceramide mediates many cell-stress responses that include the regulation of apoptosis (Obeid *et al.,* 1993) and cell senescence (Venable *et al.*, 1995), by modulating the activity of ceramide-activated protein kinases (e.g. PKC) and phosphatases (CAPP, PP1 and PP2A) (figure 3). On the other hand, S1P promotes cell proliferation and survival by acting in an autocrine manner on S1P receptors (Hla, 2004). Consequently, it is expected that alterations in the relative

amounts of sphingosine-1-phosphate and sphingosine/ceramide have significant effects on cell physiology and metabolism and ultimately on cell fate (Spiegel and Milstein, 2003).

Other components of the family of sphingolipids include ceramide-1-phosphate (C1P), which is involved in inflammation and vesicular trafficking, glucosylceramide, mostly associated with post-Golgi trafficking and drug resistance, lyso-sphingomyelin and dihydroceramide (Hannun and Obeid, 2008).

The importance of sphingolipid signalling derives form the early recognition of their contribution in the pathobiology of human cancers and other human ailments such as diabetes and heart disease, microbial infections, neurological and immune dysfunctions (Kolter and Sandhoff, 2006; Ozbayraktar and Ulgen, 2009; Kolter, 2011; Hla and Dannenberg, 2012; Young *et al*., 2013).

## I-3. Yeast sphingolipid metabolism

The general pathways governing sphingolipid metabolism are well characterized in yeast (figure 4). It is very similar to the mammalian counterpart (figure 2), and shares a similar spatial organization, with the early steps taking place in the endoplasmic reticulum (ER) and the subsequent processes occurring in the Golgi compartment for the synthesis of more complex sphingolipids (Futerman and Riezman, 2005).

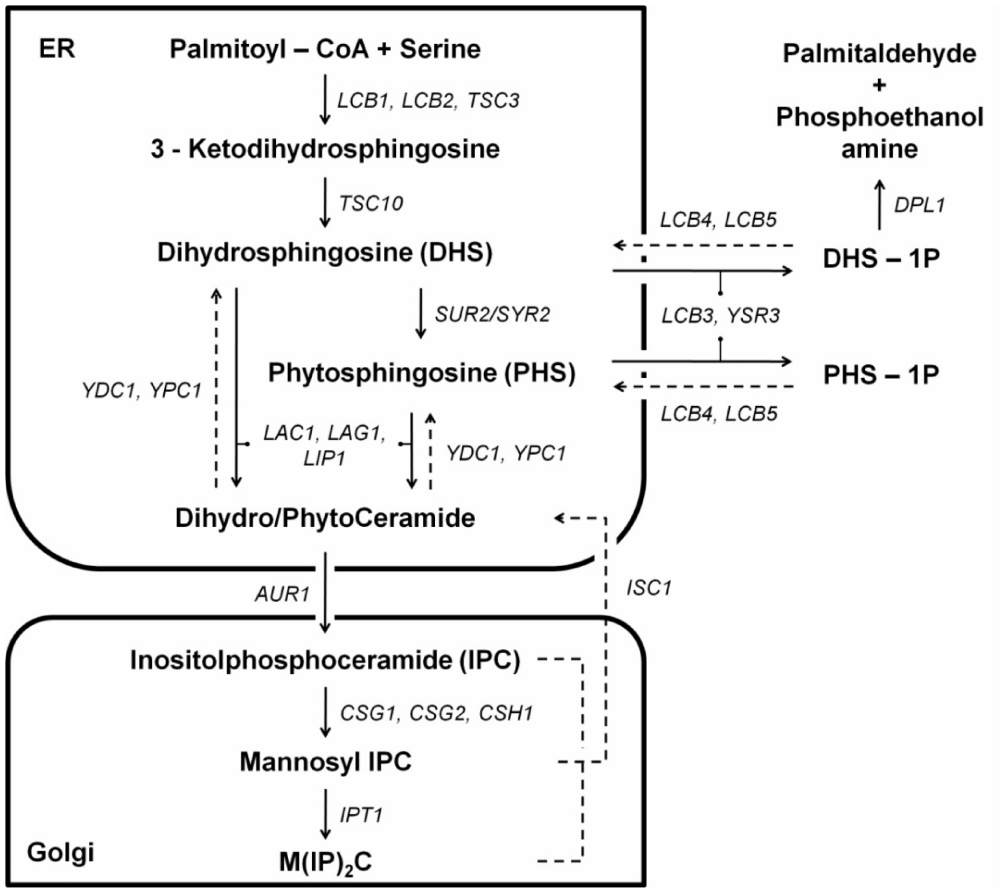



**Figure-4. Schematic overview of yeast sphingolipid metabolism displaying the metabolic intermediates, genes involved and cell location of the enzimatic reactions.** The image was modified from reference (Vallee and Riezman, 2005).

### I-3.1. *De novo* biosynthesis in the ER

As in mammalian cells, the first and rate-limiting step in yeast sphingolipid metabolism involves the condensation of serine and palmitoyl-CoA in the endoplasmic reticulum (ER) by a process catalyzed by serine palmitoyl-transferase (SPT), yielding 3-ketodihydrosphingosine (Dickson, 1997). This first step is the only entry route in the sphingolipid metabolism and several studies suggest that the subtract availability in this reaction regulates the flux through the pathway (Alvarez-Vasquez *et al*., 2005). SPT was shown to have two homologous subunits, Lcb1p and Lcb2p, both of which are required for its activity (Nagiec *et a*l.*,* 1994). In yeast, a third small subunit of SPT, Tsc3p (temperature-sensitive suppressor of calcium sensitivity) is required for the activity by forming a heterodimer with Lcb1p and Lcb2p (Gable, 2000). Tsc3p function is unknown but it influences Lcb2p in the Tsc3p-Lcb2p-Lcb1p complex (Monaghan *et al.,* 2002).

### I-3.2. Long chain base formation

After the initial condensation of serine and palmitoyl-CoA to produce 3-keto dihydrosphingosine, this intermediate is converted to the LCB dihydrosphingosine (DHS) by Tsc10p in an NADPH-dependent manner. Sur2p/Syr2p catalyzes the hydroxylation of DHS at the C4 position to produce phytosphingosine (PHS) (Haak *et al.,* 1997; Grilley *et al*., 1998). Structurally, DHS and PHS also vary in the chain length: DHS contains 16, 18 or 20 carbons while PHS presents 18 or 20 carbons (Lester and Dickson, 2001). Together, DHS and PHS constitute the LCBs in yeast, and both can potentially undergo either phosphorylation at C-1 or N-acylation. DHS and PHS can be phosphorylated by two LCB kinases, encoded by *LCB4* and *LCB5* genes, forming DHS-1-phosphate and PHS-1-phosphate, respectively. Finally, these phosphorylated products can either be dephosphorylated back to DHS and PHS by the phosphatases Lcb3p/Ysr2p and Ysr3p or catabolized by dihydrosphingosine-1-phosphate lyase (Dpl1p) to release palmitaldehyde and phosphoethanolamine (Sims *et al*., 2004). The production of these non-sphingoid molecules constitutes the only known exit route from the sphingolipid metabolism and possibly regulates the overall sphingolipid levels (Cowart and Obeid, 2007).

### I-3.3. Ceramide generation

Apart from phosphorylation, DHS or PHS can be N-acylated to produce the correspondent dihydro- and phytoceramides. This requires two ceramide synthases, encoded by *LAG1* (longevity assurance gene 1) and *LAC1* (longevity assurance gene 1 cognate) (Guillas *et al*., 2001). These enzymes are highly homologous, present redundant function and the double deletion of *LAG1* and *LAC1* is required to prevent *de novo* biosynthesis of ceramide (Guillas *et al.,* 2001; Schorling *et al.,* 2001). In addition, Lip1p forms a heteromeric complex with Lac1p and Lag1p and is essential for ceramide synthase activity *in vivo* and *in vitro* (Vallée and Riezman, 2005). Phytoceramides and dihydroceramides can be cleaved back into LCBs and free fatty acid by ceramidase Ypc1p and Ydc1p, respectively (Mao *et al.,* 2000).
Once ceramide is generated, it can follow several metabolic fates. In fact, ceramide can be deacylated to form DHS/PHS, by one of many ceramidases (figure 4).

### I-3.4. Biosynthesis of complex sphingolipids

Ceramide can also become the substrate for the production of complex sphingolipids, namely inositol-phosphoceramide (IPC), mannosyl-inositol phospho-ceramide (MIPC) and mannosyl-diinositol-phospho-ceramide [$M(IP)_2C$]. The first complex sphingolipid, IPC, is formed by transferring a myo-inositol phosphate group from phosphatidylinositol (PI) to ceramide with the concomitant release of diacylglycerol (DAG). This step is catalyzed by the IPC synthase encoded by *AUR1*, an essential gene (Nagiec *et al*., 1997). The second complex sphingolipid, MIPC, is generated by transferring the mannose from GDP-mannose onto the inositol 2-OH moiety of IPC. The enzyme inositol phosphoceramide mannosyl transferase catalyzes this reaction and has two forms, one containing Csg1p and Csg2p, and the other Csh1p and Csg2p. The Csg1p and Csh1p appear to be the catalytic subunits, whereas Csg2p performs a regulatory function (Uemura *et al*., 2003). Therefor, The $Ca^{2+}$-binding protein Csg2 can form a complex with either Csg1 or Csh1 and is considered to act as a regulatory subunit (Uemura *et al*., 2007). The terminal yeast complex sphingolipid made in the Golgi apparatus is $M(IP)_2C$. It is the most abundant complex sphingolipid in yeast and it is synthesized by the addition of another inositol phosphate group to MIPC by a process catalyzed by inositol-phosphotransferase (Ipt1p) (Dickson *et al*., 1997).

### I-3.5. Sphingolipid catabolism

#### *I-3.5.1. The Inositol phosphosphingolipid phospholipase C*

Ceramide can be produced during the catabolism of the aforementioned complex sphingolipids. This reaction is performed by inositol phosphosphingolipid phospholipase C (Isc1p), which has phospholipase-C type activity and catalyzes the removal of the polar head groups from complex sphingolipids, releasing dihydroceramide and phytoceramide (figure 4). It was previously demonstrated that Isc1p overexpression results in an increase of ceramide levels, whereas *ISC1* deletion results in an accumulation of complex sphingolipids (Sawai *et al*., 2000).

Isc1p is the yeast homologue of mammalian neutral sphingomyelinase type 2 (nSMase2) and shares 30% identity in sequence to its counterpart (Sawai *et al.*, 2000). It is activated by phosphatidylserine (PS), phosphatidylglycerol (PG), and cardiolipin (CL), and is dependent on the presence of $Mg^{2+}$ for optimal activity (Almeida *et al.,* 2008, Sawai *et al.,* 2000). Isc1p contains P-loop-like domains, found in nucleotide-binding proteins. Mutations in the P-loop-like domain significantly reduce Isc1p activity, and it was proposed that might be involved in $Mg^{2+}$ binding and function in the interaction with the substrate through an $Mg^{2+}$/phosphate bridge (Okamoto *et al.,* 2003).

Interestingly, Isc1p is post-translationally regulated by translocation from the ER into mitochondria upon the transition from fermentative to the respiratory metabolism during the so-called post-diauxic shift (PDS) (Vaena de Avalos *et al*., 2004). This appears to be associated with the regulation of mitochondrial sphingolipid metabolism and function, namely the production of α-hydroxylated-phytoceramides, which are necessary for proper the function of this organelle (Kitagaki *et al*., 2007).

## I-4. Sphigolipids signalling on Cellular Biology

Studies using the budding yeast *Saccharomyces cerevisiae* have shown that sphingolipids play an important role in the regulation of cell cycle, cell integrity, endocytosis, cytoskeleton dynamics and protein turnover (Hannun and Obeid, 2008; Dickson, 2008). Additionally, sphingolipids have been implicated in the regulation of stress responses and longevity. For instance, yeast mutants lacking Ydc1p (dihydroceramidase) are characterized by increased chronological lifespan (CLS) whereas the overexpression of *YDC1* triggers mitochondria and vacuolar fragmentation, apoptosis and accelerated aging in yeast (Aerts *et a*l., 2008). Genes

involved in sphingolipid metabolism (*LAG1*, *YPC1*, *YSR3*, *IPT1*, and *LCB5*) show variable expression in senescent and apoptotic cells (Laun *et al.*, 2005). Importantly, it was shown that the downregulation of sphingolipid synthesis increases yeast CLS in part due to a reduction in long-chain bases (LCBs) mediated activation of Sch9p, the yeast homologue of mammalian ribosomal S6K protein kinase (Huang *et al.*, 2012). Furthermore, ceramide synthase (Lag1p) and LCB kinase (Lcb4p) activities decrease upon entry into the stationary phase, leading to a large increase in the levels of LCBs (Lester *et al*., 2013).

Initial studies have also demonstrated that Isc1p is implicated on the regulation of important cellular processes, namely responses to osmostress (Betz *et al*., 2002), heat stress (Cowart *et al.*, 2006) and genotoxic agents (Matmati *et a*l*., 2009*). More recently, our lab have shown that Isc1p also regulates oxidative stress resistance, mitochondria function and chronological lifespan (CLS). In fact, *isc1*Δ cells display shortened CLS and increased hydrogen peroxide sensitivity, which appear to be associated with mitochondrial dysfunction (Almeida *et al*., 2008).
In an attempt to dissect possible signalling pathways governing *isc1*Δ phenotypes, important downstream targets of Isc1p were identified and implicated in the regulation of mitochondrial function and CLS. Lipidomic analysis showed specific changes in sphingolipids during the premature aging of Isc1p-deficient cells, such as a decrease of dihydrosphingosine levels and an increase of very long chain ceramide species, namely dihydro-$C_{26}$-ceramide and phyto-$C_{26}$-ceramide, the latter raising the possibility of activation of ceramide-dependent protein phosphatases (Barbosa *et al.*, 2011). On this basis, it was recently shown that Isc1p regulates cell signalling through modulation of ceramide levels and proteins activated by ceramide such as the Sit4p protein, the catalytic subunit of type 2A ceramide-activated protein phosphatases (Barbosa *et al.*, 2011). In fact, the deletion of *SIT4* supresses mitochondrial dysfunctions, therefore increasing oxidative stress resistance and extending CLS in Isc1p-deficient cells (Barbosa *et al.,* 2011). More recently, it was also demonstrated that sphingolipid are also able to modulate the osmosensing machinery of the HOG pathway, for instance in response to the inibition of the *de novo* biosynthetic pathway or depletion of ergosterol (Tanigawa *et al.*, 2012 )**.** It was also demonstrated that the activation of Hog1p is deleterious for *isc1*Δ cells since ceramide signalling increase the phosphorylation of Hog1p and the deletion of *HOG1* abolishes *isc1*Δ phenotypes (Barbosa *et al.*, 2012).

In the past decades, many studies contributed to the characterization of the role of sphingolipids in signal transduction. However, the mechanisms by which sphingolipids control many aspects of cell physiology and metabolism remains to be characterized.

### I-4.1. Interplay between sphingolipids and the Target of Rapamycin pathway

Recent studies have linked ceramide to important signalling pathways involved in the regulation of cell growth and survival, namely the TOR (Target of Rapamycin) pathway. This pathway is highly conserved among organisms, ranging from flies, nematodes, protozoa alongside with mammals (Raught *et al.,* 2001; De Virgilio and Loewith, 2006; Dann and Thomas, 2006; Laplante and Sabatini, 2012; Johnson *et al.*, 2013; Markaki and Tavernarakis, 2013). The TOR pathway belongs to a conserved group of serine/threonine kinases from the phosphatidylinositol kinase-related kinase (PIKK) family that is highly conserved from yeast to mammals (Bjornsti and Houhton, 2004; De Virgilio and Loewith, 2006; Wllschleger, *et al*., 2006). In *S. cerevisiae*, the TOR pathway is controlled by two Ser/Thr protein kinases, Tor1p and Tor2p, which assemble into two protein complexes with distinct subunit composition and regulatory roles (Loewith *et al.*, 2002; Loewith and Hall, 2011; Kim and Guan, 2011). The rapamycin-sensitive TOR complex 1 (TORC1) contains either Tor1p or Tor2p and is mostly associated with the regulation of cell growth (nutrient sensing), autophagy, ribosomal and protein turnover and cell proliferation (Kim and Guan, 2011; Evans *et al.*, 2011). The TORC1 is mostly influenced by nutrients, mainly by nitrogen (Shamji *et al.*, 2000) but is also responsive to the energetic metabolic status of the cell (Wullschleger *et al.*, 2006). The TOR complex 2 (TORC2) contains Tor2p, but not Tor1p, and mediates the proper maintenance of the cell cytoskeleton (Cybulski and Hall, 2009) and was recently implicated in the regulation of ceramide biosynthesis by a Ypk2p-dependent mechanism (Aronova *et al.*, 2008).

The TORC1 pathway has been linked to mitochondrial function and yeast CLS (Bonawitz *et al.*, 2007, Pan *et al.*, 2011). In fact, the deletion of *TOR1* or pharmacological inhibition of TORC1 with rapamycin extends CLS in yeast and other organisms (Powers *et al.*, 2006; Bonawitz *et al*., 2007; Kaeberlein and Kennedy, 2011). TORC1 is active during early stages of growth and represses the induction of stress responses and entry into the stationary phase, in part by inhibiting the Rim15p protein kinase and consequently the translocation of Msn2p/4p and Gis1p transcription factors into the nucleus to induce adaptive response required for CLS extension (Wanke *et al.*, 2005; Wei *et al.*, 2008). Reducing TORC1 signalling at early stages of growth extends CLS by an intrinsic mechanism involving enhanced mitochondrial membrane potential and superoxide production. This in turn induces an adaptive response that contributes to decrease ROS production in the stationary phase and promotes longevity in yeast (Pan *et al.*, 2011). Moreover, reduced TORC1 signalling derepresses Rim15p and triggers the expression of genes regulated by the mitochondrial signalling pathway known as the retrograde response (Komeili *et al*., 2000; Dilova *et al.*, 2004; Liu and Butow, 2006) as well as stress-related genes under the control of

Msn2p/Msn4p (Beck and Hall, 1999; Wei *et al.*, 2008), which mimetic some aspects observed under calorie restriction (CR), associated with lifespan extension and improvement of mitochondrial fitness (Lin *et al.*, 2004).

Some authors have identified downstream targets of TORC1 involved in the regulation of stress response and aging, including the AGC protein kinase Sch9p (Jacinto and Lorberg, 2008). It is a serine-threonine kinase with homology to the mammalian ribosomal S6 kinase (S6K) (Urban *et al.*, 2007) and protein kinase B (PKB/AKT) (Geyskens *et al.*, 2000). Like other AGC proteins, Sch9p has several conserved functional regions: a central catalytic domain, an activation loop, a turn motif (TM) and a C-terminal regulatory domain, which contains a hydrophobic motif (HM) that is phosphorylated by TORC1 (Urban *et al.*, 2007) At the N-terminal side of the activation loop, Sch9p has a calcium-dependent C2 domain with unknown function (Jacinto and Logberg, 2008). Sch9p acts as a signalling mediator, relaying upstream signals from intracellular and extracellular cues, to downstream targets by phosphorylating them on serine/threonine residues (Roelants *et al*., 2004; Urban *et al*., 2007; Smets *et al*., 2010; Stichternoth *et al*., 2011).

Importantly, Sch9p has a pivotal role in oxidative stress resistance, chronological lifespan (CLS) and mitochondrial function (Huang *et al*., 2012). In fact, the deletion of SCH9 gene leads to better mitochondrial coupling, which contributes to improve oxidative resistance and extend CLS in yeast (Urban *et al*., 2007; Wei *et al*., 2008; Burtner *et al*., 2009; Pan *et al*., 2012). Apart from sensing nutrient and stress signals from TORC1, Sch9p also regulates CLS by integrating sphingolipid signalling. In addition to phosphorylation in the C-terminus mediated by TORC1, Sch9p is phosphorylated in a Thr570 residue in the activation loop by Pkh1/2p protein kinases, homologues of mammalian phosphoinositide-dependent protein kinase 1 (PDK1), in response to LCBs (Voordeckers *et al*., 2011; Huang *et al*., 2012). Huang *et al*. has recently demonstrated that the downregulation of sphingolipid synthesis induced by myriocin (an inhibitor of the first step of *de novo* biosynthetic pathway) or the deletion of *PKH2* enhances CLS and improves mitochondrial function and oxidative stress resistance by Sch9p-dependent mechanisms (Huang *et al*., 2012), which involves a decrease in the activation of the Pkh1/2p-Sch9p axis.

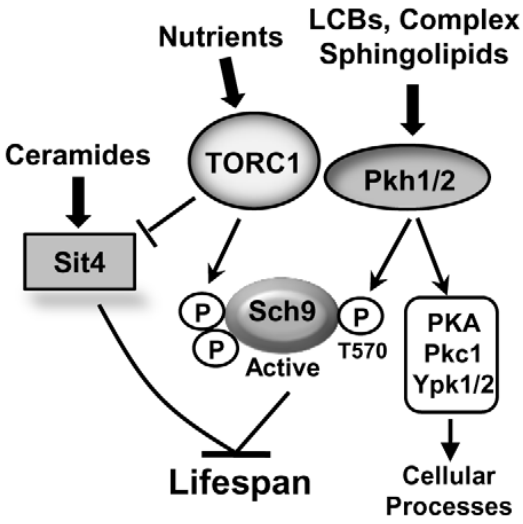



**Figure-5. Crosstalk between nutrient and sphingolipids signalling pathways that control mitochondrial function, redox homeostasis and lifespan in yeast.** TORC1 is activated by nutrients. This protein activates Sch9p by phosphorylation at the C-terminus. In addition, Sch9p is phosphorylated in a Thr570 residue in the activation loop by Pkh1/2p protein kinases in response to LCBs. Sch9p governs redox homeostasis and lifespan by acting as a physiological core center integrating nutrient and stress signal from TORC1 and sphingolipid signalling derived from LCB-Pkh1/2p axis. Ceramide-mediated activation of the Sit4p protein phosphatase may also play roles in regulating lifespan. Adapted from reference (Huang *et al.,* 2012).

Additionally, TORC1 plays a major role in regulation of autophagy (Yorimitsu *et al*, 2007), a major lysosomal/vacuolar degradative pathway for bulk proteins and damaged and/or unnecessary organelles (Mizushima and Klionsky, 2007). How this signaling pathway coordinate with sphingolipid dynamics in the regulation of cell metabolism and survival remains poorly characterized.

## I-5. Autophagy

In order to maintain viability during starvation periods, yeast undergoes a degradative process of its own cellular components by a "self-eating" process via the vacuole named autophagy (Klionski and Erm, 2000). Autophagy is an evolutionarily conserved process in eukaryotic cells that involves the engulfment of cytoplasmic cargo into double-membrane organelles called autophagosomes. After their formation, autophagosomes fuse with lysosomes (or the vacuole in yeast), within which the inner membrane and the cargo are degraded (Mizushima, 2007; Klionsky *et al*., 2007; Nakatogawa *et al*., 2009).

A basal level of constitutive autophagy is crucial for routine clearance of the cytosol under normal conditions. Basal autophagy is critical for protein and organelle homeostasis and

quality control in post-mitotic differentiated cells, such as neurons (Mizushima and Levin, 2010). In addition, autophagy becomes activated in response to low nutrient availability, (nitrogen and carbon starvation) providing a source of nutrients and energy (Blommaart *et al*., 1997; Mizushima *et al*., 2002). Autophagy is also triggered as an adaptive response to a broad range of other extracellular or intracellular stressors such as hypoxia, heat, reactive oxygen species (ROS) and accumulation of damaged cytoplasmic components (Levine and Klionsky, 2004).

Three major subtypes of the autophagy have been described: macroautophagy (the most common subtype), microautophagy, and chaperone-mediated autophagy (Ravikumar *et al*., 2010). In addition, a number of specific subtypes exist. The different forms of autophagy are shown in figure 6 and are discussed below in more detail.

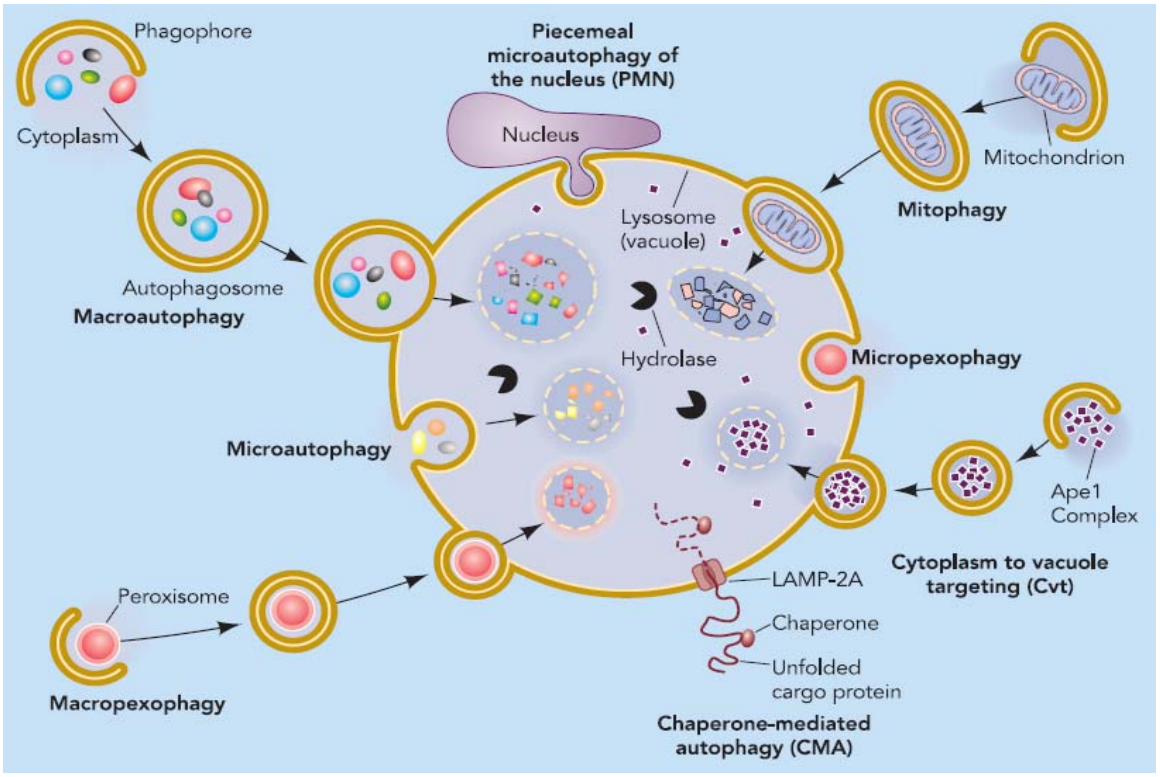



**Figure-6. Different autophagic-like processes in cell metabolism and physiology.** Macroautophagy, microautophagy (selective degradation of organelles) and chaperone-mediated autophagy (CMA) are shown. The image was altered from (Yen and Klionsky, 2008).

(i) **Macroautophagy**, where proteins or entire organelles are engulfed in a double membrane vesicle termed the autophagosome and subsequently degraded by vacuole enzymes, is the most prevalent form of autophagy and will be herein referred as to autophagy. Macroautophagy plays many roles in the cell, namely in starvation adaptation and metabolism as well as development and differentiation (Yang *and* Klionski, 2009; Farre *et al.*, 2009; Kroemer, *et al.,* 2010; Ravikumar *et al*., 2010;);

(ii) **Microautophagy** is a process in which cytoplasm is directly engulfed at the surface of the degradative organelle (the vacuole or lysosome) without the production of autophagosomes. The membrane invaginates, and pinches off to form an internal autophagic vesicle containing cytoplasmic material (Kunz *et al.*, 2004). The selective autophagy of particular organelles has been described, for example “mitophagy” is the selective degradation of mitochondria by autophagy, and “pexophagy” describes the selective turnover of peroxisomes by micro- or macroautophagy and ribophagy the selective turnover of ribosomes (figure 6) (Tuttle and Dunn, 1995; Dunn *et al*., 2005; Kanki and Klionsky, 2008).

(iii) **Chaperone-mediated autophagy** (CMA) is a selective form of autophagy, so far only detected in mammalian cells, that is activated during long-term nutrient deprivation. CMA does not involve the formation of a double membrane vesicle and targets chaperones to proteins that contain a motif biochemically related to the pentapeptide KFERQ. The chaperone-KFERQ-containing protein complex then binds LAMP (lysosome-associated membrane protein)- 2A receptors on the lysosome membrane, and translocates the target protein into the lysosomes for degradation (review in Bejarana and Cuervo, 2010).

Finally, the **cytoplasm to vacuole (Cvt) targeting pathway** is an example of a selective, autophagy-like pathway that is specific to yeast, in which the hydrolases aminopeptidase 1 and α-mannosidase are selectively transported to the vacuole (Huang and Klionsky, 2002).

Thirty-five **a**u**t**opha**g**y-related genes (*ATG*) in yeast have been so far identified, and, many of them present homologues in higher eukaryotes (Yang and Klionsky, 2009). ATG proteins are organized in functional complexes that mediate the diverse steps of macroautophagy and other selective forms of autophagy: induction/initiation, vesicle nucleation, cargo recognition and packaging, vesicle expansion and sealing, fusion with the lysosome, vesicle breakdown and recycling of the resulting macromolecules (figure 7).

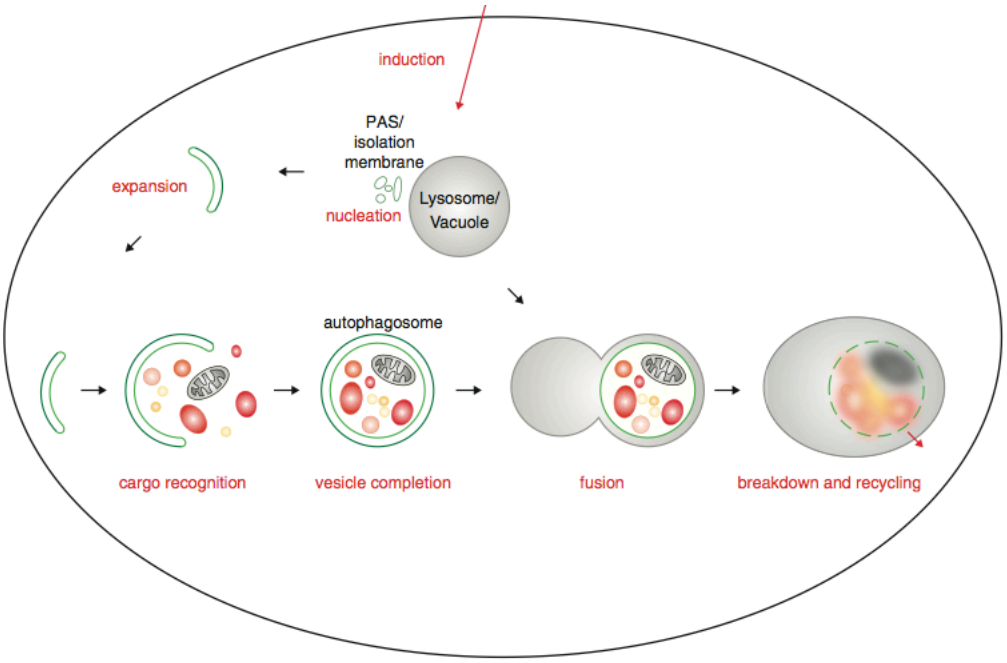



**Figure-7. Schematic representation of autophagy.** Autophagy undergoes several processes: nucleation, elongation, formation of autophagosomes, maturation, formation of autolysosomes and degradation of cargo. The image was obtained from (Kraft and Martens, 2012).

### I-5.1. Autophagy and signalling pathways

Some signalling pathways have been characterized as playing a role in the regulation of autophagy. The regulatory proteins of these pathways are the Target of Rapamycin (TOR), Sch9p, Ras/cAMP-dependent protein kinase A (PKA), and Pho85p (Budovskaya *et al*., 2004; Yorimitsu *et al*., 2007; Yang *et al*., 2010). Under nutrient-rich conditions, autophagy is inhibited because TORC1 is activated and drives the hyperphosphorylation of the protein Atg13p, resulting in a lower affinity for Atg1p and Atg17p to begin the induction of the process (figure 8) (Kamada *et al*., 2010). In this process, it is also known that PKA and Sch9p are involved in the regulation of Atg13p phosphorylation and localization to the preautophagosomal structure (Stephan *et al*., 2009), although the mechanisms involved are yet to be understood.

When TORC1 activity is inhibited, either by rapamycin or starvation, Atg13p is rapidly dephosphorylated (to yield a hypo-phosphorylated form of Atg13p) and can interact with the Atg1p serine/threonine kinase. The Atg1-Atg13 protein complex then associates with Atg17p, which is part of a ternary complex with Atg29p and Atg31p (Cebollero and Reggiori, 2009; Nakatogawa *et al*., 2009; Chang and Neufeld, 2010; Kamada *et al*., 2010). The Atg1-Atg13 protein complex then recruits other Atg proteins to the phagophore assembly site (PAS) and controls their dynamics (Kabeya *et al.,* 2005; Cheong *et al.,* 2008, Kawamata *et*

*al.,* 2008). Autophagosome nucleation requires a complex containing Atg6p and the class III phosphatidylinositol 3-kinase Vps34p, the latter generating phosphatidylinositol 3-phosphate.

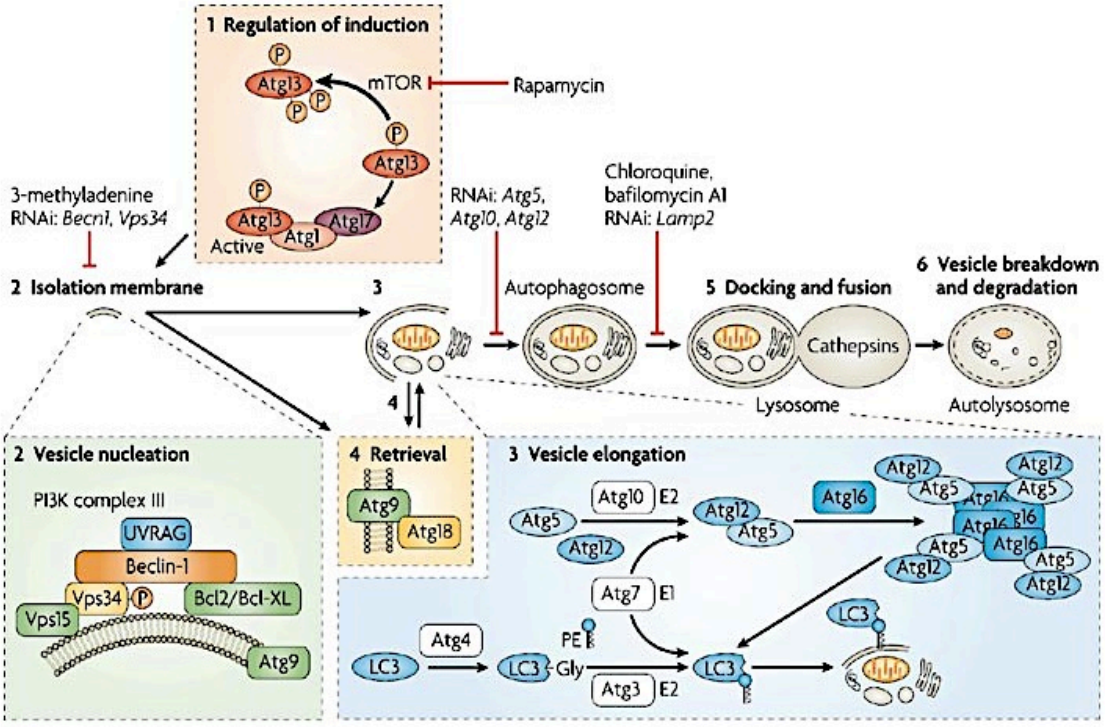



**Figure-8. Schematic diagram of the various stages of autophagy.** Stage 1 involves the regulation of autophagy induction, in which mTOR is inactivated, allowing for the activation of the Ulk1 kinase complex. In stage 2, nucleation, the Class III PI(3)K complex forms which is necessary for formation of the isolation membrane. The membrane expands to engulf cytosolic contents. In stage 3, vesicle elongation, a process that requires the two ubiquitin-like conjugation steps of Atg5–Atg12 and LC3/Atg8p–PE. In stage 4, vesicle retrieval, the transport of Atg9p between the PAS and non-PAS sites is necessary for autophagosome formation and requires Atg18. Stage 5, vesicle maturation, involves trafficking and fusion of the fully enclosed double-membrane autophagosome to various endosomal compartments, which finally fuses with the lysosome to form the autolysosome. In the final stage, stage 6, degradation (the contents of the autolysosome are degraded by resident lysosomal enzymes). Although the process is described for mammalian cells, similar features are also observed for yeast cells. The image was obtained from (Maiuri *et al*., 2007).

The expansion of autophagosomal membranes involves two ubiquitin-like molecules, Atg12p and Atg8p, an E1 ubiquitin activating enzyme (Atg7p), two analogues of ubiquitin-conjugated enzymes (Atg10p and Atg3p), an Atg8p modifying protease (Atg4p), the protein target of Atg12p attachment (Atg5p) and Atg16p. In the first ubiquitination reaction, the E1-like Atg7p and the E2-like Atg10p promote the association of Atg12p with Atg5p (Suzuki *et al*., 2001; Suziki *et al*., 2007). This conjugate subsequently interacts with Atg16p to generate pre-autophagosomal structures (PAS) (Mizushima *et al*., 1999). In the second ubiquitin reaction, Atg8p is cleaved by the protease Atg4p and conjugated to phosphatidylethanolamine (PE) by Atg7p (E1-like) and Atg3p (E2-like) (Kim *et al*., 1999;

Kirisako *et al*., 2000;). This lipidated form of Atg8p is essential to drive proper autophagosome biogenesis (Nair *et al*., 2012). Upon completion of autophagosome formation, the Atg12–Atg5–Atg16 protein complex is released into the cytosol, whereas Atg8-PE remains stably associated with the autophagosomal membranes (Kirisaki *et al*., 2000). Lysosome docking and fusion occurs when the outer autophagosomal membrane fuses with the lysosomal membrane to produce an autophagic body (autolysosome in mammalian cells). The remaining single-membrane that envelops the cargo is lysed and the population of Atg8-PE together with the enclosed cargo are released into the lysosome lumen and degraded by resident vacuolar hydrolases (proteases, lipases, nucleases and glucosidases) (Kirisako *et al.*, 1999; Kabeba *et al.*, 2000). The resulting degradation products are released back into the cytosol through the activity of specific membrane permeases for recycling.

### I-5.2. Autophagy and aging

The relationship between CLS and autophagy is extremely complex and not fully understood. Autophagy appears to be a common downstream target of multiple cellular pathways with well-known roles in longevity regulation (Madeo *et al*., 2010). The upregulation of autophagy extends chronological lifespan in mice, *Caenorhabditis elegans*, yeast and other organisms (Eisenberg *et al*., 2009). Importantly, the TOR/Sch9p and the Ras/cAMP-dependent protein kinase proteins, which integrate the network of nutrient-sensing pathways and regulate autophagy, are known to be involved in proper regulation of longevity pathways (Kaeberlein *et al*., 2005; Gomes *et al*., 2007; Hen and Klionsky, 2011). Recently, Hansen *et al*. found that dietary restriction and TOR inhibition in *C. elegans* produce an autophagic phenotype and that inhibiting genes required for autophagy prevents dietary restriction and TOR inhibition from extending lifespan, corroborating with this conception (Hansen *et al*., 2008).

Screenings performed in yeast have demonstrated that genes encoding proteins of the autophagic machinery are necessary to extend lifespan during nitrogen starvation (Tsukada, 1993). Suppression of autophagy by knockdown of essential autophagy genes triggers apoptosis or necrosis in cells that would otherwise survive under stress conditions (reviewed in Kourtis and Tavernarakis, 2009; Mathew *et al*., 2009). Autophagy appears to serve primarily a cytoprotective function by maintaining nutrient and energy homeostasis during starvation or by degrading damaged cellular components and invasive pathogens (review in Lionaki *et al.,* 2013). Paradoxically, although autophagy is a predominantly homeostatic mechanism, it can also play a role in cell death, which is not restricted to developmental

programmed cell death, but extends to cell death that occurs in many pathological conditions. Excessive autophagy induced by extreme conditions such as toxins and necrosis-triggering insults might cause uncontrollable degradation or sequestration of cells contents resulting in undesirable cell death if not properly regulated (Samara and Tavernarakis, 2008; Kourtis and Tavernarakis, 2009; Yang and Klionsky, 2010).

### I-5.3. Mitophagy: the autophagic-like selective degradation of mitochondria process

The view of mitochondrial dynamics has expanded into an integral cell biological process influencing many cellular functions and ultimately contributing to cell death and aging (Braun and Westermann, 2011). Mitochondria are dynamic structures that migrate throughout the cell, fuse and divide, and undergo regulated turnover (Westermann, 2010). On this basis, the regulation of mitochondrial dynamics (fusion/fission cycles) and the selective degradation of mitochondria by an-autophagic-like process (mitophagy) are important on the regulation of mitochondrial function and cell physiology by allowing mitochondrial recruitment to critical subcellular compartments, mitochondrial communication, regulation of the mitochondrial shape and to the mitochondrial quality control (Liesa and Shirihai, 2013).

The mitochondrial theory of aging predicts that an accumulation of oxidative stress and mtDNA mutations eventually is associated with the onset of age-associated pathologies and cell death (Cadenas and Davies, 2000). Apparently, mitophagy is associated with the removal of damaged/dysfunctional or oxidized mitochondria and therefore contributes to the homeostatic maintenance of sustainable mitochondrial function, allowing an efficient process for ATP production and cellular energetics (Kissova *et al*., 2004). There are several lines of evidence in yeast studies suggesting that damaged mitochondria are eliminated by mitophagy. For example, interference with $F_1F_o$-ATPase biogenesis in a temperature sensitive *fmc1* mutant (Priault *et al*., 2005), or osmotic swelling of mitochondria caused by depletion of the mitochondrial $K^+/H^+$ exchanger Mdm38 (Nowikovsky *et al*., 2007) induce mitophagy.

It is also conceivable to assume that this process allows complementation of mtDNA gene products in heteroplasmic cells that have accumulated different somatic mutations, thus diluting the effect of mtDNA mutations and depolarized mitochondria during aging. Furthermore, Mao et al. have recently disclosed an important link between mitophagy and mitochondrial dynamics. On this basis, both processes may act in a coordinate manner to assure the proper connectivity of the mitochondrial network, which is an important factor that determines the cell's response to calcium and other pro-apoptotic signals and ultimately cell fate (Mao *et al*., 2011). In addition, mitophagy is also an essential step in certain

developmental processes such as embryonic development and spermatogenesis (Al Rawi *et al*., 2011; Sato and Sato, 2011).

The core autophagic machinery used is common with other types of autophagy. The requirement of several *ATG* genes for mitophagy has been reported from several groups (Kissova *et al*., 2004; 2007; Tal *et al*., 2007; Zhang *et al*., 2007; Kanki and Klionsky, 2008) and some *atg* mutants strains in *S. cerevisiae* screenings were identified to be selectively involved in mitophagy, namely *ATG32* and *ATG33* genes (Kanki *et al.*, 2009). Their function is not completely understood in the process.

In yeast studies, there are several ways to induced mitophagy. The most common are the incubation in nitrogen starvation conditions (Kissova *et al*., 2007; Mao *et a*l., 2011; Suzuki *et al*., 2011, Kurihara *et al*., 2012), treatment with the TORC1 inhibitor, rapamycin, after pre-culturing yeast in a non-fermentable medium that induces the proliferation of mitochondria (e.g. lactate) (Tal *et al*., 2007; Kanki and Klionsky, 2008; Kanki *et al*., 2009). In more physiological conditions, mitophagy is induced at stationary phase when yeast cells are cultured in a medium with a non-fermentable carbon source (Tal *et al.*, 2007; Kanki and Klionsky, 2008).
Mitophagy has recently become the subject of much scientific interest. This is due in part to the central role of this organelle in various cellular processes, as well as the association of mitochondrial dysfunction with pathological conditions in humans such as the neurodegenerative Alzheimer's and Parkinson's diseases (Abeliovich, 2010).

# Chapter II
# Aim of the work

Previous studies have reported that the Sch9p protein kinase negatively regulates mitochondrial function (Pan and Shadel, 2009), autophagy (Yorimitsu *et al*., 2007) and CLS by integrating nutrient signals from TORC1 with stress signals from sphingolipids (Huang *et al*, 2012). Indeed, Sch9p can also be phosphorylated in the C-terminus by TORC1 or in the Thr570 residue located in the activation loop by the Pkh1/2p protein kinases, the last in response to LCBs (Voordeckers *et al*., 2011, Huang *et al*., 2012). Our lab has recently revealed that TORC1 is a negative regulater of *isc1*Δ phenotypes (Teixeira *et al.*, unpublished results), thus we hypotheside that Sch9p may act downstream of TORC1 and be implicated in *isc1*Δ phenotypes.

The present work aimed to unravel the role of the Sch9p kinase in mediating phenotypes of Isc1p-deficient cells such as oxidative stress sensitivity, shortened CLS, mitochondrial dysfunction, and impaired autophagy-like mechanisms. The following studies were performed using the *S. cerevisiae* BY4741 parental strain and its isogenic *isc1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ mutant strains:

- Assessment of hydrogen peroxide resistance and antioxidant defense levels, namely superoxide dismutase and catalase activities;
- Characterization of mitochondrial function, by measuring oxygen consumption, cytochrome *c* oxidase activity, mitochondrial membrane potential and reactive oxygen species levels, and assessment of the mitochondrial network organization;
- Characterization of autophagy and mitophagy processes.

# Chapter III
# Material and Methods

## III-1. Yeast strains, and growth conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cells were grown aerobically at 26°C in a gyratory shaker (at 140 rpm), with a ratio of flask volume/medium volume of 5:1, to exponential phase ($OD_{600}$=0.6) or to post-diauxic phase ($OD_{600}$=7-8). The growth media used were yeast peptone dextrose, YPD (1 % (wt/vol) yeast extract, 2% (wt/vol) bactopeptone, 2% (wt/vol) glucose); synthetic complete (SC) drop-out medium containing 2% (wt/vol) glucose and 0.67% yeast nitrogen base without aminoacids (BD BioSciences) and supplemented with appropriate aminoacids or nucleotides (0.008% (wt/vol) histidine, 0.008 % (wt/vol) tryptophan, 0.04% (wt/vol) leucine and 0.008% (wt/vol) uracil); minimal medium (0.67% (wt/vol) yeast nitrogen base without aminoacids, 2% (wt/vol) glucose), supplemented with appropriate amino acids and nucleotides (0.004% (wt/vol) histidine, 0.004% (wt/vol) methionine, 0.008% (wt/vol) leucine and 0.004 % (wt/vol) uracil); and synthetic drop-out medium containing 2% (wt/vol) lactate, 0.67% (wt/vol) yeast nitrogen base without amino acids, and supplemented with appropriate amino acids or nucleotides (0.008% (wt/vol) histidine, 0.008% (wt/vol) tryptophan, and 0.008% (wt/vol) uracil) with pH adjusted to 5.5.

**Table 1. Yeast strains used in this work**

| Strain | Genotype | Reference/source |
|---|---|---|
| BY4741 | Mata, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* | EUROSCARF |
| *isc1*Δ | BY4741 *isc1*Δ::*KanMX4* | EUROSCARF |
| *sch9*Δ | BY4741 *sch9*Δ::*KanMX4* | EUROSCARF |
| *isc1*Δ*sch9*Δ | BY4741 *isc1*Δ::*LEU2 sch9*Δ:.*KanMX4* | This study |
| BY4741 pYX222 | BY4741 carrying pYX222 | Teixeira, V. |
| *isc1*Δ pYX222 | *isc1*Δ carrying pYX222 | Teixeira, V. |
| *sch9*Δ pYX222 | *sch9*Δ carrying pYX222 | This study |
| *isc1*Δ*sch9*Δ pYX222 | *isc1*Δ*sch9*Δ carrying pYX222 | This study |
| BY4741 p*GFP-ATG8* | BY4741 carrying pRS416-*GFP-ATG8* | Teixeira, V. |
| *isc1*Δ p*GFP-ATG8* | *isc1*Δ carrying pRS416-*GFP-ATG8* | Teixeira, V. |
| *sch9*Δ p*GFP-ATG8* | *sch9*Δ carrying pRS416-*GFP-ATG8* | This study |
| *isc1*Δ*sch9*Δ p*GFP-ATG8* | *isc1*Δ*sch9*Δ carrying pRS416-*GFP-ATG8* | This study |
| BY4741 *pho8*Δ pmt*PHO*8 | BY4741 *pho8*::*HPH* carrying pYX242-pmt*PHO8* | Teixeira, V. |
| *isc1*Δ*pho8*Δ pmt*PHO*8 | *isc1*Δ *pho8::HPH* carrying pYX242-pmt*PHO8* | Teixeira, V. |
| *sch9*Δ*pho8*Δ pmt*PHO*8 | *sch9*Δ *pho8::HPH* carrying pYX242-pmt*PHO8* | This study |
| *isc1*Δ*sch9*Δ *pho8*Δ pmt*PHO*8 | *isc1*Δ::*URA3 sch9*::*KanMX4 pho8*Δ*::HPH* carrying pYX242 -pmt*PHO8* | This study |

## III-2. Genomic DNA isolation

Cells (10 mL) were cultured overnight and harvested by centrifugation during 5 min at 4000 rpm. The pellet was collected, washed once and ressuspended in 100 µL of lysis buffer (2% (vol/vol) Triton X-100, 1% (wt/vol) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 100 µL of phenol:chloroform:isoamyl alcohol [50:48:2 (vol/vol/vol)]. Cells were lysed by vigorous shaking of the cell suspension in the presence of glass beads for 3 min (short pulses of 1 min were used, with 1-min intervals on ice). The aqueous phase was recovered after centrifugation at 4000 rpm for 5 min, and 100 µL of chloroform were added. The mixture was homogenized by vortexing 3 min (as described previously), supplemented with 100 µL TE buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA) and centrifuged for 5 min at 14000 rpm. The aqueous phase was washed with 1 mL of 100 % ethanol. After centrifugation (14000 rpm, 3 min), the pellet was ressuspended in 400 µL of TE buffer. It was added 30 µg of RNAse and the mixture was incubated for 5 min at 37°C. Then, 10 µL of 4 M ammonium acetate and 1 mL of 100 % ethanol were added. The DNA was collected by centrifugation (14000 rpm, 3 min), washed twice with 70 % (vol/vol) ethanol, dried and ressuspended in water. The genomic DNA was quantified using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific) and analyzed by gel electrophoresis in 1% (wt/vol) agarose and add ethidium bromide (EtBr) to a final concentration of 0.5µg/mL and evaluate the quality of the isolated DNA.

## III-3. Polymerase Chain Reaction (PCR) procedure

A mix of 20 µL containing 1 x Reaction Buffer (Thermo Scientific), 1.5 mM $MgCl_2$ (Thermo Scientific), 0.2 mM sense primer, 0.2 mM antisense primer, 0.2 µM dNTPs (Thermo Scientific), 1 U Taq Polymerase (Thermo Scientific), and 300 ng genomic DNA was prepared. For the amplification of the *LEU2* cassette used for the disruption of *ISC1*, the annealing temperature was 50ºC and the elongation time was 108 seconds during 30 cycles. For the confirmation of *ISC1* deletion with a *LEU2* cassette, the annealing temperature was 51ºC and the elongation time was 85 seconds for 30 cyles. For the amplification of the *URA3* cassette used for the disruption of *ISC1*, the annealing temperature was 50ºC and the elongation time was 108 seconds during 30 cycles. For the confirmation of this disruption, the annealing temperature was 52ºC, with an elongation time of 75 seconds for 30 cycles. For the amplification of the hygromycin (*HPH*) cassette used for the disruption of the *PHO8* gene and the confirmation of the deletion, the annealing temperature was 57ºC and the elongation time was 80 seconds, which was performed

during 30 cycles. PCR products were analyzed in 1% (wt/vol) agarose gel using 0,55µg/mL EtBr and TAE 1x as buffer, and DNA bands were compared to Gene Ruler Ladder Mix (Thermo Scientific)

## III-4. Gene disruption

The disruption of *ISC1* using *LEU2* cassette was performed by homologue recombination in *sch9*Δ. The deletion fragment containing *LEU2* and the flanking regions of *ISC1* was amplified from genomic DNA isolated from the BY4741 *isc1*Δ::*LEU2* strain stored in the lab using primers ISC1_Amp_Fw and ISC1_Amp_Rv (Table 2). The disruption of *ISC1* using a *URA3* cassette was amplified from genomic DNA isolated from the BY4741 *isc1*Δ::*URA3* strain stored in the lab using the same primers as described above. The purification of DNA from TAE agarose gels was performed with GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare). Cells were transformed by electroporation and selected in minimal medium lacking leucine and uracil, respectively. Gene disruption was confirmed by PCR (figure 9), using the following pair of primers: LEU2_Conf_Fw + LEU2_Conf_Rv and URA3_Conf_Fw + URA3_Conf_Rv, respectively (Table 2).

The disruption of the *PHO8* gene in BY4741, *isc1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells was performed using a deletion fragment that contains a hygromycin cassette and the flanking regions of *PHO8*, as reported (Sampaio-Marques *et al.*, 2012). The deletion fragment was amplified by PCR using the next set of primers: Pho8_Amp_Fw + Pho8_HPH_Rv and Pho8_Amp_Rv + Pho8_HPH_Fw (Table 2). Cells were transformed by electroporation and selected in YPD medium supplemented with hygromycin (150 µg/mL). The correct insertion of cassette was confirmed by PCR using the subsequent set of primers: Pho8_Conf_Fw + Pho8_HPH_Rv and Pho8_Conf_Rv + Pho8_HPH_Fw. These strains were then transformed with plasmid pYX242-mt*PHO8* and selected in minimal medium lacking leucine.

For the analysis of mitochondrial morphology, yeast cells were transformed with a plasmid expressing mitochondrial DsRed (pYX222-mtDsRed) and selected in minimal medium lacking histidine. For autophagy analysis, BY4741, *isc1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ were transformed with pRS416-*GFP-ATG8* and selected in minimal medium lacking uracil.

**Table 2. Primers used in this work**

*Fw-Forward primer/Rv-Reverse primer

| Primers | Sequence |
|---|---|
| **ISC1_Amp_Fw** | 5´-CTTTCCGCGTAAAAAGGGAA-3´ |
| **ISC1_Amp_Rv** | 5´-TTGCTTTGCATCTATTGACGA-3´ |
| **LEU2_Conf_Fw** | 5´-AGACGATTGCTAACCACCTA-3´ |
| **LEU2_Conf_Rv** | 5´-CGAACGAGGCAGTAGTCATGTT-3´ |
| **URA3_Conf_Fw** | 5´-ATCATCGCCGAATACGAAAC-3´ |
| **URA3_Conf_Rv** | 5´-CCCGCAGAGTACTGCAATTT-3´ |
| **Pho8_Amp_Fw** | 5´-GCCAGCAAGTGGCTACATAAA-3´ |
| **Pho8_HPH_Rv** | 5´-AAAGCATCAGCTCATCGAGA-3´ |
| **Pho8_Amp_Rv** | 5´-CAGTACGTGTCATGCGGTTAG-3´ |
| **Pho8_HPH_Fw** | 5´-CGCAAGGAATCGGTCAATAC-3´ |
| **Pho8_Conf_Fw** | 5´-CGACATGAATAGCAGCATTGA-3´ |
| **Pho8_Conf_Rv** | 5´-TCACGCTATAGAATGCACCT-3´ |

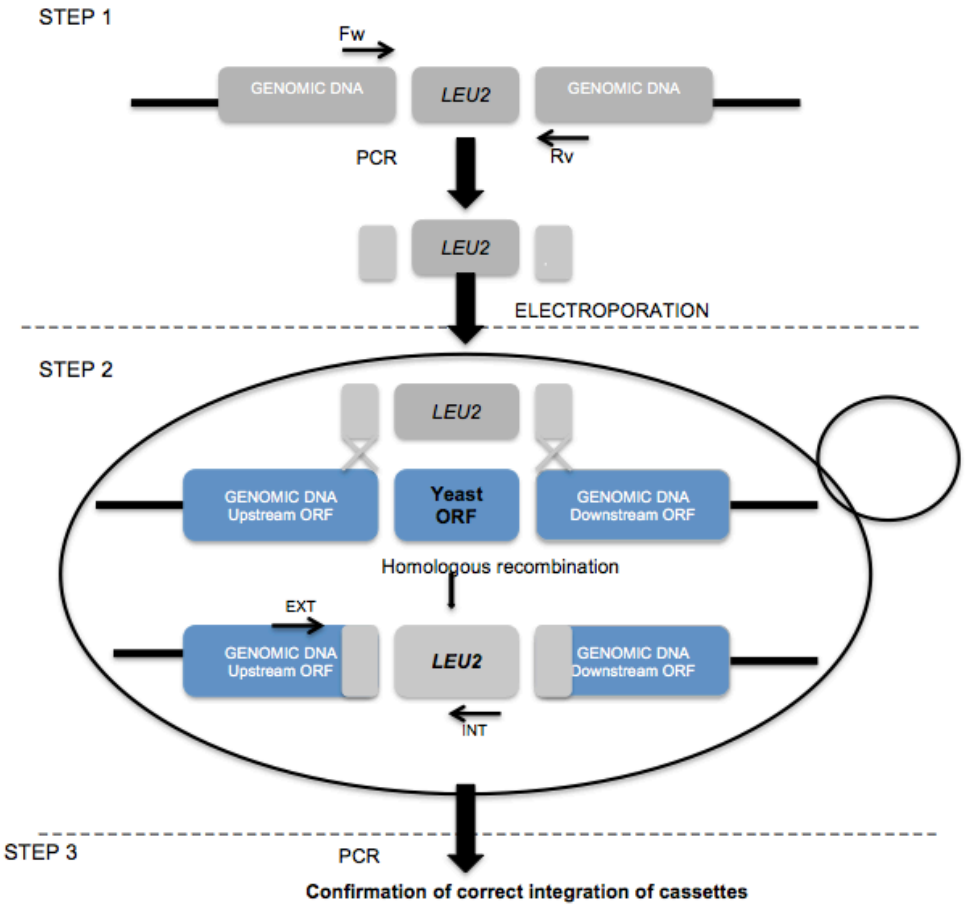



**Figure-9. General scheme of the strategy for the construction of yeast mutants.** Step 1 represents the procedure used for the generation of the *LEU2* cassette for the disruption of the *ISC1* gene, step 2 accounts for the homologous recombination mechanism for proper integration on the desired region and the step 3 exemplifies the comfirmation of the correct integration of the disruption cassette on the genome.

## III-5. Yeast electroporation

### III_5.1. Preparation of electro-competent cells

Cells were grown in 50 mL of YPD medium to an $OD_{600}$= 1.3 – 1.5, harvested, and ressuspended in 10 mL of a solution containing 10 mM Tris-HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5, and gently shacked during 45 min at 26ºC. Then, 250 µL of 1 M DTT was added and cells were shacked for 15 min at 26ºC. Ice-cold sterile water was added for a final volume of 50 mL and cells were centrifuged at 4ºC. Cells were firstly washed with 25 mL of ice-cold sterile water and then ressuspended in 50 µL of 1 M sorbitol (maintained at 4ºC).

### III-5.2. Electro-transformation and plating

Electro-competent cells (40 µL) were mixed with 5 µL of deletion fragment (containing approximately 0.5 µg of DNA). The mixture was transferred to pre-chilled sterile 2 mm electroporation cuvette. An electric pulse (1.5 kV, 25 µF and 200 Ω) was applied in parallel using an electroporation system (BioRad). After the pulse delivery, 1 mL of selective minimal medium was immediately added and cells were allowed to recover in appropriate media for 30 min (replicative plasmids) or 4 hours (integrative cassettes) at 26 ºC. Cells were then plated in selective medium and grown for 3 days at 26 ºC.

## III-6. Oxidative stress resistance

For the analysis of oxidative stress resistance, yeast cells were grown in SC-medium to exponential phase ($OD_{600}$=0.6) and treated with $H_2O_2$ (Merck) for 1 hour. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % agar (w/v). Colonies were counted after growth at 26 ºC for 3 days. Viability was expressed as the percentage of the colony-forming units (CFUs) (treated cells vs. untreated cells).

## III-7. Enzymatic activities and oxygen consumption

For enzymatic activities, yeast cells were harvested by centrifugation for 5 min at 4000 rpm (4ºC). Cells were then ressuspended in 50 mM potassium phosphate buffer (pH 7.0)

containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannhein) and total protein extracts were obtained by mechanical disruption through vigorous shaking of the cell suspension in the presence of glass beads for 5 min. Short pulses of 1 min were applied followed by 1-min incubation on ice. Cell debris was removed by centrifugation at 13000 rpm for 15 min and protein content was determined by the method of Lowry, using bovine serum albumin as a standard. Catalase activity was analyzed *in situ*, in the presence of 3,3′-diaminobenzidine tetrahydrochloride, using the $H_2O_2$/peroxidase system (Conyers and Kidwell, 1991). Superoxide dismutase activity was determined *in situ*, as described by e Flohe and Otting (1984). Cytochrome *c* oxidase (COX) activity was determined by measuring cytochrome *c* oxidation (Poyton et al., 1995). Oxygen consumption rate was measured for 3 x $10^8$ cells in PBS buffer (pH 7.4), using an oxygen electrode (Oxygraph, Hansatech). Data was analyzed using the Oxyg32 V2.25 software.

## III-8. Mitochondrial membrane potential and ROS levels

The mitochondrial membrane potential was assessed by flow cytometry, using cells labeled with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probes), a mitochondrial-specific voltage-dependent dye (Rottenberg and Wu, 1998). Briefly, 2x$10^6$ cells were ressuspended in sample buffer [10 mM 2-(N-morpholino) ethanesulfonic acid, 0.1 mM $MgCl_2$ and 2% (w/v) glucose, pH 6.0]. $DiOC_6(3)$ was added to a final concentration of 1 nM. The cell suspension was then incubated for 30 min at 26°C, collected by centrifugation and washed twice with PBS. Fluorescence was measured on the FL-1 channel with excitation and emission settings of 488 nm and 525 nm, respectively, without compensation. Data was analyzed using FlowJo software (Tree Star).

For the quantification of ROS levels, 5x$10^6$ cells were ressuspended in PBS and the superoxide anion sensitive probe dihydroethidium (DHE, Molecular Probes) was added to a final concentration of 5 µM. Cells were incubated for 10 min at 26°C, pelleted by centrifugation, washed twice with PBS and analyzed by flow cytometry with excitation and emission settings of 488 nm and ≥670 nm (FL-3 channel), without compensation. Data was analyzed using FlowJo software (Tree Star).

## III-9. Fluorescence microscopy

For mitochondrial morphology analysis, cells carrying the plasmid expressing a mitochondria-targeted DsRed fluorescent protein (pYX222-mtDsRed) were grown in SC-medium lacking histidine to the post-diauxic shift phase. Live cells were observed by fluorescence microscopy (AxioImager Z1, Carl Zeiss). Data image stacks were deconvolved by QMLE algorithm of Huygens Professional v3.0.2p1 (Scientific Volume Imaging B.V.). Maximum intensity projection was used to output final images using ImageJ 1.45v software.

## III-10. Western blot analysis

To assess alterations in autophagic flux, cells harboring the plasmid pRS416-*GFP-ATG8* were grown to the exponential phase in SC-medium lacking uracil and treated with either rapamycin (200 ng/mL, (Sigma-Aldrich)) or DMSO (vehicle, Sigma-Aldrich) for 3 h. Total protein extracts (30 µg) were separated by SDS-PAGE using 10% SDS-polyacrylamide gels at 16 mA and transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare) at 0.8 $mA/cm^2$ during 1 h. After blotting, the nitrocellulose membranes were stained with Ponceau S (0,6 % (wt/vol) of Ponceau S, 3 % (wt/vol) TCA and 3 % (wt/vol) sulfosalicylic acid acid) to visualize proteins.

Membranes were blocked for at least 2 h in TTBS [TBS supplemented with 0.05% (v/v) Tween-20 (Merck)] containing 5 % (w/v) non-fat dry milk and then incubated overnight with the primary antibody anti-GFP (1:3000; Roche). After washing twice with TTBS for 15 min, membranes were incubated with the secondary $\alpha$-mouse IgG (1:3000; Molecular Probes) for 1 hour and then washed with TTBS and TBS twice. Immunodetection of bands was revealed by chemiluminescence, using a kit from GE Healthcare (RPN 2109).

## III-11. Alkaline phosphatase assay

For the alkaline phosphatase assay, cells were harvested and ressuspended in 100 µL of assay buffer (250 mM Tris, 10 mM $MgSO_4$, 10 mM $ZnSO_4$, pH 9.0) and supplemented with 5 µL of Complete Mini protease inhibitor cocktail. The cells were lysed by vortexing with glass beads for 5 min. After centrifugation at 14000 rpm for 15 min, the supernatant was collected and the protein concentration was measured by the method of Lowry, using bovine serum albumin as a standard. 10 µg of total protein extract was added to reaction buffer (250 mM

Tris, 10 mM $MgSO_4$, 10 mM $ZnSO_4$, 4.56 mM nitrophenyl-phosphate). Samples were incubated for 15 min at 30°C before terminating the reaction by adding 500 µL of stop buffer (2 M glycine, pH 11.0). The production of nitrophenol was monitored by measuring the absorbance at 400 nm, and the nitrophenol concentration was calculated using Beer's law with $\varepsilon_{400}$ = 18,000 $M^{-1}cm^{-1}$. One activity unit was defined as nmol nitrophenol/min/mg protein.

## III-12. Statistical analyses

Data were analysed in GraphPad Prism Software v5.01 (GraphPad Software) and expressed as mean values ± SD from at least three independent experiments. Values were compared by Student's t-test $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$; ****, $p<0.0001$ or two-way ANOVA with Bonferroni correction, as properly referred.

# Chapter IV
# Results

# IV-1. Characterization of oxidative stress resistance response

### IV-1.1. *SCH9* disruption suppresses the oxidative stress sensitivity of *isc1*Δ cells

Our lab has recently demonstrated that TORC1 activity is increased in Isc1p-deficient cells, as monitored by the TORC1-dependent phosphorylation of Sch9p at the C-terminus (Teixeira *et al*., unpublished results). Since Sch9p is a downstream effector of TORC1 in the regulation of mitochondrial function, oxidative stress and chronological lifespan in yeast (Wei *et al*., 2008) we evaluated if *SCH9* deletion could abolish *isc1*Δ phenotypes, such as oxidative stress hypersensitivity and mitochondrial dysfunctions (Almeida *et al.,* 2008). To assess oxidative stress resistance, cells were grown in SC-medium to the exponential (fermentative) phase and treated with 1.5 mM $H_2O_2$ for 1 h.

As reported, *sch9*Δ cells were more resistant to oxidative stress than parental cells (Wei *et al*., 2008). Moreover, the deletion of *SCH9* suppressed the hydrogen peroxide sensitivity of *isc1*Δ cells, increasing cell survival to levels similar to the observed for *sch9*Δ mutant: cell viability was approximately 27% in parental cells, 7% in *isc1*Δ cells and 35% in *sch9*Δ and *isc1*Δ*sch9*Δ cells (figure 10). These results suggest that Sch9p contributes to increased oxidative stress in Isc1p-deficient cells.

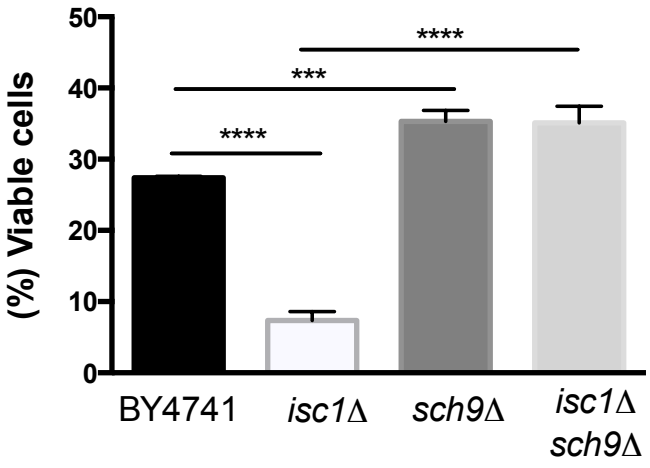



**Figure-10. *SCH9* disruption suppresses the oxidative stress sensitivity of *isc1*Δ cells.** Yeast cells grown to exponential phase and exposed to 1.5mM $H_2O_2$ for 1 hour. Cell viability was expressed as the percentage of CFUs (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s t-test. ***, p< 0.001; ****, p<0.0001.

# IV-2. Characterization of mitochondrial function

## IV-2.1. *SCH9* deletion relieves mitochondrial dysfunction of *isc1*Δ cells

It was previously shown that *sch9*Δ cells have extended chronological lifespan and this is attributed, in part, to improved and better coupled mitochondrial respiration at early stages of growth, which ultimately preconditions yeast to better survive on the stationary phase (F; (Fabrizio *et al.,* 2001; Wei *et al.*, 2008, Pan *et al.*, 2011). Since *isc1*Δ cells display severe mitochondrial dysfunction (Almeida *et al.*, 2008, Barbosa *et al.*, 2011), we evaluated if *SCH9* disruption could improve mitochondrial fitness of this mutant strain. To address this hypothesis, we have analyzed different mitochondrial parameters, namely cell growth in medium containing glycerol, a non-fermentable carbon source, oxygen consumption and cytochrome *c* oxidase (COX) activity.

To monitor respiratory capacity, we have firstly analyzed growth in glycerol, which requires functional mitochondria to metabolize it. For this purpose, yeast cells were grown to exponential phase, diluted to an $OD_{600}$ = 0.1 and five-fold serial dilutions were performed in media containing either glucose or glycerol as carbon source. As expected, parental (BY4741) and *sch9*Δ cells were able to grow in glycerol whereas *isc1*Δ mutant cells were unable to grow in such conditions (Almeida *et al.*, 2008, Barbosa *et al.*, 2011). Importantly, it was observed that the growth defect of *isc1*Δ cells on glycerol medium was suppressed in the *isc1*Δ*sch9*Δ double mutant (figure 11).

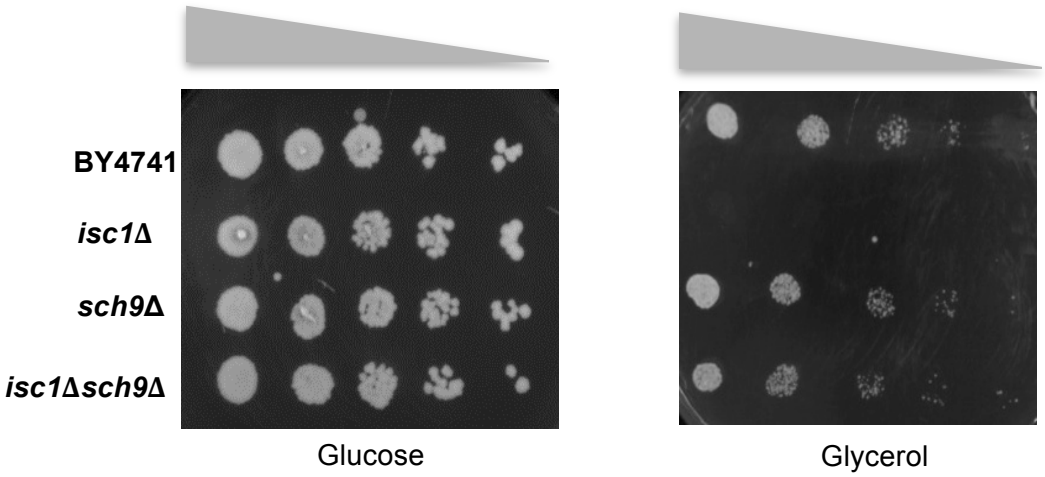



**Figure-11. Deletion of *SCH9* restores respiratory capacity of *isc1*Δ cells.** Yeast cells were grown to exponential phase, diluted to a $OD_{600}$=0.1 and fivefold dilutions were plated in SC-medium containing glucose or glycerol as carbon source.

Consistent with published data (Barbosa *et al.*, 2011), the deletion of *ISC1* almost completely abolished oxygen consumption and COX activity in cells grown to the post-diauxic shift (PDS) phase (figure 12). In *sch9*Δ cells, both COX activity and oxygen consumption at PDS phase were increased when compared to parental cells, in agreement with previous reports (Pan *et al.*, 20011). Notably, *SCH9* disruption suppressed the defects observed in *isc1*Δ cells: both oxygen consumption and COX activity were significantly increased to *sch9*Δ and wild-type levels, respectively (figure 12). These results suggest that Sch9p also contributes to mitochondrial dysfunction in *isc1*Δ cells.

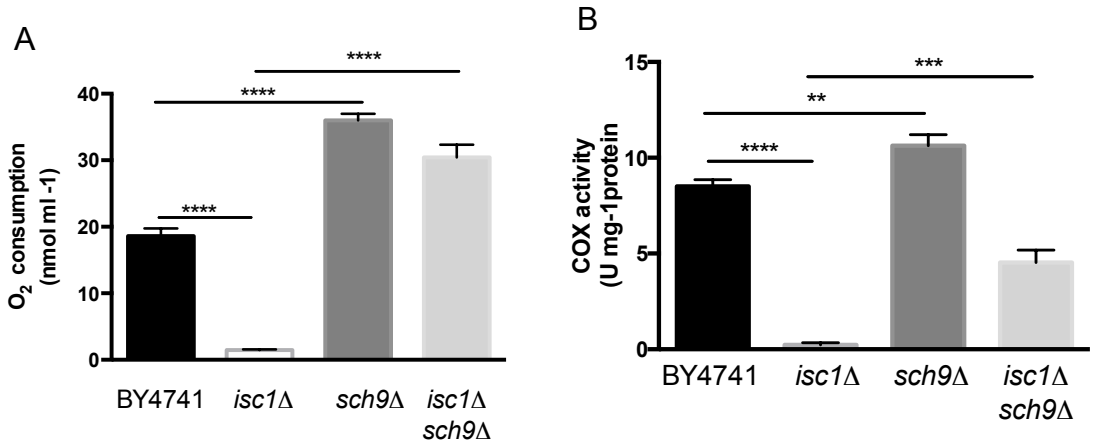



**Figure-12. *SCH9* disruption abolishes mitochondrial dysfunction in *isc1*Δ cells.** *S. cerevisiae* BY4741, *isc1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cells were grown in YPD medium to the post-diauxic shift phase. A- Oxygen consumption rates were measured as described in Material and Methods. B- Cytochrome *c* oxidase (COX)-specific activity. Cells were expressed as mean values ± SD pf at least three independent experiments. Values were compared by Student's t-test. **,p<0.01;***,p<0.001;****,p<0.0001

## IV- 2.2. Hyperpolarization and fragmentation of the mitochondrial network in *isc1*Δ cells are suppressed by *SCH9* deletion

To get additional insights into alterations in mitochondrial function, we have also assessed the mitochondrial membrane potential ($\Delta\psi_m$), a parameter that has been used to monitor changes on bioenergetics and functions as a key indicator of cell health or injury (Nicholls, 2004). For this purpose, yeast cells were labeled with a mitochondria-specific voltage-dependent dye, 3,3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], which aggregates and preferentially accumulates into functional mitochondria, and analyzed by flow cytometry (figure 13). When the mitochondrial membrane depolarizes, the dye no longer accumulates into mitochondria and becomes distributed throughout the cell, resulting in a decrease in

green fluorescence. At the PDS phase, cells lacking Isc1p displayed enhanced $\Delta\psi_m$ when compared to parental cells (figure 13), which is consistent with mitochondrial hyperpolarization. This has been associated with the activation of a mitochondrial dependent apoptotic pathway, which initially involves a transient hyperpolarization followed by depolarization of the mitochondrial membrane and release of cytochrome *c* from the mitochondria into the cytosol (Kroemer *et al.*, 2007). In contrast, *sch9*Δ cells had a slightly lower $\Delta\psi_m$, which has been related with mild mitochondrial uncoupling (Pan *et al.*, 2011). The *SCH9* disruption in *isc1*Δ cells reversed mitochondrial hyperpolarization and decreased the $\Delta\psi_m$ to values similar to those observed in parental cells (figure 13).

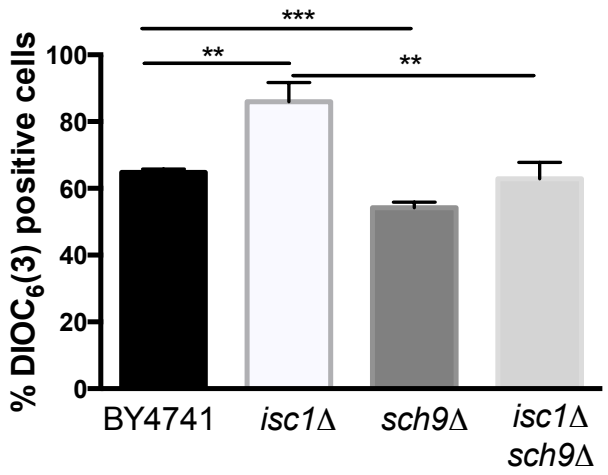



**Figure-13. The *SCH9* disruption reversed mitochondrial hyperpolarization in *isc1*Δ cells.** *S. cerevisiae* BY4741, *isc1*Δ, *sch9*Δ and *isc1*Δ*sch9*Δ cell were grown to PDS phase and treated for 30 minutes with 1nM $DIOC_6(3)$ and analyzed by flow cytometer as described in Materal and Methods. The percentage of $DIOC_6(3)$- positive cells was determined by FlowJo software analysis. Data are mean ± SD of at least three independent experiments. Values were compared by Student´s t-test. **,p<0.01;***,p<0.001

The mitochondrial membrane potential is known to play a key role in the regulation of mitochondrial morphology and alterations on this parameter were demonstrated to impact on mitochondrial dynamics (Detmer and Chan, 2007; Berman *et al.*, 2008). To assess the integrity of the mitochondrial network, cells were transformed with a plasmid expressing a mitochondria-targeted DsRed fluorescent protein (pYX222-mtDsRed) and grown in SC-medium lacking histidine. The mitochondrial network was analyzed in live cells by fluorescent microscopy. At PDS phase, *isc1*Δ cells showed the formation of a typically punctuated pattern contrasting with tubular and well-organized network observed in healthy parental cells (figure 14). This structural alteration has been associated with fragmentation and observed cells undergoing apopotic cell death.

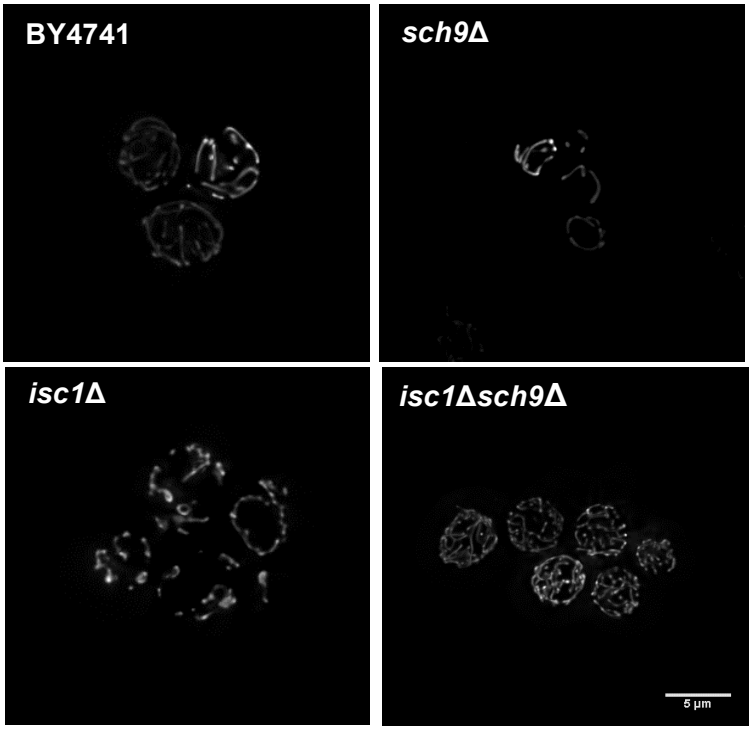



**Figure-14. Sch9p is implicated in the regulation of mitochondrial dynamics.** Yeast cells expressing a mitochondria-targeted DsRed flourescent protein (pYX222-mtDsRed) were grown to PDS phase in SC-glucose lacking histidine. Live cells were observed by flourescent microscopy.

The normal tubular mitochondrial network was restored in the double mutant *isc1*Δ*sch9*Δ (figure 14), suggesting that Sch9p is also implicated in the regulation of mitochondrial dynamics. The overall results support the hypothesis that the activation of Sch9p-driven signalling is detrimental for overall mitochondrial function and dynamics.

## IV-3. *SCH9* disruption decreases ROS production and improves antioxidant defense mechanisms in *isc1*Δ cells

Apoptosis and aging has been extensively associated with enhanced ROS production (Simm and Brömme, 2005, Raftopoulou, 2005, Kregel and Zhang, 2007, Marchi *et al., 2012*). Thus, the improvements of mitochondrial function and/or antioxidant defenses may decrease mitochondrial ROS production or increase its detoxification, leading to lifespan extension. To test this hypothesis, ROS levels were measured by flow cytometry using PDS and early stationary phase cells stained with dihydroethidium (DHE), a molecular probe particularly sensitive to superoxide radicals. The results show that ROS levels were low in all

strains at the PDS phase. Upon the transition to the stationary phase, ROS levels remained low in parental and *sch9*Δ cells but approximately 50% of *isc1*Δ cells were DHE-positive at this stage (figure 15). In *isc1*Δ cells with reduced Sch9p signalling, ROS levels were higher than in parental cells but significantly lower when compared to Isc1p-deficient cells (by approximately one-half), suggesting that *SCH9* deletion decreases ROS generation.

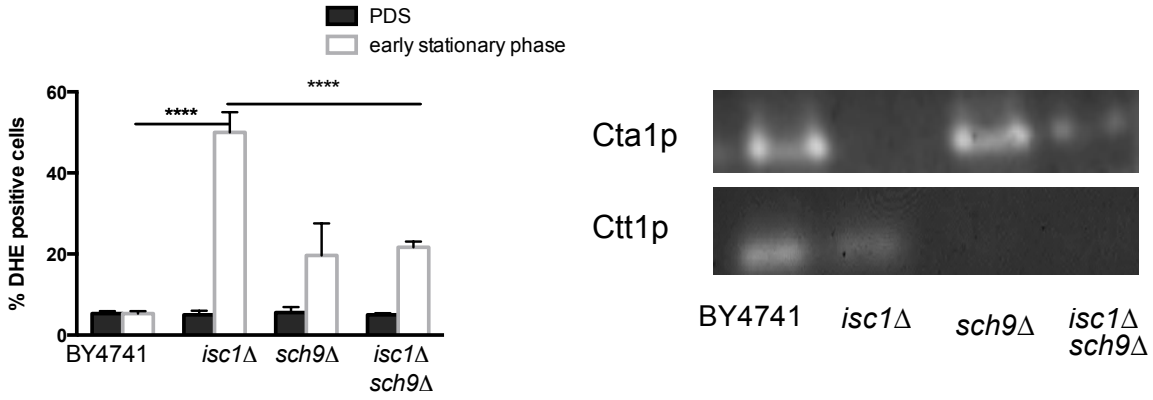



**Figure-15. *SCH9* disruption decreases ROS production and improves antioxidant mechanisms in *isc1*Δ cells.** A- Quantification of intracellular ROS. Yeast cells were grown to PDS phase and early stationary phase, labeled with DHE and analyzed by flow cytometer as described in Material and Methods. The percentage of positive cells was quantified by FlowJo software analyses. Data are means ± SD of at least three independent experiments. Values were compared by Student´s t-test. ****,p<0.0001. B. Catalase activity was detected *in situ* after non-denaturing polyacrylamide gel electrophoresis, using the $H_2O_2$/peroxidade system. A representative result is shown (out of 3 independent experiments).

Increased ROS levels have been associated with homeostatic imbalance partially dictated by impaired cellular antioxidant defences. Hence, we hypothesized that the improvement of antioxidant defence mechanisms could also contribute to decrease ROS levels in *isc1*Δ*sch9*Δ cells. The *isc1*Δ cells fail to induce *CTA1* gene expression in the post-diauxic phase (Kitagaki et al., 2009) and display low activity of Cta1p (Barbosa et al., 2011; figure 15B), the catalase A form present in mitochondria and peroxisomes (Petrova *et al*., 2004). In *isc1*Δ*sch9*Δ cells, Cta1p activity was restored, suggesting that Cta1p activity decreases in *isc1*Δ cells by a Sch9p-dependent mechanism. The *isc1*Δ*sch9*Δ cells, similarly to *sch9*Δ mutants, did not exhibit Ctt1p activity, which is consistent with the fact that the Sch9p kinase is directly or indirectly involved in the transcriptional control of *CTT1* expression in yeast (Pascual-Ahuir *et al.*, 2007).

## IV-4. Quantification of autophagy and mitophagy

Autophagy is a lysosomal/vacuolar degradative process that provides substrates upon energy demand during nutrient starvation and in basal conditions removes damaged organelles and biomolecules (Mizushima and Klionsky, 2007; Yorimitsu *et al*, 2007). Previous studies have demonstrated that autophagy has an important role in maintaining proper mitochondrial function and dynamics since autophagy-defective mutants present severe mitochondria dysfunctions, namely increased ROS production, growth defect on non-fermentable carbon sources, reduced oxygen consumption and general defects related to mitochondrial biology (Zhan *et al.*, 2007). Since *isc1*Δ cells present similar phenotypic features, we intended to evaluate if these cells present any autophagy defect impacting on various aspects of mitochondrial function. To begin to understand in more detail this complex crosstalk between mitochondria and autophagy, we have also assessed if the protein kinase Sch9p was also involved in the modulation of autophagy in Isc1p-deficient cells.

To monitor autophagy, we evaluated the processing of GFP-Atg8p in inducible conditions. Atg8p is an ubiquitin-like protein involved on autophagosome biogenesis and membrane elongation. When autophagy is induced, GFP-Atg8p is recruited to the phagophore where it is converted to its lipidated form, Atg8p-PE, to drive autophagosome biogenesis (Kirisako *et al*., 1999). It is then delivered to the vacuole inside the autophagic body. Whereas Atg8p is degraded after lysis of the autophagic body by resident vacuolar hydrolases, the GFP moiety is relatively resistant to proteolysis. Therefore, the appearance of free GFP signal is indicative of autophagy induction (Shintani and Klionsky, 2004). For this purpose, cells were treated with rapamycin (200 ng/mL), a well-known inducer of autophagy (Alvers *et al*., 2009; Noda and Ohsumit, 1997) and GFP-Atg8p processing was then analyzed by Western Blotting. As observed in figure 16, *isc1*Δ cells presented reduced autophagic flux (35%) when compared to the parental strain (60%), therefore corroborating that this defect in autophagy may contribute to the severe mitochondrial dysfunctions displayed by the mutant strain. To further substantiate this observation, we have also evaluated autophagic flux in *isc1*Δ cells upon the deletion of *SCH9*. The results demonstrate that the double mutant *isc1*Δ*sch9*Δ restored autophagic flux levels to values close to those observed for the *sch9*Δ single mutant, demonstrating that Sch9p is also involved in the deregulation of autophagy, ultimately contributing to *isc1*Δ phenotypes.

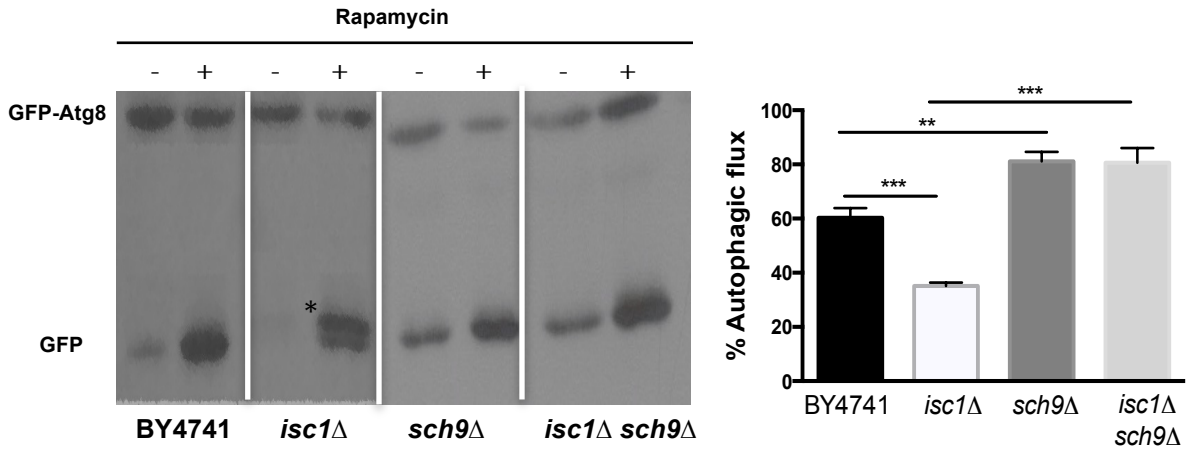



**Figure-16. *SCH9* disruption restoresd impaired autophagic flux in *isc1Δ* cells.** *S. cerevisiae* BY4741, *isc1Δ, sch9Δ*, *isc1Δsch9Δ* cells were grown to the exponential phase and treated with rapamycin (200 ng/ml) or DMSO (vehicle) for 3 hours. A- Proteins extracts wete separated by SDS-PAGE followed by imnoblotting with anti-GFP as primary antibody. B. Autophagic flux was calculated by teh ratio between free GFP signal and the sumo f the free GFP and the GFP-Atg8p signal. The quantification of the bands was performed by densitometry. **, p<0.01;***,p<0.001. * unspecific band.

More recently, the selective degradation of mitochondria by mitophagy and mitochondrial dynamics have been ascribed as key quality control mechanisms in age-related diseases by allowing the maintenance of a healthy mitochondrial network and proper overall cellular energetics. Since *isc1*Δ cells present mitochondrial dysfunction, it is conceivable to assume that mitophagy should be active in an attempt to counteract the impairment of mitochondrial function and increased mitochondrial fragmentation, which is usually associated with increased induction of the mitochondrial fission machinery (Jheng *et al.*, 2012).

In order to monitor mitophagy, we took advantage of the well-established alkaline phosphatase (ALP) assay (Campbell and Thorsness, 1998; Noda *et al.*, 1995). Cells lacking the endogenous vacuolar alkaline phosphatase Pho8p but expressing an inactive Pho8p proenzyme targeted to the mitochondrial matrix (mtPho8p) were used in this assay. When mitophagy is induced, this enzyme is relocated to the vacuole and converted to its active form after processing by resident hydrolases. Therefore, an increase in alkaline phosphatase activity reflects the induction of mitophagy. For this purpose, we have deleted *PHO8* and replaced it by a hygromycin cassette followed by transformation with a plasmid containing the inactive Pho8p proenzyme version targeted to the mitochondrial matrix (pYXXX-mtPho8).

Firstly, we have assessed mitophagy in inducible conditions. In this case, cells were grown to early exponential phase in SC-medium containing glucose ($OD_{600}$=0.1) and then shifted to SC-medium containing lactate, which induces profuse mitochondrial proliferation and mitophagy (Kurihara *et al.*, 2012*)*. As expected, mitophagy induction was increased in

parental cells upon shift to lactate-growing conditions (figure 17). Notably, *isc1*Δ cells presented enhanced alkaline phosphatase activity when compared to parental cells in similar conditions, suggesting that mitophagy is increased in the mutant strain. On the other hand, mitophagy induction was restored to values similar to parental cells in the *isc1*Δ*sch9*Δ double mutant. These results suggest that Sch9p also mediates mitophagy induction in *isc1*Δ cells.

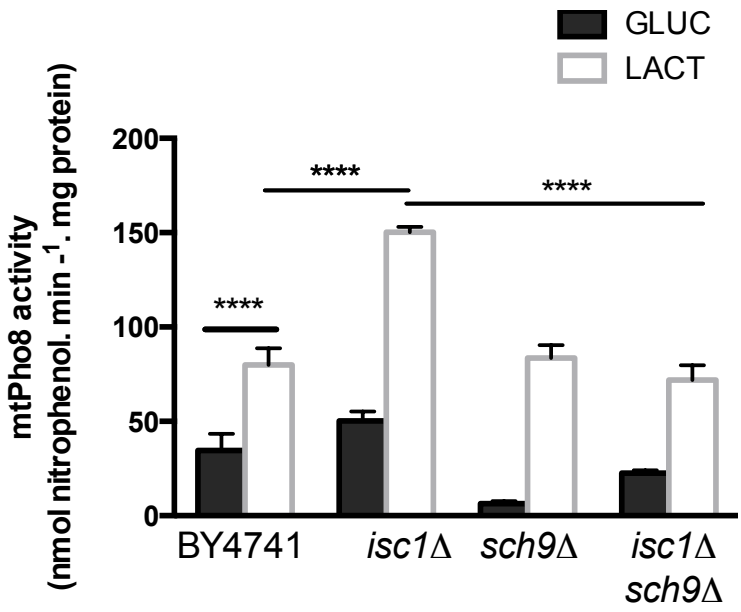



**Figure-17. Mitophagy induction is enhanced in *isc1*Δ cells by Sch9p-dependent mechanisms.** Yeast cells were grown in SC-medium containing glucose to early exponential phase (GLUC) and then shifted to SC-medium containing lactate (LAC) for 48 hours. The mtPho8 assay was performed as described in Material and Methods. Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s t-test. ****,p<0.0001.

We have also monitored alteration in mitophagy induction during chronological aging (figure 18). In this case, cells were grown in SC-medium to the PDS phase and then transferred to water overtime. We observed that mitophagy is only significantly induced after a 7-day incubation period in such conditions in parental cells. However, mitophagy induction was already higher in *isc1*Δ cells at the PDS phase and became significantly induced in cells aged for 5 days, maintaining steady levels at day 7. Importantly, mitophagy induction reached a peak at day 3 in the double mutant *isc1*Δ*sch9*Δ and then decreased to levels similar to those observed for parental cells and this was correlated with the period when Isc1p-deficient cells reached maximum induction.

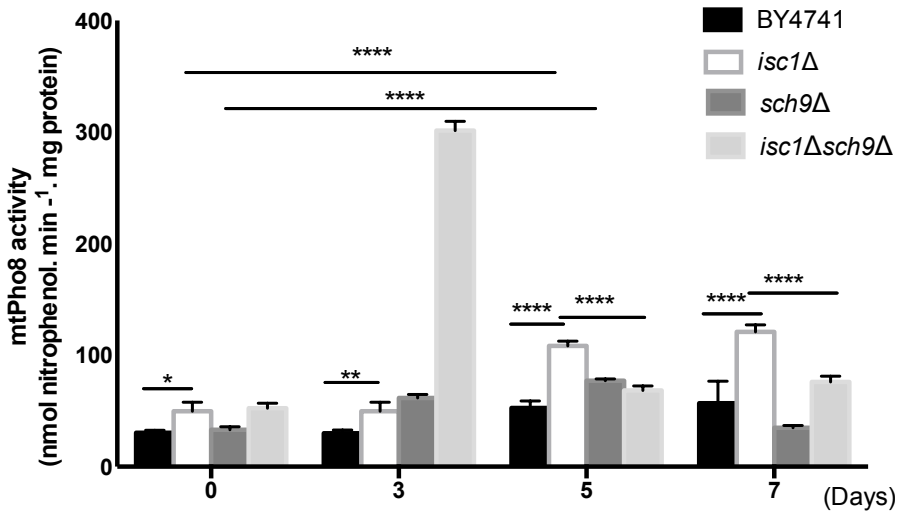



**Figure -18. Mitophagy induction during chronological lifespan.** *S.cerevisiae*, *isc1*Δ, *sch*9Δ and *isc1*Δ*sch9*Δ cells were grown in SC-medium to the PDS phase, shifted to water and then kept throughout the assay. The mtPho8 activity was determined as described in Material and Methods. Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by two-way ANOVA with Bonferroni correction.*,p<0.05;**,p<0.01;****,p<0.0001.

# Chapter V
# Discussion

Sphingolipids, such as ceramide, sphingosine and sphingosine-1-phosphate, have emerged in the last decades as key bioactive molecules, with roles in differentiation, senescence, cell cycle arrest, apoptosis and stress responses (Hannun and Obeid, 2008). The ability of these lipids to modulate cell signalling through activation of downstream effectors (protein kinases and phosphatases) and modulation of protein trafficking and intracellular localization suggests complex roles for sphingolipids in cell metabolism and physiology (Cowart and Obeid, 2007). The link between sphingolipid signalling and redox regulation has been established in some studies. For example, sphingolipids regulate cellular redox homeostasis and the activity of sphingomyelinases and ceramidase can be modulated by ROS and glutathione levels (Won and Singh, 2006).

In *S. cerevisiae,* Isc1p, an inositolphosphosphingolipid phospholipase C, is required for oxidative stress resistance, chronological lifespan and proper mitochondrial function (Almeida *et al.,* 2008, Barbosa *et al.*, 2011). However, the role of Isc1p in signal transduction during oxidative stress response and chronological aging is not fully understood. In this work, we explored the involvement of the Sch9p protein kinase on these signalling pathways. Some studies have reported that Sch9p regulates mitochondrial function and CLS (Pan and Shadel, 2009) and autophagy (Yorimitsu *et al.* 2007) by integrating nutrient signals from TORC1 with stress signals from sphingolipids (Huang *et al,* 2012). On this basis, we hypothesized that the activation of the TORC1-Sch9p axis could be implicated in *isc1*Δ phenotypes.

Our lab has recently disclosed that TORC1 is activated in cells lacking Isc1p (Teixeira *et al*., unpublished results), thus we postulated that Sch9p may act downstream of TORC1 and contribute to mitochondrial dysfunction, hydrogen peroxide sensitivity and premature aging of *isc1*Δ cells. Here we provided evidence that Sch9p signalling is impaired in Isc1p-deficient cells. In fact, the deletion of *SCH9* alleviates the oxidative stress sensitivity and mitochondrial dysfunctions of *isc1*Δ cells, which is compatible with Isc1p acting upstream of Sch9p. Such features are in agreement with previous studies showing that *SCH9* deletion contributes to better mitochondrial coupling and fitness during active growth in yeast cells by Rim15p-dependent mechanisms, eliciting an adaptive response that preconditions yeast cells to better survive in the stationary phase and promote longevity (Pan *et al.*, 2011). This is associated with increased translation of both nuclear- and mtDNA-encoded subunits of the oxidative phosphorylation system (Bonawitz *et al.*, 2007), which is consistent with higher oxygen consumption and COX activity observed in this study. In addition, *SCH9* disruption improves oxidative stress resistance already at the exponential phase, which is further extended at later stages of growth (stationary phase), as reported by others (Wei *et al.*,

2009). Importantly, the restoration of proper mitochondrial function and oxidative stress resistance mechanisms (Cta1p activity) in *isc1*Δ*sch9*Δ cells was correlated with a decrease in ROS levels during the stationary phase. Since the deletion of *SCH9* suppresses the shortened CLS of *isc1*Δ cells, this decrease probably contributes to lifespan extension in *isc1*Δ*sch9*Δ cells (Teixeira *et al*., unpublished results).

Our data suggest that the protein kinase Sch9p, the yeast homologue of mammalian Akt and pS6K proteins, acts downstream of TORC1 since the disruption of both *TOR1* (Teixeira *et a*l., unpublished results) and *SCH9* (this study) abolishes *isc1*Δ phenotypes. However, such regulation may also occur by TORC1-independent mechanisms. Lipidomic analysis showed specific changes in sphingolipids that accompanied the premature ageing of Isc1p-deficient cells, including increased basal levels of phytosphingosine (PHS), raising the possibility that Sch9p may also be activated in response to sphingolipid metabolism, as reported by others (Huang *et al.,* 2011). In addition to the phosphorylation of C-terminus by TORC1, Sch9p is phosphorylated in Thr570 in the activation loop by Pkh1p/Pkh2p protein kinases, in response to LCBs (Liu *et al.*, 2005). Furthermore, the downregulation of sphingolipid synthesis enhances CLS and improves mitochondrial function and oxidative stress resistance through the modulation of the PHS-Pkh1/2p-Sch9p axis. On this basis, we hypothesize that Sch9p may also act by integrating sphingolipid signalling. Further studies are necessary to clarify this hypothesis.
Studies performed in mammalian cells also support a functional connection between sphingolipid metabolism and the activation of Akt pathway and S6K. Qin *et al.* have recently demonstrated that fibroblasts with neutral sphingomyelinase 2 deficiency presented increased hyaluronan synthesis and secretion and this was correlated with the activation of Akt/mTOR pathway (increased phosphorylation of Akt) and p70S6K (Qin *et al.*, 2012.).

Autophagy is a degradative process for bulk proteins and damaged and/or unnecessary organelles, induced primarily in response to nutrient starvation (Mizushima and Klionsky, 2007). Recent studies have reported that autophagic defects have a functional impact on various aspects of mitochondrial functions, suggesting a critical role of autophagy in mitochondria functional integrity and maintenance (Zhang *et al.*, 2007; Twig *et al.*, 2008). The deletion mutants for essential autophagy genes exhibit various defects related to mitochondrial biology. For instance, Zhang Y *et al.* demonstrated that mutants defective in *ATG* genes (autophagy related genes) presented lower oxygen consumption rates, higher levels of ROS and they were more prone to accumulate dysfunctional mitochondria (Zhang *et al.*, 2007).

TORC1 plays a major role in the regulation of autophagy and recent data have demonstrated that Sch9p is a negative regulator of the process (Yorimitsu *et al.*, 2007). In this study, we show that Isc1p-deficient cells presented reduced autophagic flux, which could be on the etiology of mitochondrial dysfunctions exhibited by the mutant strain. Remarkably, *SCH9* disruption reestablishes the autophagic flux in *isc1*Δ cells, which is consistent with the suppression of mitochondrial dysfunction, $H_2O_2$ hypersensitivity and premature aging in the double mutant.

Mitochondria are dynamic structures that migrate throughout the cell, fuse and divide, and undergo regulated turnover by mitophagy. These highly coordinated processes are important on the regulation of mitochondrial function and cell physiology by allowing mitochondrial recruitment to critical subcellular compartments, mitochondrial communication, regulation of the mitochondrial shape and mitochondrial quality control. However, alterations in these quality control mechanisms affect overall cellular metabolism and cell fate. For instance, when mitochondrial dynamics is disrupted, mitochondrial dysfunction ensues and this is usually related with ageing and age-associated pathologies, such as cancer and neurodegenerative diseases (Lin and Beal, 2006; Anandatheerthavarada *et al.*, 2003).
On this basis, we have studied alterations in mitochondrial dynamics and mitophagy. In this study, we demonstrate that Isc1p-deficient cells have disrupted mitochondrial network, consistent with mitochondrial fragmentation, and that the deletion of *SCH9* abolishes this phenotype. These results suggest that Sch9p is also implicated in the regulation of mitochondrial dynamics in *isc1*Δ cells, accounting for mitochondrial dysfunction displayed by this mutant strain.
Mitophagy in yeast can be induced by transfering cells initially grown in a lactate medium for mitochondrial proliferation to nitrogen starvation medium with glucose (Kurihara *et al.*, 2011*).* However, we already observed a significant induction of mitophagy in *isc1*Δ cells during growth on lactate medium, comparing with parental cells. Consequently, we have decided to study mitophagy only by shifting the cells from glucose to lactate medium. The increased mitophagy observed in *isc1*Δ cells is consistent with the fact that this mutant exhibits dysfunctional mitochondria. Thus, the induction of mitophagy may account as a clearance mechanism in an attempt to remove damaged mitochondria and possibly maintain a healthy population of organelles to fulfill cellular bioenergetics demands. However, some lines of evidence have demonstrated that mitophagy can also be activated in response to alterations in mitochondrial dynamics. For instance, Mao *et al.* have recently demonstrated that mutants cells lacking Dnm1p and Fis1p, which are involved in mitochondrial fission in yeast, present lower levels of mitophagy in inducible conditions, unraveling an important link between these control quality mechanisms (Mao *et al.*, 2011). Thus, we also hypothesize that the increased

mitophagy observed in *isc1*Δ cells could be attributed to increased mitochondrial fission since *SCH9* deletion suppressed mitochondrial fragmentation in Isc1p-deficent cells and this was correlated with lower mitophagy induction in the double mutant *isc1*Δ*sch9*Δ. Further studies are necessary to substantiate this hypothesis.

In order to obtain a more general framework, we have also analysed mitophagy during CLS. Aging can be defined as a multifactorial phenomenon characterized by a time-dependent decline in physiological function that results in a gradual structural and functional deterioration of biomolecules and impairment of stress resistance mechanisms. Mitochondria, as major sites of ROS production, contribute to the accumulation of damage to macromolecules, which in turn overwhelm the capacity of biological systems to repair themselves, resulting in an inevitable functional decline. To monitor the magnitude of these alterations during aging, we have firstly grown cells in SC-medium to the PDS phase and then transferred to water throughout. Such severe caloric restriction (CR) regime (incubation in water) was used to extend the monitoring of this process during CLS, since *isc1*Δ cells present premature aging and this regime results in the longest survival for wild-type yeast strains (Fabrizio and Longo, 2003). Our results suggest that Sch9p activation is also detrimental in the regulation of mitophagy in *isc1*Δ cells during aging, which is intimately related with the involvement of the protein kinase in mitochondrial dysfunction and impairment of mitochondrial dynamics in the mutant strain. Importantly, the diminished induction of mitophagy at late stages of growth (when mitophagy induction reaches a maximum in Isc1p-deficient cells) is associated with the extended CLS of *isc1*Δ cells upon deletion of *SCH9* (Teixeira *et al*., unpublished results).

# Chapter VI
# Conclusion

In summary, our data suggest that Isc1p functions upstream of Sch9p and implicate the protein kinase in mitochondrial dysfunction, premature ageing and oxidative stress sensitivity of Isc1p-defcient cells. In fact, *SCH9* deletion restores mitochondrial function and proper morphology and the functional integrity of related biological processes, such as autophagy, mitophagy and oxidative stress response in *isc1*Δ cells. These results offer new insights on the regulation of redox homeostasis and cell quality control mechanisms by sphingolipid signalling, a link that has been suggested in several studies.

# Chapter VII

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