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Aging-related elevation of sphingoid bases shortens yeast chronological life span by compromising mitochondrial function

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by

Jae Kyo Yi

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Abstract of the Dissertation

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Aging is the biological process of growing older in a deleterious sense, senescence. However, what we have gained only comprises a partial and incomplete understanding of the fundamental molecular mechanisms. Increasing evidence suggests that dysregulation of the metabolism of sphingolipids including sphingoid bases (SBs, long-chain bases) might play an important role in the aging. We know neither how SBs are regulated during yeast aging nor how they, in turn, regulate it. Herein, I demonstrate that the yeast alkaline ceramidases (YPC1 and YDC1) and SB kinases (LCB4 and LCB5) cooperate in regulating SBs during the aging process and that SBs shorten chronological life span (CLS) by compromising mitochondrial functions. With a lipidomics approach, I found that SBs were increased in a time-dependent manner during yeast aging. I also demonstrated that among the enzymes known for being responsible for the metabolism of SBs, YPC1 was upregulated whereas LCB4/5 were downregulated in the course of

aging. This inverse regulation of YPC1 and LCB4/5 led to not only the aging-related upregulation of SBs in yeast but also a reduction in CLS. With the proteomics-based approach (SILAC), I revealed that increased SBs altered the levels of proteins related to mitochondria. Further mechanistic studies demonstrated that increased SBs inhibited mitochondrial fusion and caused fragmentation, resulting in decreases in mtDNA copy numbers, ATP levels, mitochondrial membrane potentials, and oxygen consumption. As the metabolism of sphingolipids is highly conserved between yeast and mammalian cells, these important findings would also facilitate our understanding of the role and mechanism of the action of sphingolipids and their metabolizing enzymes in physiological and pathological aging of humans and other mammals.

Dedication page

I would like to dedicate my thesis to my beloved parents

who always encourage me to pursue my dream and to go on every adventure,

especially this one.

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List of Abbreviations

ACER1	Alkaline ceramidase 1
ACER2	Alkaline ceramidase 2
ACER3	Alkaline ceramidase 3
aSMase	acid sphingomyelinase
ATP	adenosine triphosphaste
C-4 OHase	C-4 hydroxylase
CDases	ceramidases
CERK	ceramide kinase
Cer-P-Pase	ceramide phosphate phosphatase
CerS	Ceramide synthases
CS I	ceramide synthase I
CS II	ceramide synthase II
DHCD	dihydroceramide desaturase
DHS	dihydrosphingosine
ECR	enoyl-CoA reductase
Ecs	endothelial
ELO	fatty acid elongase
ER	endoplasmic reticulum
FA α-OHase	fatty acid α-hydroxylase
GCS	glucosylceramide synthase
GCS	glucosylceramide synthase

GIPC	glycosyl inositolphosphoceramid
HAD	hydroxyacyl-CoA dehydrase
IPCS	inositol phosphoceramide synthase
JNK1	c-Jun NH2-terminal kinase 1
KCS	3-ketoacyl-CoA reductase
KDSR	3-ketodihydrosphingosine reductase
KSR	3-ketosphinganine reductase
LCBK	LCB kinase
LCB-P-Pase	LCB phosphate phosphatase
LCS	lactosylceramide synthase
MAPK	mitogen-activated protein kinase
MCRMs	mitochondrial ceramide-rich macrodomains
n-9 DES	n-9 desaturase
NF-κB	nuclear factor-κB
nSMase	neutral sphingomyelinase
PHS	phytosphingosine
PI	phosphatidylinositol; DAG
PKB	protein kinase B
PKC	potent protein kinase C
PKCζ	protein kinase Cζ
PPAR	peroxisome proliferators-activated receptors
RC	mitochondrial respiratory chain
ROS	reactive oxygen species

S1P	form sphingosine-1-phosphate
SK	sphingosine kinase
SMase	sphingomyelinase
SMCs	smooth muscle cells
SMS	sphingomyelin synthase
SPH	sphingosine
SPHK	sphingosine kinase
SPT	serine palmitoyltransferase
SPT	serine palmitoyltransferase
TAP	Tandem affinity purification
TNF	tumor necrosis factor-α
TNFα	Tumor necrosis factor alpha
VLCFA	very long chain fatty acyl
αS	α-synuclein
Δ4 DES	Δ4 desaturases
Δ8 DES	Δ8 desaturases

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Chapter 1. Background and Significance

1.1 Aging

1.1.1 Introduction of aging

Aging is an inherently complex process that is manifested by an organism at genetic, molecular, cellular, organ, and system levels. From birth, aging begins and continues along with growth. Due to aging, various physiological phenomena occur in relation to reduction in the number of cellular tissues as well as body fluid, a decrease of the metabolic rate, loss of biological adaptability, and an increase of diseases (Kregel and Zhang 2007). Such physiological phenomena further lead to several vital impairments such as cardiopulmonary, neurological, endocrine function, immune function, as well as motor function impairments (Sartoris *et al.* 1985, Evers *et al.* 1994, Kajiya *et al.* 1997, Ardeljan and Chan 2013).

Due to the complexity of the aging process, it has been suggested that an integrative approach is required to better understand the mechanisms of aging (Jin 2010). In this regard, omics such as genomics, transcriptomics, proteomics, lipidomics and metabolomics, can play a very important role in the elucidation of the complex, interconnected changes that take place at the different levels of the biological hierarchy during the aging process (Barja 2013). However, the current knowledge of these molecular interactions is still very limited.

Although the fundamental mechanisms of the cause of aging need to be revealed, research findings addressed several variables and/or conditions such as, accumulated DNA damage, dysregulation of mitochondrial functions, and oxidative damage, as the causes of aging (Bohr and Anson 1995, Tatar *et al.* 2003, Trifunovic *et al.* 2004). Determining the underlying cellular and molecular processes that deteriorate with age and lead to increased disease susceptibility is critical because

there are increasing numbers of aging human populations and growing healthcare needs. For the reasons, it is necessary to develop novel therapeutic strategies to treat age-onset disorders.

1.1.2 Roles of mitochondria in aging

While the aging process is poorly understood, a number of aging theories have been proposed, such as a somatic DNA damage theory, a cross-linking theory, a wear and tear theory and a programmed theory (Harman 1956, Kirkwood 1977, Jin 2010, Mitteldorf 2012). The mitochondrial theory of aging has taken center stage for several decades. Mitochondria are intracellular organelles and present in most of the mammalian cells. Their primary role is the production of adenosine triphosphate (ATP) through oxidative phosphorylation. Mitochondria contain their own small 16.5 kb circular chromosome of DNA encoding several key proteins of the mitochondrial respiratory chain (RC) (Lee and Wei 2012). The mitochondrial theory is based on several observations. First of all, mitochondrial reactive oxygen species (ROS) production increases with age because mitochondrial function and activity of several ROS-scavenging enzymes decline with age. Additionally, mutations of mitochondrial DNA (mtDNA) accumulate during aging, which can lead to a vicious cycle because somatic mtDNA mutations impair respiratory chain function. Impaired mitochondrial function, in turn, increases ROS production, resulting in accumulation of oxidative damage to proteins, lipids, and DNA (Marnett *et al.* 1985, Fraga *et al.* 1990, Stadtman 1992).

It is well established that various alterations in mitochondrial structure and functions contribute to aging from yeast to humans (Breitenbach *et al.* 2012, Lee and Wei 2012). Mitochondrial function has been reported to decline during aging along with the appearance of mitochondrial

morphological alterations (Shigenaga *et al.* 1994). First, the number of mitochondria decreases with age in liver cells of mice, rats, and humans (Herbener 1976, Stocco and Hutson 1978, Yen *et al.* 1989). With advanced age, the mitochondrial density in skeletal muscle was also shown to decline gradually (Crane *et al.* 2010), suggesting a decrease in mitochondrial biogenesis. Additionally, RC capacity is reduced in human tissues such as skeletal muscles, brains and liver (Ojaimi *et al.* 1999, Perez-Carrera *et al.* 1999, Short *et al.* 2005). Although the reasons for the decline in mitochondria numbers and RC functions are currently not clear, the decrease in mtDNA and increased rates of mtDNA mutation have been suggested as putative causes (Nicholls 2002).

However, the observations of a decline in mtDNA numbers, mtDNA mutation and RC capacities do not completely explain the causality between age-associated changes in mitochondrial function and the aging process. An animal study provided us with a clue of it using the progeroid phenotype of the mtDNA mutator mice (Ahlqvist *et al.* 2012). Ahlqvist *et al.* demonstrated that development of neural and hematopoietic progenitor cells of the mtDNA mutator mice showed reduced self-renewal in vitro. The data suggest that mtDNA mutagenesis affects reconstitution capacity and long-term sustenance of somatic stem cells. Another mouse study also showed that the accumulation of mtDNA mutations caused by the mutated mtDNA polymerase show the aging-related phenotypes in mice (Trifunovic *et al.* 2004). Although there is a lack of abundant cellular and animal models in the field of aging study, it is noticeable that the intact mitochondrial function and their maintenance may at least partly be required to extend the life span.

In recent years, the mitochondria have been again proposed to have a prominent role in aging research by a mouse model (Trifunovic, Wredenberg *et al.* 2004, Kujoth *et al.* 2005). In the studies, they found that accumulation of mtDNA mutations promote the aging process by knocking-in a proofreading-deficient version of the mitochondrial DNA polymerase, suggesting a causative link

between mitochondria and aging. Although there is a critical point of view on the mitochondrial theory of aging (Sanz *et al.* 2006, Gruber *et al.* 2008, Sanz and Stefanatos 2008), we ironically could have a chance to better understand the sophisticated nature of mitochondria and the aging process. Nevertheless, there are a number of unresolved controversies and contradictory observations.

1.1.3 The oxidative stress and aging

There is increasing evidence that points toward ROS as one of the primary determinants of aging (Kregel and Zhang 2007). ROS, such as $O_2\cdot$, and H_2O_2, and $\cdot OH$, are produced in the mitochondria of most all organisms as a byproduct of normal oxidative phosphorylation. These free radicals can damage macromolecules, including proteins, lipids, and DNA, thereby imposing oxidative stress (Martindale and Holbrook 2002). Accumulated ROS can potently cause loss of mitochondrial membrane potential and mitochondrial fragmentation (Wu *et al.* 2011), resulting in significant mitochondrial dysfunction including decreased oxygen consumption, decreased ATP production, and increased ROS production. Then, the mitochondrial dysfunction leads to unregulated apoptosis and premature cellular senescence, thus contributing to aging process.

In addition, ROS, as signaling molecules, activate various signaling pathways implicated in the aging process. These include protein kinase networks, calcium signaling, cellular metabolic networks and redox responses. A good example for an ROS-activated signaling network is the mitogen-activated protein kinase (MAPK) cascade. ROS accumulation can activate many different MAPKs cascades such as MAPKKK, MEKK1, MPK4 and MPK6 (Jammes *et al.* 2009). Furthermore, ROS signaling is highly integrated with hormonal signaling networks, thereby

allowing organisms to regulate developmental processes, as well as adaptive responses to environmental cues (Chamnongpol *et al.* 1998).

Mammalian models also show the relation between oxidative stress and aging. Exposure of wild-type mice to caloric restriction resulted in a decrease of oxidative DNA damage and protected the mice from age-related phenotypes (Someya *et al.* 2007). In this study, wild-type mice showed higher levels of oxidative DNA damage than catalase-overexpressing mice. Another example that links oxidative stress to aging was presented by (Someya *et al.* 2009). Overexpression of catalase in the mitochondria of mice resulted in a reduction of age-related loss of neurons in the spinal ganglion. It was also suggested that the lifespan is positively correlated to mitochondrial hydrogen peroxide production by the interspecies comparison (Lambert *et al.* 2007, Cocheme *et al.* 2011). Low rates of hydrogen peroxide production by isolated mitochondria associate with long maximum lifespan in insect, avian, and mammalian species, potentially implicating ROS levels as a determining factor for life span regulation.

However, recent studies cast doubt on the rule that oxidative stress promotes the aging process. For example, the very long-lived rodent, the naked mole rat, actually shows increased ROS compared with other rodents. In addition, naked-mole rats do not show age-dependent variations in terms of antioxidant enzyme expression (Andziak *et al.* 2006, Rodriguez *et al.* 2011). The further findings are consistent with the recent observation that increased ROS levels even may result in lifespan extension in worms, flies, and mice (Copeland *et al.* 2009, Ungvari *et al.* 2009, Yang and Hekimi 2010), suggesting that ROS are not simply unwanted byproducts but that they also act as important signaling molecules to delay the aging process. Several models have tried to determine if an anti-oxidant is helpful. A study showed that the general antioxidant such as beta-carotene, vitamin A, and vitamin E showed no effect (Bjelakovic *et al.* 2007), whereas a targeted

antioxidant, such as catalase, appeared to result in an attenuated rate of aging (Lee *et al.* 2010). In summary, the age-related increases in oxidative damage and ROS production may not explain all of the severe physiological alterations occurring during aging. However, it might be impetuous to decide that the oxidative damage does not play an important role in age-related diseases as long as there is a clear correlation between oxidative stress and the aging process at the cellular level.

1.2 Sphingolipids

1.2.1 Sphingolipid metabolism

Sphingolipids are lipids defined by their amino-alcohol backbones which are synthesized in the ER from serine and palmitoyl-CoA. Various modifications occur on the backbones, which produces a variety of sphingolipids that play important roles in cell biology as bioactive metabolites or components of the membrane structure. Although the sphingolipid family includes many different species, their synthesis and degradation are regulated by common pathways such as *de novo*, salvage, or sphingomyelinase pathways (Hannun and Obeid 2008). In this regard, we can deem sphingolipid metabolism an interconnected network that regulates cell signaling as bioactive lipids.

1.2.1.1 Sphingolipid metabolism in human

In human (Fig 1.1), the first step in the *de novo* biosynthesis of sphingolipids is the condensation of serine and palmitoyl-CoA, a reaction catalyzed by the serine palmitoyltransferase (SPT) to produce 3-ketodihydrosphingosine which is further reduced by 3-Ketosphingosine reductase (3-KSR) to form DHS (Hanada 2003). Following DHS formation, ceramides can be produced by

ceramide synthases (CerS) with fatty acyl-CoAs. Human has six CerSs (CerS1-6). Each CerS has a high specificity to the length of a fatty acyl-CoA, indicating that they are responsible for the length of fatty acid in ceramides and have distinctive features (Pewzner-Jung *et al.* 2006). Because ceramide is considered as a molecule central to sphingolipid metabolic pathway and it serves as a branch point in the pathway (Pralhada Rao *et al.* 2013), many studies were conducted on roles of ceramide in both cell biology and sphingolipid metabolism. As a result, ceramide became the best-characterized sphingolipid.

The synthesis of complex sphingolipids such as sphingomyelin (SM) and glycosphingolipids (GSLs) are also key regulatory steps. Ceramide is a substrate for sphingomyelin synthase to produce SM (Huitema *et al.* 2004). In addition, SM levels are maintained by the catabolic action of sphingomyelinases (SMases), either neutral or acidic, releasing ceramide (Schussel *et al.* 1996). GSLs contain dozens of different sphingolipid species determined by both the number and type of sugar residues attached to their headgroups. GLSs are further divided into two categories: glucosphingolipids and galactosphingolipids. Each one has glucose or galactose attached to their headgroups by glucosylceramide synthase (GCS) or galactosylceramide synthase (GalCerS), respectively. The sphingomyelin species are the most abundant lipids among GSLs defined by a phosphocholine headgroup rather than the addition of sugar residues. Each GSL and sphingomyelin have a different acyl chain length and their function may differ in accordance with the chain length (Hernandez-Corbacho *et al.* 2011, Gonzalez-Covarrubias *et al.* 2013, Liang *et al.* 2013, Zhu *et al.* 2015).

In addition to the *de novo* synthesis, salvage pathways modulate cellular levels of sphingolipids. In the salvage pathway, ceramides can be hydrolyzed by the ceramidases (CDases) to form SPH, which in turn is phosphorylated by SPH kinases SK1 and SK2 to form sphingosine-1-phosphate

(S1P), which are further degraded by the S1P lyase to ethanolamine-1-phosphate and hexadecenal (Siow *et al.* 2011).

1.2.1.2 Sphingolipid metabolism in yeast

Sphingolipid metabolism in yeast is very similar to the one in human (Fig 1.2). In the *de novo* pathway of yeast cells, condensation of fatty acyl-coA and serine is catalyzed by the serine palmitoyltransferase (SPT) complex (Nagiec *et al.* 1994). The reaction of SPT produces 3-ketodihydrosphingosine, a short-lived metabolite that is rapidly converted to DHS (Beeler *et al.* 1998). DHS can be further converted to phytosphingosine (PHS) by the Sur2p/Syr2p hydroxylase (Grilley *et al.* 1998). The N-acylation of SBs to form ceramides requires the gene products encoded by LAG1 or LAC1 (Schorling *et al.* 2001).

In yeast, the complexity of sphingolipid is diminished. Yeast has only three types of complex sphingolipids including inositol phosphorylceramide (IPC), mannose inositol phosphorylceramide (MIPC), and mannose (inositol phosphoryl)$_2$-ceramide (M(IP)$_2$C) (Nagiec *et al.* 1997). Ceramides serve as substrates for the synthesis of complex lipids. IPC synthase Aur1p attaches a phosphoinositol headgroup to ceramide, forming IPC (Nagiec *et al.* 1997), which can then undergo mannosylation by the enzymes encoded by CSG1, CSG2, and CSH1 (Beeler *et al.* 1997, Uemura *et al.* 2003).

In the salvage pathway, phytoceramide (PHC) can be hydrolyzed by the alkaline ceramidase Ypc1p to form PHS, which in turn is phosphorylated by SPH kinases Lcb4p and Lcb5p to form PHS-1-phosphate (PHS1P), which are further degraded by the DHS and PHS phosphate lyase Dpl1p to ethanolamine-1-phosphate (Perry and Hannun 1998). Dihydroceramide also can be hydrolyzed by the yeast alkaline ceramidase Ydc1p to produce DHS.

1.2.2 Sphingolipid structure

The SBs are the simplest sphingolipids. Ceramides contain a fatty acid linked to SBs by an amide bond. The fatty acids of sphingolipids consist of very-long-chain (up to C26) odd- and even-numbered saturated or monoenoic and related 2-R-hydroxy components, while even longer fatty acids (C28 to C36) occur in spermatozoa and the epidermis (Sandhoff 2010). In plants, 2-hydroxy acids predominate and there are small amounts of 2,3-dihydroxy acids. Most (~90%) sphingolipids in plants are in a complex form with a polar headgroup linked to C-1 of the SBs (Markham *et al.* 2013) (Fig. 1.2). The polar headgroup and non-polar ceramide give complex sphingolipids their amphipathic and bilayer-forming properties. Skin ceramides also contain unusual fatty acids, while yeast sphingolipids are distinctive in containing mainly C26 fatty acids (Obeid *et al.* 2002, Holleran *et al.* 2006).

Sphingomyelin is made up of a long-chain sphingoid base, predominantly SPH in mammalian cells, an amide-linked acyl chain which may be of long (C22:0, C24:0 and C24:1) or intermediate (C16:0 and C18:0) length and a phosphorylcholine headgroup. Sphingomyelins of testes and spermatozoa contain polyunsaturated fatty acids, which are even longer in chain length (up to 34 carbon atoms) (Slotte 1999).

Very-long-chain saturated and monoenoic fatty acids are produced by specific elongases (ELOVL), and there is increasing evidence that specific isoforms are involved in the biosynthesis of certain ceramides. For example, ELOVL1 has been linked to the production of ceramides with C24 fatty acids (Ohno *et al.* 2010). Hydroxylation is effected by a fatty acid 2-hydroxylase in mammals, such as an NAD(P)H-dependent monooxygenase, which is an integral membrane protein of the endoplasmic reticulum (Hama 2010). It converts free long-chain fatty acids to free 2-hydroxy acids

in vitro and also in vivo. An animal study showed that 2-hydroxylation occurs at the fatty acid level prior to incorporation into ceramides in the brain of mice (Alderson *et al.* 2006). In plants, two fatty acid 2-hydroxylases have been found in Arabidopsis, with one specific for very-long-chain fatty acids and one for palmitic acid (Mitchell and Martin 1997, Mayer *et al.* 1999). In addition, it is possible in plants that 2-hydroxyl groups are inserted into fatty acyl chains while they are linked to ceramide, as ceramide synthase does not accept hydroxy fatty acids *in vitro* (Nagano *et al.* 2012).

1.3 Roles of sphingolipids in biological processes

1.3.1 Roles of sphingolipids in differentiation

The cellular differentiation is the process of a cell changing from one cell type to another (Slack 2007). The balance between various sphingolipid metabolites is reported to regulate either cellular differentiation or proliferation (Spiegel and Milstien 1995). The role of sphingolipid in differentiation is especially implicated in skin cells. Predominantly, S1P was suggested to stimulate cell proliferation (Xu *et al.* 2000), while short chain ceramides (C2, C6) as well as long-chain ceramides (C18) were reported to cause growth inhibition and induce differentiation in normal keratinocytes (Wakita *et al.* 1994, Di Nardo *et al.* 2000) or even apoptosis in human HaCat keratinocytes (Takeda *et al.* 2006). Lehmann *et al.* suggested that the downstream effects of the sphingolipid rheostat involve modulation of PKC, MAPK, and peroxisome proliferators-activated receptors (PPAR) (Lehmann *et al.* 2004). It was also suggested that PHS plays an important role in the regulation of keratinocyte differentiation (Pillai *et al.* 1988).

Alkaline ceramidase 1 (ACER1), a member of alkaline ceramidase family is highly expressed in the skin. The upregulation of ACER1 and aCDase was found to be essential for mediating the differentiation-inducing effect of calcium in keratinocytes (Sun *et al.* 2008). In the study, it was demonstrated that ACER1 is upregulated in proliferating epidermal keratinocytes in response to a shift of a low to the high extracellular concentration of calcium which is a key physiological inducer for keratinocyte differentiation, suggesting that ACER1 play a role in epidermal differentiation. Although there is evidence on the effect of single sphingolipid species in keratinocytes, to my knowledge, a genome-wide analysis and quantitative comparison of their effects on gene expression during the early stages of keratinocyte differentiation is still required.

1.3.2 Roles of sphingolipids in programmed cell death

Apoptosis is executed via mitochondrial (or intrinsic) and death receptor (or extrinsic) pathways, and it culminates with the activation of caspases, which are proteases that execute cell death (Vucic *et al.* 2011). Sphingolipids have been shown to have direct effects on regulators of the extrinsic pathway of apoptosis. For instance, ceramide has been reported to activate protein kinase Cζ (PKCζ), which regulates the activation of c-Jun NH2-terminal kinase 1 (JNK1) and inhibition of protein kinase B (PKB or Akt) to induce apoptosis (Bourbon *et al.* 2000, Bourbon *et al.* 2002, Fox *et al.* 2007). Ceramides are also able to directly bind and activate the lysosomal protease cathepsin D, a direct effector of apoptosis (Heinrich *et al.* 2004). Upon stimulation with tumor necrosis factor (TNF)-α, increased levels of ceramide stimulate cathepsin D-mediated cleavage of BID to activate the apoptotic pathway (Dumitru *et al.* 2009). SPH acts also as a pro-apoptotic signaling lipid via suppression of the MAPK/ERK signaling pathway (Jarvis *et al.* 1997). In contrast, S1P is a suppressor of ceramide-mediated activation of JNK1 by activating pro-survival Akt/mTOR

complex 1 (mTORC1), MAPK/ERK, and NF-κB signaling pathways (Cuvillier *et al.* 1996). Thus, regulators of the extrinsic pathway of apoptosis are affected in response to various sphingolipids.

Several studies have highlighted the regulatory roles of sphingolipids in the intrinsic pathway of apoptosis. Ceramides promote the intrinsic pathway by the formation of channels in the outer membrane of the mitochondria to promote the release of cytochrome c resulting in apoptosis (Siskind *et al.* 2002). On the other hand, it was also reported that DHC inhibits ceramide channel formation (Stiban *et al.* 2006). In addition, the intrinsic pathway can also be stimulated by the inhibitory action of ceramide on mitochondrial complex III to generate reactive oxygen species (ROS) (Gudz *et al.* 1997). It has been shown that ceramide released by aSMase is able to bind directly to lysosomal protease cathepsin D, leading to cathepsin D activation. The activated cathepsin D subsequently cleaves BH3-only protein BID and promotes the mitochondrial apoptotic pathway (Heinrich, Neumeyer *et al.* 2004). Furthermore, while ceramide-mediated activation of the pro-apoptotic protein, BAD, promotes apoptosis, S1P suppresses apoptosis via BAD inactivation (Basu *et al.* 1998). SPH was also suggested to downregulate expression of anti-apoptotic proteins Bcl-2 and Bcl-xL to enhance apoptosis in human leukemia cells (Sakakura *et al.* 1996).

Ceramide is known to induce apoptosis in cells while S1P counterbalances ceramide and promotes cell survival in what is referred to as the sphingolipid 'rheostat'. These effects are significant in many diseases which are related to the dysregulation of apoptosis.

1.3.3 Roles of sphingolipids in cell adhesion and migration

Cell migration affects all morphogenetic processes and contributes to numerous diseases, including cancer and cardiovascular disease (Parsons *et al.* 2010). Studies in endothelial (ECs) and smooth muscle cells suggested that S1P has a key role in regulating cell migration (Waeber *et al.* 2004). S1P stimulates the migration of ECs from different vessels through the activation of S1P1 and S1P3 receptors. Subsequently, the receptors activate pertussis toxin-sensitive G proteins, which results in the activation of Rac1, a critical factor involved in cytoskeletal rearrangement during cell migration (Gonzalez *et al.* 2006). Gonzalez *et al.* suggested that PI3K and Akt might be involved in the mechanisms that S1P mediates the cell migration. In addition, Lee *et al.* provided evidence that S1P-induced Rac1 activation requires direct phosphorylation of the S1P1 receptor by Akt (Lee *et al.* 2001). These data indicate the important role of S1P in cell migration.

Tumor necrosis factor alpha (TNFα) is known to promotes the expression of adhesion molecules on endothelial cells (Walsh *et al.* 1991). Knock-down studies have implicated SK1 in mediating the effects of TNFα on regulating cyclooxygenase-2 (COX2), the production of prostaglandins, and the induction of adhesion molecules (Lee *et al.* 1999, Pettus *et al.* 2003, Billich *et al.* 2005). Additionally, knock-down of S1P phosphatase or S1P lyase increased prostaglandin production, along with augmentation of S1P levels (Pettus, Bielawski *et al.* 2003), verifying the role of S1P in cell adhesion. Another study demonstrated that nSMase2 induces the effects of TNFα on cell adhesion and migration (Clarke *et al.* 2007), which suggests the requirement of further studies to reveal the potential roles of other sphingolipids in cell migration and adhesion.

1.3.4 Roles of sphingolipids in angiogenesis

Angiogenesis is the formation of new blood vessels. Sphingolipid is also involved in angiogenesis because endothelial cell migration is essential to angiogenesis as mentioned above (Lamalice *et al.* 2007). S1P stimulates angiogenesis *in vivo* mainly via an S1P1 receptor, and to a lesser extent, S1P3 receptor. The first discovery of *in vivo* angiogenesis induced by S1P came from the observation that S1P stimulated angiogenesis in matrigel implants in mice (Lee *et al.* 1999). This effect was inhibited by anti-sense oligonucleotide-mediated downregulation of either S1P1 or S1P3 receptor. Subsequently, it has been demonstrated that S1P1 receptor gene ablation in mice impairs accumulation of pericytes (Liu *et al.* 2000). An S1P1-selective antagonist inhibited VEGF-induced angiogenesis in a corneal model, which suggests that endogenous S1P is involved in VEGF-induced angiogenesis (Yonesu *et al.* 2010). However, it was also demonstrated that S1P1 receptor inhibits sprouting angiogenesis promoted by VEGF to prevent excessive sprouting and fusion of neovessels (Ben Shoham *et al.* 2012), which suggests another role of the S1P-S1P1 pathway in angiogenesis as a vascular-intrinsic stabilization mechanism, protecting developing blood vessels against aberrant angiogenic responses.

The SK1–S1P pathway has been known to also regulate developmental angiogenesis. It was demonstrated that homozygous deletion of S1P1 gene in mice results in intrauterine death between E12.5 and E14.5 due to lack of normal angiogenesis (Liu, Wada *et al.* 2000). S1P1-null embryos showed that the coverage of vessels by smooth muscle cells (SMCs) is abnormal, whereas SMCs are present only on the ventral surface of the entire length of the dorsal aorta, but deficient on the dorsal surface of the aorta in S1P1-null embryos at E12.5. Different from S1P1-null mice, either S1P2- or S1P3-single null mice are alive without a vascular formation defect. It has been shown

that, compared with mice null for S1P1 alone, embryos null for S1P1 and S1P2, null for S1P1 and S1P3, and null for S1P1, S1P2, and S1P3 exhibit more severe S1P1-null phenotypes, including a vascular maturation defect and hemorrhage with earlier intrauterine death (Kono *et al.* 2004). Based on these observations, S1P1 deficiency has been suggested to result directly or indirectly in defects in normal angiogenesis process.

1.3.5 Roles of sphingolipids in inflammation

The inflammation is induced and regulated by a variety of mediators such as cytokines, prostaglandins, and ROX (Pettus *et al.* 2004). Different studies demonstrated that cellular signaling in inflammatory processes is controlled by sphingolipids (Kitatani *et al.* 2009, Snider *et al.* 2010, Hoeferlin *et al.* 2013). The first indications that ceramide could play a role in inflammation were started from studies examining the TNFα (Kim *et al.* 1991). TNFα can activate aSMase resulting in ceramide production and subsequent activation of the pro-inflammatory transcription factor, nuclear factor-κB (NF-κB) (Schutze *et al.* 1992). Interestingly, TNFα can activate neutral and acid SMases, but the only activation of acid SMase results in NF-κB activation (Wiegmann *et al.* 1994). However, in several other studies, it has been subsequently shown that the activation of SMases in a variety of different cell types is not essential for TNF-induced NF-κB activation. This included endothelial cells and macrophages (Slowik *et al.* 1996, Manthey and Schuchman 1998). While SMase activity may not be required for this TNF-induced effect, these studies have demonstrated that ceramide itself can activate inflammatory pathways via NF-κB gene transcription.

Compared with ceramide, investigation of the roles of S1P has provided more direct evidence for its relative importance in inflammation. In mast cells, S1P is known to have an important role during activation and subsequent development of the inflammatory response (Olivera 2008).

Antigen engagement of the high-affinity receptor for IgE on mast cells results in the activation of SK and production of S1P (Prieschl *et al.* 1999). Another study showed that a deficiency in SK2 results in a decreased degranulation and decreased the production of eicosanoids and cytokines suggesting the importance of S1P production (Olivera *et al.* 2006). Furthermore, mast cells express S1P1 and S1P2 receptors and these mediate different but important effects of mast cell activation (Jolly *et al.* 2004). In the study, it was demonstrated that S1P1 receptor is involved in mast cell migration while S1P2 receptor is important in degranulation. These data indicate the possibility that the role of S1P in mast cell function translates into several different areas of clinical relevance.

1.3.6 Roles of sphingolipids in cellular senescence and aging

Ceramides are very strong candidates for regulating the aging process since their levels alters in aged cells and tissues (Cutler *et al.* 2004, Perez *et al.* 2005, Di Marzio *et al.* 2008, Kobayashi *et al.* 2013), and previous studies already showed their roles in many diseases such as cancer, type 2 diabetes, neurodegeneration and cardiovascular dysfunction in association with aging (Bismuth *et al.* 2008, Ford 2010, Morad and Cabot 2013, Palmer *et al.* 2015). First of all, roles for ceramides in response to oxidative stress are intriguing because the cellular responses to oxidative stress are thought to modulate the aging process (Kregel and Zhang 2007, Yan 2014). Ceramides have been shown to induce the activation of NADPH oxidase and to increase the production of ROS in various cells including human endothelial cells, macrophages, and rat mesangial cells (Zhang *et al.* 2003, Yi *et al.* 2004, Zhang *et al.* 2008). Even a novel cationic ceramide, L-threo-C6-pyridinium-ceramide bromide, activates NADPH oxidase in pancreatic β-cells (Syed *et al.* 2012). Zhang et al. indicated the role of ceramide as a membrane component by proposing that ROS production is promoted by the fusion of small raft domains to ceramide-enriched membrane platforms, thereby clustering subunits of NADPH oxidase (Zhang *et al.* 2010). However, the cell

death induced by ROS can be abolished by the ceramide-activated protein kinase (CAPK) inhibitor, suggesting that the ceramide also plays a role in response to oxidative stress as a signaling molecule (Ardestani *et al.* 2013). Since managing oxidative stress at the cellular level is critical for the longevity, strategies to properly regulate the ceramide can lower the incidence and severity of diseases caused by ROS.

Mitochondrial function is thought to be a key player in the aging process (Kong *et al.* 2014). Ceramide has been reported to be involved in mitochondrial structure and functions in a variety of studies. The membrane-permeabilizing effect of ceramide on mitochondria was first demonstrated by Obeid et al. (Obeid *et al.* 1993). In the study, the programmed cell death is induced by the mitochondrial membrane permeabilization caused by ceramide. Since then, it was further verified by studies that ceramide promotes the apoptosis through the mitochondrial membrane permeabilization (von Haefen *et al.* 2002, Ganesan *et al.* 2010, Lee *et al.* 2015). Especially, the synergetic relation between ceramide and Bax was highlighted in the studies, indicating the role of ceramide as a chemical messenger. Recent studies also emphasized the roles of ceramide in regulating membrane raft formation on mitochondria (Colombini 2016). Chang et al. suggested that ceramide channels are essential for mitochondria to release the pro-apoptotic molecules and the channels is destabilized by Bcl-xL (Chang *et al.* 2015). In addition, ceramide has been shown to interact with the mitochondrial electron transport chain leading to the generation of ROS (Corda *et al.* 2001). Because the rate of apoptosis is elevated in most types of aging cell populations and organs (Muradian and Schachtschabel 2001) and mitochondria are an important source of ROS (Murphy 2009), it is clear that ceramides play, at least in part, important roles in the aging process.

Compared with other sphingolipids, the roles of SBs in aging in mammalian systems has been less studied. The potential role of SBs in aging started from the observation that they are increased

during the aging process in many types of cells and tissues. Early studies showed that SPH is increased in aging neuronal cells of rats (Valsecchi *et al.* 1993, Valsecchi *et al.* 1996). Recent studies also supported the increase in SBs in the aging process. Our group reported that SPH levels increase in the brain in middle-aged mice compared to young adult mice (Wang *et al.* 2015). Increased levels of both of SPH and DHS are shown in human plasma, liver tissues of rats, and gastric smooth muscle cells of mice over time (Lightle *et al.* 2000, Lee *et al.* 2014, Choi *et al.* 2015). These reports suggest a reason to think that SBs are involved in aging.

Because the direct evidence showing the correlation between SBs and aging in mammals has not been reported yet, we can only speculate on how they affect the aging process. Aging is known to be regulated by a few highly conserved signaling mechanisms involved in nutrient sensing (Sengupta *et al.* 2010), mitochondrial function (Comfort 1989), redox metabolism (Majmundar *et al.* 2010), the DNA damage response (Ciccia and Elledge 2010), proteostasis (Richter *et al.* 2010) and autophagy (Kroemer *et al.* 2010). Interestingly, SBs are thought to have important functions as signaling molecules making cells to respond to those cellular stresses including heat shocks (Jenkins and Hannun 2001, Olivera and Spiegel 2001, Meier *et al.* 2006), nutrient depletion (Alvarez-Vasquez *et al.* 2007), mitochondria-induced apoptosis (Cuvillier *et al.* 2001), and oxidative stress (Abrahan *et al.* 2010). These studies suggest SBs as putative strong targets for studying aging process.

Studies in budding yeast, *Saccharomyces cerevisiae* show further evidence that SBs play important roles in aging because both the metabolism and signaling functions of sphingolipids are well conserved between yeast and mammals (Mao *et al.* 2000). Aerts et al. demonstrated that overexpression of YDC1, a ceramidase that catalyzes the hydrolysis of dihydroceramides to generate DHS, shortens chronological life span (CLS) (Aerts *et al.* 2008). Although it is not clear

if the reduction in CLS is due to decrease in ceramides or increase in SBs, it is hard to exclude SBs from the responsible candidates. Furthermore, it was demonstrated that PHS promoted the phosphorylation of Sch9p which is a target of Tor1p and also known to reduce the yeast lifespan (Liu *et al.* 2005). SBs were reported to be increased during the diauxic shift (Alvarez-Vasquez *et al.* 2007). The diauxic shift is inevitably included in the aging process of the yeast system. However, the study showed the SBs regulation during the relatively short time period, less than 24 hours. Also, it was not suggested how an increase in SBs during the diauxic shift affect yeast aging. Recently our group reported that SBs shorten the yeast CLS by compromising the mitochondrial functions (Yi *et al.* 2016). In the study, SBs inhibit mitochondrial fusion along with a decrease in their functions which are required to be maintained properly for the yeast longevity. Taken together, although it is still required to reveal a clear role of SBs in the aging process, their role in aging should not be overlooked.

Compared with other SBs, more studies have been conducted showing that S1P is supposed to be strongly involved in aging in response to oxidative stress. The free-radical theory of aging suggests that accumulated reactive oxygen species (ROS) causes the slow deterioration of cellular processes, or aging (Harman 1956, Kirkwood 1977, Lee and Wei 2012). S1P has an important role in oxidative stress response (Van Brocklyn and Williams 2012) and ROS can activate SK1 which catalyzes the synthesis S1P (Ader *et al.* 2008). In addition, it has been shown that PHS1P attenuates H_2O_2-induced growth arrest of fibroblasts through regulation of the signal molecules Akt and JNK (Lee *et al.* 2012). Tao et al. has reported that SK1 protects cardiac myocytes from hypoxia-induced apoptosis (Tao *et al.* 2007). Furthermore, pretreatment of isolated mouse hearts with S1P protects from ischemic injury (Jiang *et al.* 2002) and S1P receptor 1 (S1PR1) agonism attenuates lung ischemia-reperfusion injury (Stone *et al.* 2015). Although the mechanism is still required to further

studied, the studies indicate that S1P plays an important role in aging not only promoting cell survival but maintaining organs.

1.3.7 Roles of sphingolipids in aging-related diseases

Importantly, ceramide currently gains much attention as a pivotal player in age-related neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Yadav and Tiwari 2014). Elevated levels of ceramide species are measured in serum (Mielke *et al.* 2012), in plasma (Savica *et al.* 2016), in cerebrospinal fluid (Fonteh *et al.* 2015), or in brains of AD patients in comparison to age-matched normal controls (Filippov *et al.* 2012). Interestingly, it was recently reported that there are the stage-specific associations between ceramides and neuropsychiatric symptoms of AD (Xing *et al.* 2016). In the study, the C16:0-Cer and C20:0-Cer levels are positively associated with delusions, and those of C22:0-Cer and C24:0-Cer are positively associated with depression, suggesting that the mechanism study for each ceramide species is further required. Moreover, ceramide-dependent apoptosis was suggested to be a putative mechanism of PD (FranceLanord *et al.* 1997). It appears that C2-ceramide-induced neuronal death is inhibited in a murine cathecolaminergic cell by overexpression of DJ-1 of which mutation is highly associated with PD (Jaramillo-Gomez *et al.* 2015). Abbott et al. also revealed that there is a significant change in the ceramide acyl chain length in the PD anterior cingulate cortex compared with the normal brain tissues (Abbott *et al.* 2014). Overall, the data for ceramides are intriguing, yet they raise many questions and point to the unforeseen and multiples roles that ceramides are likely to have in aging-related diseases.

SM metabolism also represents a promising area of research on AD or PD-associated processes. Interesting data was obtained from AD plaque analysis. The senile plaques isolated post-mortem from the brains of AD patients contained an increased concentration of predominantly C20:0-Cer

in comparison with surrounding neuropil. On the other hand, the level of C20:0-SM was only slightly reduced. Authors suggested that elevated Cers could partially originate from the SM hydrolysis since the expressions of both acid sphingomyelinase (a-SMase) and neutral sphingomyelinase (n-SMase) were found in the corona, and in some cases core, of senile plaques (Panchal *et al.* 2014). The highly altered lipid compositions of lipid rafts were found in frontal and entorhinal cortices in non-symptomatic and mild dementia phases, in AD brains (Fabelo *et al.* 2014). In addition, the levels of SMs associated with the lipid rafts were significantly reduced in frontal cortex at the early stages of AD. Authors hypothesized that such reorganization of lipid components at the beginning of the AD might provoke the Aβ structural changes toward senile plaque creation. Indeed, mutations in a-SMase coding sequence were also found to correlate with PD incidence (Foo *et al.* 2013, Dagan *et al.* 2015). Moreover, it was reported that mutations in the SMPD1 gene led to abnormalities in the lysosome functions, which resulted in disturbed α-synuclein (αS) (Gan-Or *et al.* 2013). The dysregulated SM metabolism can also influence the process of neurotransmission in PD. It is well known that αS, by aggregation in PM of neuronal cells, causes a deterioration of the synaptic functions (Das *et al.* 2015).

Sphingolipids affect various aspects of cell physiology like cell proliferation, cell death, differentiation, and cell signaling and are known to contribute to key cellular pathologies like ER stress, insulin resistance, inflammation, and drug resistance. However, analyzing different disease is required to be distinct from each disease because sphingolipid metabolism separately contributes towards the pathology of different diseases. Understanding the nature of changes in the sphingolipid metabolic pathway and their overall pathological effect under various disease conditions would be very useful in order to design therapeutic strategies.

1.3.8 Roles of sphingolipids in signal transduction

De novo production of ceramide is implicated in cell cycle arrest (Lee *et al.* 1998, Villasmil *et al.* 2016) and apoptosis (von Haefen, Wieder *et al.* 2002, Ganesan, Perera *et al.* 2010). Other signaling events in which ceramide has been implicated include activation of MAPKs (Jarvis, Fornari *et al.* 1997), inhibition of PI3K activation (Hanna *et al.* 1999), regulation of SMC tone (Johns *et al.* 2000), oxidative stress responses (Hannun and Luberto 2000) and nitric oxide signaling (Pahan *et al.* 1998). Additionally, the evidence that ceramide is an important second messenger in many cellular functions in response to diverse stimuli has been also reviewed extensively (Hannun and Luberto 2000, Mouton and Venable 2000, Obeid and Hannun 2003, Yang *et al.* 2004, Ben-David and Futerman 2010, Nikolova-Karakashian and Rozenova 2010, Mencarelli and Martinez-Martinez 2013, Park and Pewzner-Jung 2013). Although there are conflicting reports concerning the sphingomyelinases involved (Levade and Jaffrezou 1999) and the role of ceramide in apoptosis (Hofmann and Dixit 1998), overwhelming evidence supports the view of ceramide as a second messenger. However, its mechanisms of action are less clear.

S1P is unique in the sphingolipid signaling system in its ability to act as an extracellular stimulus through specific cell surface receptors and as an intracellular second messenger through direct activation of signaling proteins. Extracellular S1P signals through G protein-coupled receptors belonging to the endothelial-differentiating gene (EDG) family (Liu, Wada *et al.* 2000). MAPK, PKC, and Rho pathways can be activated by S1P and they are implicated in diverse cellular responses including differentiation, migration, mitogenesis and apoptosis (Huwiler *et al.* 2000). S1P has also been proposed to play a second messenger role, following the observation that sphingosine-induced calcium release was dependent on SK activation (Ghosh *et al.* 1994).

Lipid rafts are emerging as important centers for cell signaling. The roles of sphingolipid in signaling as a component of lipid rafts has been also highlighted. It has been shown that sphingomyelin hydrolysis and the generation of ceramide at the extracellular face of the plasma membrane is required for CD95 receptor clustering in Jurkat cells (Grassme *et al.* 2001). Disruption of lipid rafts with cholesterol prevented ceramide generation and receptor aggregation. Another study conducted by Cremesti *et al.* showed that intact lipid rafts and ceramide generation were essential for capping of Fas and induction of apoptosis following anti-Fas antibody treatment (Cremesti *et al.* 2001). These studies suggest an important function for sphingolipid in lipid raft-mediating receptor aggregation and subsequent signaling.

1.4 Figures and tables

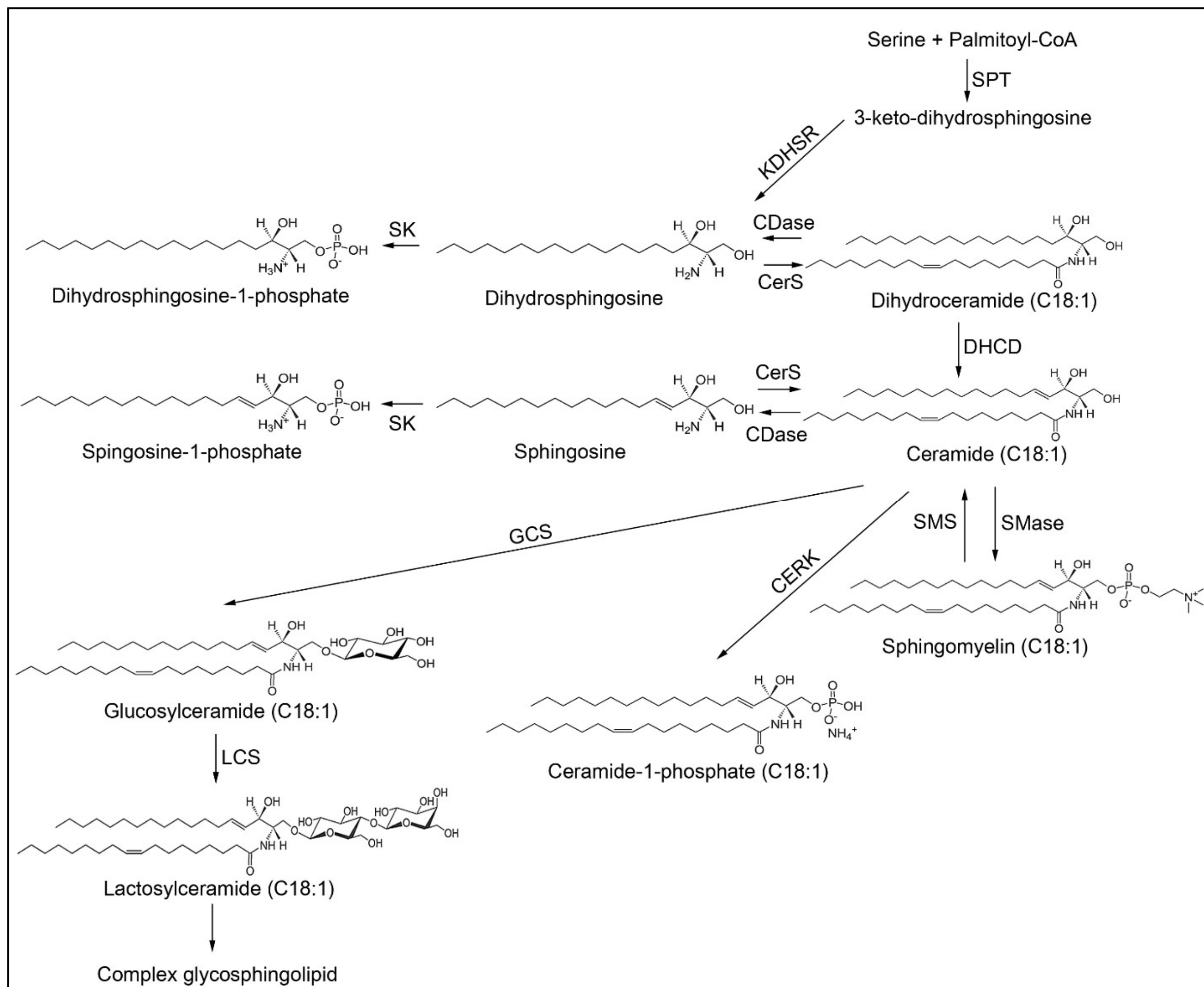


Figure 1.1 Sphingolipid metabolism in human

Ceramide is central to sphingolipid metabolism. It can be broken down to create other metabolites. Ceramidases are enzymes which can metabolize ceramides by breaking the amide bond and turning them into sphingosines. The sphingosines can then be phosphorylated by sphingosine kinase (SK) to create S1P. Ceramide synthases (CerS) are enzymes that catalyze the formation of ceramides from SB and acyl-CoA substrates. Furthermore, ceramide can serve as a substrate for the synthesis of sphingomyelin or glycolipids.

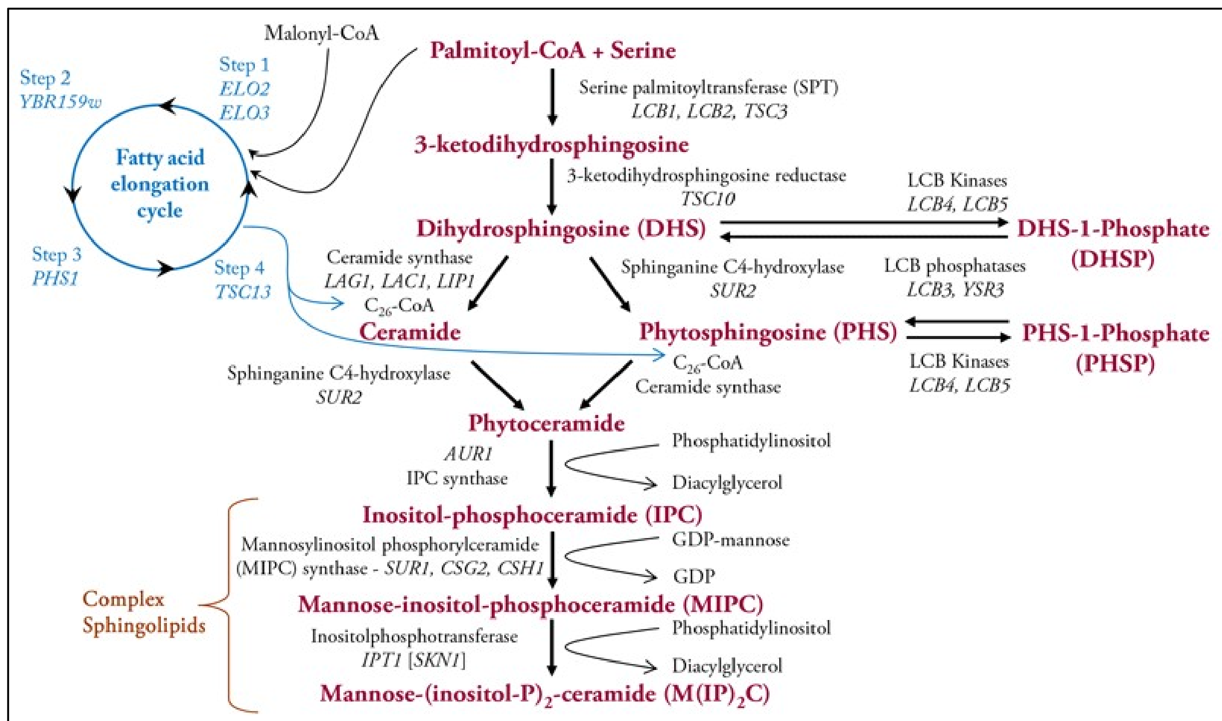


Figure 1.2 Sphingolipid metabolism in yeasts

Diagram of sphingolipid metabolism in *Saccharomyces cerevisiae* primarily showing de novo synthesis reactions. Metabolic intermediates and the complex sphingolipids are indicated in bold maroon lettering while enzyme names genes are in black lettering with gene names indicated by italics. The reaction cycle that produces the very long-chain C26 fatty-acid, characteristic of yeast sphingolipids, is indicated in blue. When grown aerobically the fatty acid in complex sphingolipids is often hydroxylated at C2. Ceramides can be hydrolyzed by two ceramidases, Ydc1, and Ypc1, to yield a fatty acid and an SB. (Adopted from (Dickson 2010))

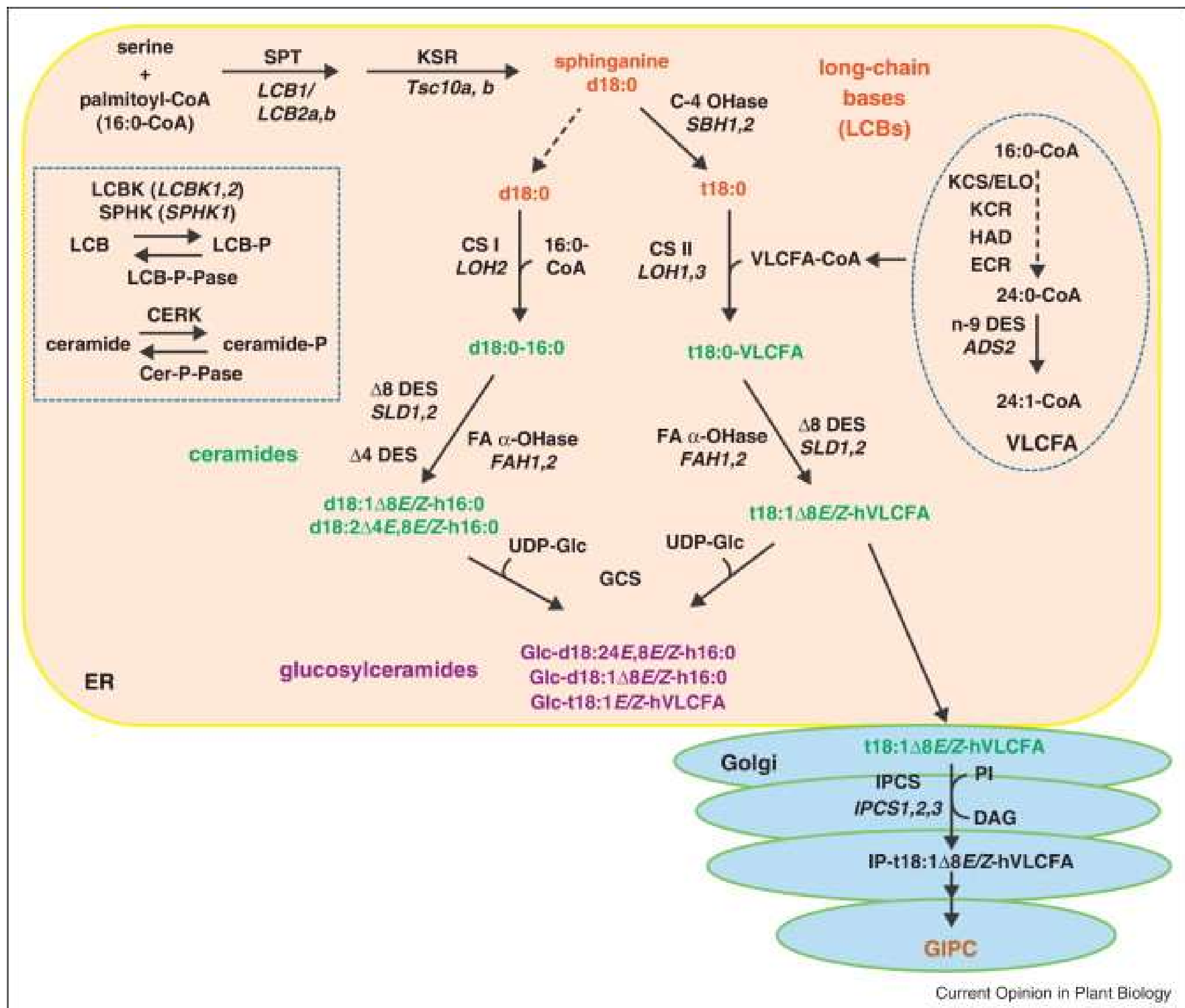


Figure 1.3 Sphingolipid metabolism in plants

Simplified sphingolipid biosynthetic pathway in plants highlighting SBs (long-chain base, LCB) and FA modification reactions and the spatial division of the pathway between the endoplasmic reticulum (ER) and Golgi. As shown, SBs are preferentially acylated with C16 FAs (16:0-CoA) by ceramide synthase I (CS I) primarily for glucosylceramide synthesis. Trihydroxy SBs are preferentially acylated with very long chain fatty acyl (VLCFA)-CoAs by ceramide synthase II (CS II) for glucosylceramide and glycosyl inositolphosphoceramide (GIPC) synthesis. As also indicated, evidence to date is consistent with free SBs as substrates for the C-4 hydroxylase (C-4

OHase) and ceramides as substrates for Δ4 and Δ8 desaturases (Δ4 DES, Δ8 DES) and fatty acid α-hydroxylase (FA α-OHase) (Adopted from (Markham, Lynch *et al.* 2013))

Chapter 2. Sphingoid bases are accumulated in yeast cells during the aging process.

2.1 Introduction

SBs have been shown to be increased by different forms of stress, including oxidative stress, key risk factors of aging in mammalian cells (Mao and Obeid 2008), suggesting that SBs may be increased during the aging process and that their increase may be a cause of yeast aging. However, two important issues remain to be resolved. First, it remains unclear whether the SB rise in aging or aged yeast cells is a general or strain-specific phenomenon. If the former is the case, what is the mechanism for the SB rise? Answering these two questions is a prerequisite to testing my hypothesis that dysregulation of SBs may lead to yeast aging. Therefore, I 1) confirmed that SBs including PHS and DHS are increased in yeast cells aged in the synthetic complete medium; and 2) defined the metabolic enzyme(s) responsible for the accumulation of SBs during aging process in this chapter.

2.2 Results

2.2.1 Sphingoid bases and their metabolizing enzymes are regulated in yeast cells during aging process

Since the metabolism of sphingolipids has been shown to change with age in different species, I wondered if this also occurs in the yeast *Saccharomyces cerevisiae* during the aging process. To this end, I applied a lipidomics approach to measuring the levels of sphingolipids in yeast cells during aging that was induced by nutrient depletion. Wild-type (WT, JK93dα) yeast cells were cultured in the synthetic medium including 2% glucose and harvested at 8 h (corresponding to

mid-log phase), 16 h (diauxic shift, DS), 48 h (day 0 in the stationary phase), and 4, 8, 12, and 16 days post the stationary phase before sphingolipid profiling by LC-MS/MS. I found that the levels of PHS were constantly increased from DS up to Day 16 compared to the log phase and that the levels of DHS were increased from Day 0 up to Day 16 (Fig. 2.1). The levels of phytoceramide (PHC) were decreased from the log phase to Day 0, returned to the log phase levels Day 4, were slightly increased from day 4 to Day 8 and maintained constant from Day 8 to Day 16 (Fig. 2.1.B). No significant changes were observed in the levels of dihydroceramides (DHC) during the entire time course (Fig. 2.1.B). These results suggest that the levels of SBs (PHS and DHS) are increased in yeast cells markedly and constantly during the entire course of aging.

Following the above finding, we investigated whether enzymes responsible for the metabolism of SBs are also regulated during the aging process. As shown in Fig. 2.2, the metabolism of SBs involves multiple enzymes, including YPC1, YDC1, LAC1, LAG1, LCB4, LCB5, YSR2/LCB3, and YSR3, so I performed qPCR to determine the mRNA levels of these enzymes in yeast cells collected at different time points as described in Fig. 2.1.A and 2.1.B. I found that the mRNA levels of YPC1 were significantly increased in yeast cells at the diauxic shift and the early stationary phase compared to the log phase whereas the mRNA levels of LCB4, LCB5, LAC1, and LAG1 were decreased in yeast cells at the late stationary phase compared to the log phase (Fig. 2.3). These results suggest that SBs and their producing enzyme YPC1 are upregulated whereas SB-degrading enzymes LCB4/5 are downregulated in yeast cells during the aging process.

2.2.2 Inverse regulation of alkaline ceramidases and sphingoid base kinases leads to aging-induced elevation of sphingoid bases in yeast cells

Following the above findings, I investigated if increasing YPC1 expression or inhibiting both LCB4 and LCB5 is sufficient to increase the levels of SBs in yeast cells whereas knocking down YPC1 inhibits the aging-related increase in SBs.

First, I determined if YPC1 overexpression could increase endogenous SBs in yeast cells because Ypc1p catalyzes the hydrolysis of phytoceramide to generate PHS. To exclude the possibility that Ypc1p protein *per se* may affect the expression of other sphingolipid metabolic enzymes, a catalytically inactive mutant of this enzyme was constructed as a negative control by site-directed mutagenesis as described in the experimental procedure. I mutated Ypc1p by replacing Cys^{27} with Phe to generate the $Cys^{27} > Phe^{27}$ mutant, named Ypc1p-C27F because it was reported that Cys^{27} is essential for Ypc1p's reverse activity (Ramachandra and Conzelmann 2013). I generated a yeast strain that overexpresses Ypc1p or Ypc1p-C27F under the control of a Gal1 promoter. These yeast cells were cultured in galactose-containing medium for 8 h to induce gene expression. Protein expression levels and enzymatic activities were examined in microsomes isolated from yeast cells. The expression levels of Ypc1p and Ypc1p-C27F were similar (Fig. 2.4.A), and WT Ypc1p but not Ypc1p-C27F exhibited alkaline ceramidase activity (Fig. 2.4.B), suggesting that Ypc1p-C27F is a proper negative control for functional studies of the WT Ypc1p. I showed that overexpression of Ypc1p increased the levels of both PHS and DHS in yeast cells compared to overexpression of Ypc1p-C27F (Fig. 2.5.A), suggesting that YPC1 upregulation contributes to the increased generation of SBs in yeast cells during the aging process.

Second, I determined if knockout of both LCB4 and LCB5 could also increase the levels of SBs in yeast cells because both enzymes convert SBs to their phosphates (Nagiec *et al.* 1998). Indeed,

with LC-MS/MS, I demonstrated that the levels of SBs were markedly increased in the yeast mutant deficient in both LCB4 and LCB5 compared to WT yeast cells (Fig. 2.5.B), suggesting that the downregulation of these enzymes also contributes to an increase in the levels of SBs in yeast cells in response to aging.

Lastly, I determined if YPC1 knockout inhibited the increase in the levels of SBs in yeast cells during aging. Unexpectedly, I found that knocking out YPC1 increased rather decreased the levels of SBs in yeast cells (Fig. 2.5.C). Because yeast cells express the other alkaline ceramidase YDC1, which may compensate for the loss of YPC1 for making SBs, I tested if knocking out both YPC1 and YDC1 could lower SBs. Indeed, I found that knocking out both YPC1 and YDC1 markedly inhibited the increase in the levels of SBs in response to aging (Fig. 2.5.D). These results suggest that YPC1 and YDC1 have a redundant role in regulating the generation of SBs during the aging process.

2.2.3 MSN2/4 are required for YPC1 regulation during aging

Following the finding that YPC1 is upregulated during aging, I investigated the mechanism for the YPC1 upregulation. To this end, I tested if MSN2/4 are required for the regulation of YPC1 during the aging process because previous studies indicated that YPC1 is a putative target of MSN2/4, yeast transcriptional factors that regulate expression of various genes implicated in stress response by binding stress response elements (STREs) present on the targeted genes (Hasan *et al.* 2002, Doniger and Fay 2007). Interestingly, there are 3 putative STREs within a 100 nucleotide-region in the YPC1 gene (Table 2.1), indicating that MSN2/4 may regulate YPC1 under stressful conditions.

To test this hypothesis, first, I tested if both MSN2 and MSN4 are required to regulate YPC1 mRNA levels during aging. YPC1 mRNA levels were assessed by qPCR in WT, MSN2 single knockout (Msn2Δ, BY4742 background, Table 2.2), MSN4 single knockout (Msn4Δ, BY4742 background, Table 2.2), or MSN2 and MSN4 double knockout (Msn2ΔMsn4Δ, W303 background, Table 2.2) yeast strain at different time points as described earlier. YPC1 mRNA levels were increased with time in WT, Msn2Δ, or Msn4Δ cells but not in Msn2ΔMsn4Δ cells (Fig. 2.6), suggesting that MSN2 and MSN4 have a redundant role in regulating YPC1 expression during aging. Second, I tested if MSN2/4 affected SBs levels by regulating YPC1 during the aging process. I analyzed the levels of SBs in WT or Msn2ΔMsn4Δ cells that were grown for different time durations. The results showed that during the aging process, the levels of PHS were increased with time in WT cells but not in Msn2ΔMsn4Δ cells (Fig. 2.7). Furthermore, the aging-dependent increase in the levels of DHS was also attenuated in Msn2ΔMsn4Δ cells compared to WT cells. Taken together, these results support the notion that YPC1 is a key enzyme that regulates the aging-associated increase in the levels of SBs in an MSN2/4-dependent manner.

2.3 Discussion

Sphingolipids have been known to be altered in different organisms in response to the aging. Mouton *et al*. found that ceramides are increased in human fibroblasts during aging (Mouton and Venable 2000). Furthermore, it has been shown that the levels of long-chain ceramides are increased in mice during a normal aging (Cutler *et al.* 2004). Our recent studies demonstrated that SPH levels are also increased in the brain in middle-aged mice compared to young adult mice (Wang, Xu *et al.* 2015). In this study, I demonstrated that SBs were increased during the aging process in JK-93d yeast cells (Fig. 1) and several other yeast strains (data not shown). However, I

found that the levels of PHC, the yeast ceramides, were transiently decreased in yeast cells from the log phase to the stationary phase and were only slightly increased in the late phase of the aging process. These results suggest that aging process-associated increase of SBs is a highly conserved phenomenon across various species.

SBs are intermediates in the metabolic network of sphingolipids so their levels can be controlled by different sphingolipid-metabolizing enzymes. In this study, I for the first time demonstrated that upregulation of the alkaline ceramidases YPC1 and YDC1 is a major cause for the aging-associated increase in the levels of SBs in yeast cells. First, I showed that the mRNA levels of YPC1 are increased during the early phase of aging. Second, knocking out both YPC1 and YDC1 but no single ceramidase totally blocked the aging-associated increase in the levels of SBs in yeast cells, suggesting that the hydrolysis of yeast ceramides by the action of both YPC1 and YDC1 is the major source of SBs that are increased in response to aging. Interestingly, Zhao et al. demonstrated that different types of ceramidases are also upregulated in rat aging brains compared to young brains. (Zhao *et al.* 2014). Our recent study found that the alkaline ceramidase ACER3, a human homolog of the yeast alkaline ceramidases, is also significantly upregulated in aging mouse brains compared to young brains (Wang, Xu *et al.* 2015). These results suggest that it is a general phenomenon for SB-producing ceramidases to increase in different species during aging. In addition to ceramidases, SB kinases LCB4/5 are important in regulating the levels of SBs by controlling SB phosphorylation. In this study, I for the first time demonstrated that downregulation of LCB4/LCB5 also plays an important role in increasing the levels of SBs during the late phase of aging. Although Lester et al. already showed that LCB4 deletion induced the increase in SBs during the diauxic shift (Lester *et al.* 2013), it was required to check its role in SB regulation for the longer period of time in order to study aging. First, I showed that both LCB4 and LCB5 mRNA

levels were decreased in yeast cells during the aging process, especially in the late phase. Second, knocking out both LCB4 and LCB5 is sufficient to increase SBs in yeast cells even in the log phase. This explains why SBs continue to be increased in yeast cells in the late phase of aging although SB-producing enzymes YPC1 and YDC1 are not upregulated. Taken together, these results strongly suggest that an inverse regulation of the ceramidases and SB kinases is responsible for the aging –associated increase in the levels of SBs in yeast cells.

Previous studies demonstrated that the transcription factors MSN2/4 play a key role in regulating the expression of genes implicated in stress responses of yeast cells. These stress-responsive transcriptional activators, MSN2/4, are known to bind to STRE (Causton *et al.* 2001). There are several putative STREs found in the promoter region of YPC1. A previous study showed that a loss of one of such STREs leads to 36% reduction in YPC1 gene expression (Doniger and Fay 2007). Interestingly, our study showed no difference in YPC1 mRNA levels between WT and a single deletion strain of either MSN2 or MSN4 but, the deletion of both MSN2 and MSN4 totally blocked the upregulation of YPC1 in yeast cells in response to prolonged culture in nutrient-deficient medium, indicating that MSN2/4 have a redundant role in regulating the expression of YPC1 in response to nutrient depletion. Although MSN2/4 activity was shown to protect yeast cells from the detrimental effects of extreme stress (Hasan, Leroy *et al.* 2002, Kandror *et al.* 2004) and to be required to extend the lifespan (Fabrizio *et al.* 2003, Medvedik *et al.* 2007), our result showed a small but significant increase in CLS in Msn2/4Δ. There are also a couple of previous studies showing no or little difference in CLS between WT and Msn2/4Δ (Fabrizio *et al.* 2004, Medvedik, Lamming *et al.* 2007). Moreover, it was reported that deletion of SIR2 which is subsequently activated by a target of MSN2/4, PNC1, can extend chronological lifespan in several strains (Fabrizio *et al.* 2005). Because MSN2/4 regulate expressions of various genes under

different stresses (Martinez-Pastor *et al.* 1996) and the difference in yeast backgrounds may affect the outcome of an experiment, our results might be contradictory to previous studies where deletion of MSN2/4 activities was required to extend yeast CLS. Taken together, YPC1 and its lipid products SBs are upregulated by MSN2/MSN4 in yeast cells in response to nutrient depletion and possibly other forms of stress.

2.4 Materials and Methods

2.4.1 Yeast strains, media and growth conditions

Yeast strains used in this study are presented in Table 2.1. The wild-type *Saccharomyces cerevisiae* strains JK93d and YAG6B were grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose), and transformed strains a uracil dropout medium SC-ura (CLONTECH, CA). Yeast cells were cultured at 30ºC under rotational shaking at 200 rpm. The gene overexpression strains in this study constructed as follows. To overexpress Ypc1p in yeast cells under the control of the promoter Gal1, the open reading frame (ORF) of the YPC1 gene was cloned into the vector pYES2 to yield the construct pYES2::YPC1 as described in (Mao *et al.* 2000) and the resulting expression construct pYES2::YPC1 was sequenced to ensure that no errors were introduced into the YPC1 ORF by PCR before being transformed into yeast cells by the lithium acetate method (Mao, Xu *et al.* 2000). The strain containing pYES2::YPC1 was grown and maintained in SC-ura medium with 2% glucose. Expression of Ypc1p was induced in SC-ura medium with 2% galactose. To exclude the possibility that Ypc1p *per se* may induce biological effects independently of its lipid mediator, a catalytically inactive mutant of this enzyme was constructed as a negative control. Ypc1p was mutated by replacing Cys^{27} with Phe to generate the $Cys^{27} > Phe^{27}$ mutant

(Ramachandra and Conzelmann 2013), named Ypc1p-C27F, by a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technology, CT) following the manufacturer's manual. The ORF of Ypc1p-C27F was cloned into a pYES2 vector, and the resulting expression construct, pYES2::YPC1^{C27F}, was transformed into yeast cells as described previously.

2.4.2 Sphingolipid analyses

Yeast cells were harvested at various time points including the log phase, the diauxic shift, and every 4 days in the stationary phase. The cells were washed and suspended in a lipid extraction solvent consisting of ethanol, diethyl ether, pyridine, ammonium hydroxide, and water (50:10:2:25:15 by volume) in a screw-cap vial containing 0.5 ml of glass beads (Sigma, MO) before being homogenized for 10 min by vortexing at 4 °C at the maximum speed. For mitochondrial lipid analyses, mitochondria were extracted with the solvent system consisting of 70% isopropanol: ethyl acetate: pyridine: 25% ammonia (60:20:2:0.5 by volume). The lipid extracts were dried under a stream of N_2 gas and reconstituted in 150 µl of methanol before sphingolipids were determined by LC-MS/MS performed on a TSQ Quantum Ultra quadruple mass spectrometer (Thermo Finnigan, NJ) according to the method described in (Bielawski *et al.* 2006).

2.4.3 Quantitative mRNA levels of sphingolipid metabolism enzymes

Total RNA was extracted from yeast cells harvested at different time points as described in (Ares 2012). Oligonucleotide primers for RT-qPCR were described in Table 2.3. One microgram of total RNA was reverse-transcribed into cDNA in a 20 µl reaction mixture using the SuperScript® III Reverse Transcriptase (Life Technology, CA). The reaction mix (25 µl final volume) consisted of 12.5 µl of iQ SYBR Green Supermix (Bio-Rad, CA), 1.25 µl of each primer (0.5 µM final

concentration), and 10 µl of a 1/10 dilution of the cDNA preparation. The thermocycling program consisted of one hold at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C and 45 s at 60°C. Relative gene expression levels were determined by the 2ΔΔCt method (Livak and Schmittgen 2001).

2.4.4 Ceramidase activity assay

Ceramidase activity was assayed as described in (Wang, Xu *et al.* 2015) with slight modifications. C_{16}-phytoceramide (2.5 nmoles) in ethanol was dried under a stream of nitrogen gas, resuspended in 40 µl of the assay buffer A (25 mM Tris-HCl, pH 8.0, 5 mM CaCl, and 0.5% Triton X-100), boiled for 10 s, and chilled on ice, and sonicated in a water bath ultrasonicator for 2 min as described (Mitsutake *et al.* 1997). The substrate was incubated with 40 µl (approximately 400 µg of proteins) of microsomes at 37 °C for 60 min, and the reaction was terminated by adding 400 µl of methanol. The reaction mixture was dried and dissolved in 30 µl of chloroform:methanol (2:1, v/v) before PHS was quantified by LC-MS/MS as described in (Bielawski, Szulc *et al.* 2006).

2.5 Figures and tables

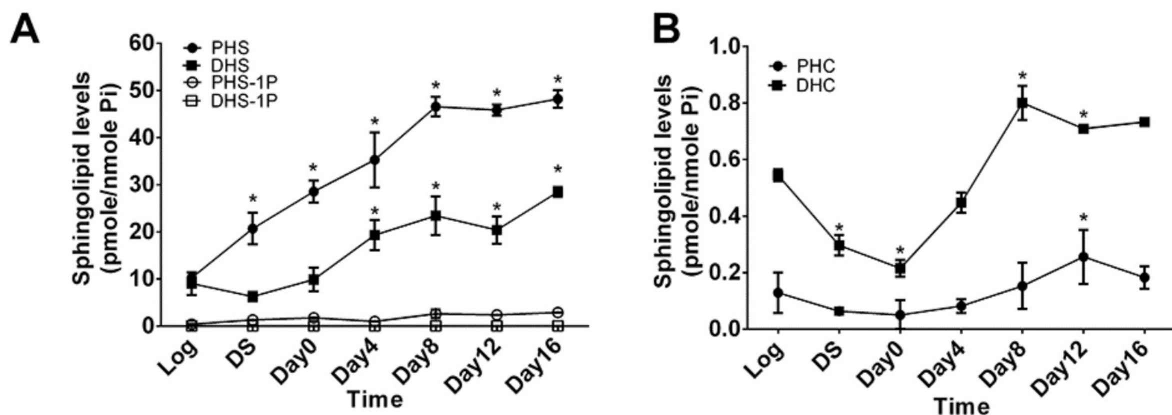


Figure 2.1 Sphingoid bases and their enzymes are altered in yeast cells during the chronological aging process.

A and **B**, aging-related changes in sphingolipid levels in yeast cells. WT JK93d cells were harvested at various time points including the log phase (log), the diauxic shift (DS), the stationary phase (Day 0), and every 4 days post the stationary phase (Day 4, 6, 8, or 16). Cells were subjected to LC-MS/MS analysis for SBs (A), PHC (B), or DHC (B) as described in (Bielawski, Szulc *et al.* 2006).

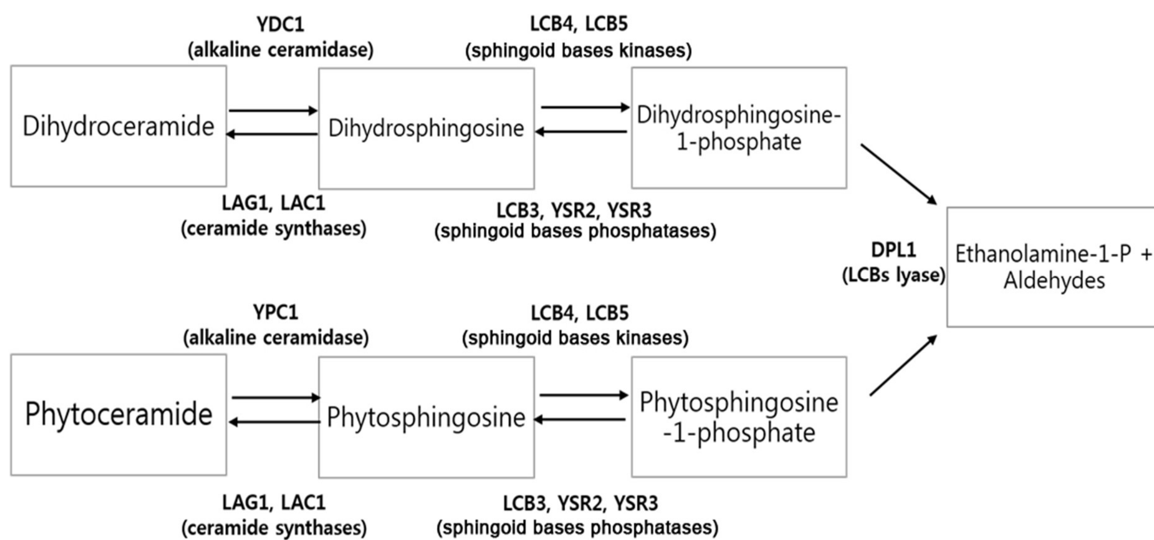


Figure 2.2 Simplified sphingolipid metabolic pathways in yeast cells.

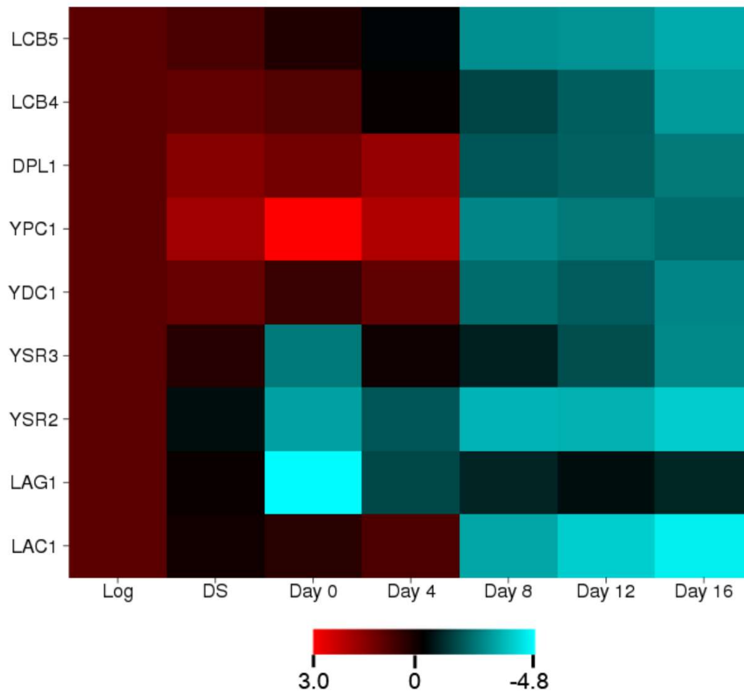
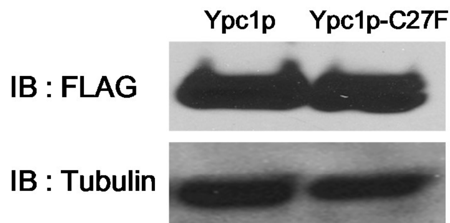


Figure 2.3 Aging-related changes in mRNA levels of sphingolipid-metabolizing enzymes in yeast cells.

JK93d cells grown at different time points were subjected to qPCR analyses for the mRNA levels of sphingolipid-metabolizing enzymes. The heat map was constructed with the log2 ratios of the mRNA levels of each of targeted genes at each post-log phase time point to those at the log phase. Data represent mean ± SD; n=3. *$p<0.05$ compared to log phase levels (two-way ANOVA)

A



B

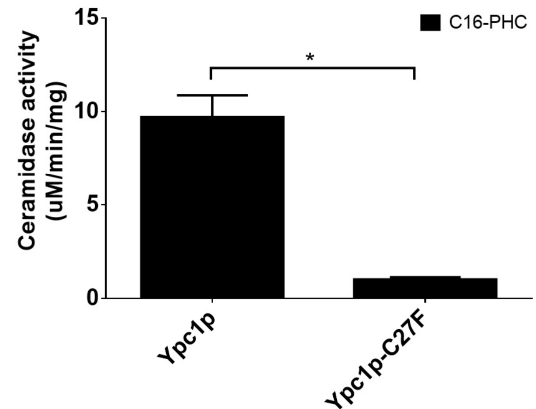


Figure 2.4 Inverse regulation of YPC1 and LCB4/5 leads to an increase in sphingoid bases in aging yeast cells.

A, Western blot analyses of Ypc1p and Ypc1p-C27F. YAG6B cells expressing the FLAG-tagged Ypc1p or its mutant, Ypc1p-C27F, were analyzed by Western blot analyses using the anti-FLAG antibody as described in Materials and Methods. **B**, Ypc1p activity assays. Microsomes from cells expressing Ypc1p or Ypc1p-C27F were subjected to alkaline ceramidase activity assays with C_{16}-phytoceramide as a substrate as described in Materials and Methods. Data represent mean ± SD; n=3. *$p<0.05$

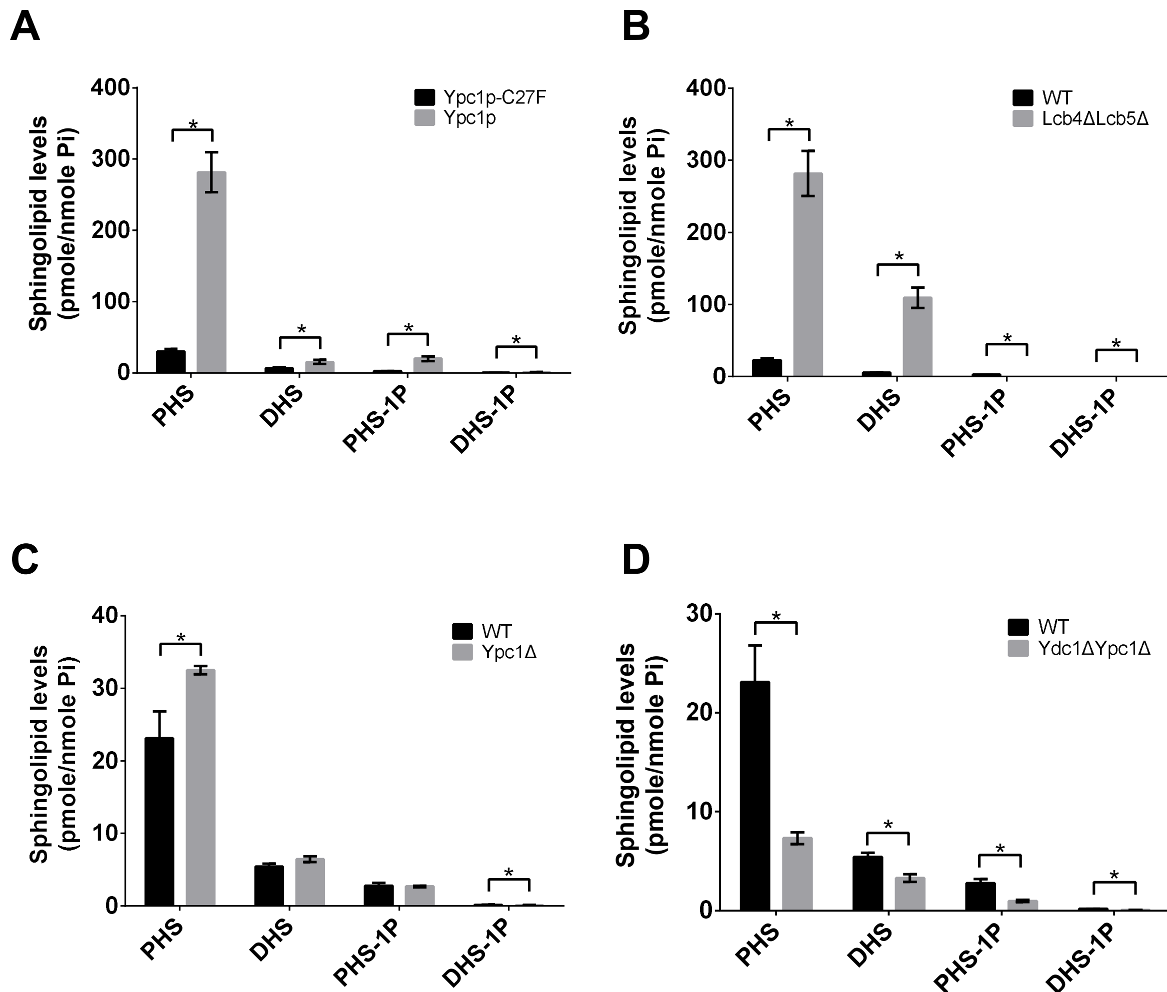


Figure 2.5 LC-MS/MS analyses of SBs.

The levels of SBs were determined by LC-MS/MS in yeast cells overexpressing Ypc1 vs Ypc1p-C27F (A), WT vs Lcb4ΔLcb5Δ (B), WT vs Ypc1Δ (C), or WT vs Ydc1ΔYpc1Δ (D) as described in Materials and Methods. The levels of SBs were normalized to total phosphate. Data represent mean ± SD; n=3. *$p<0.05$

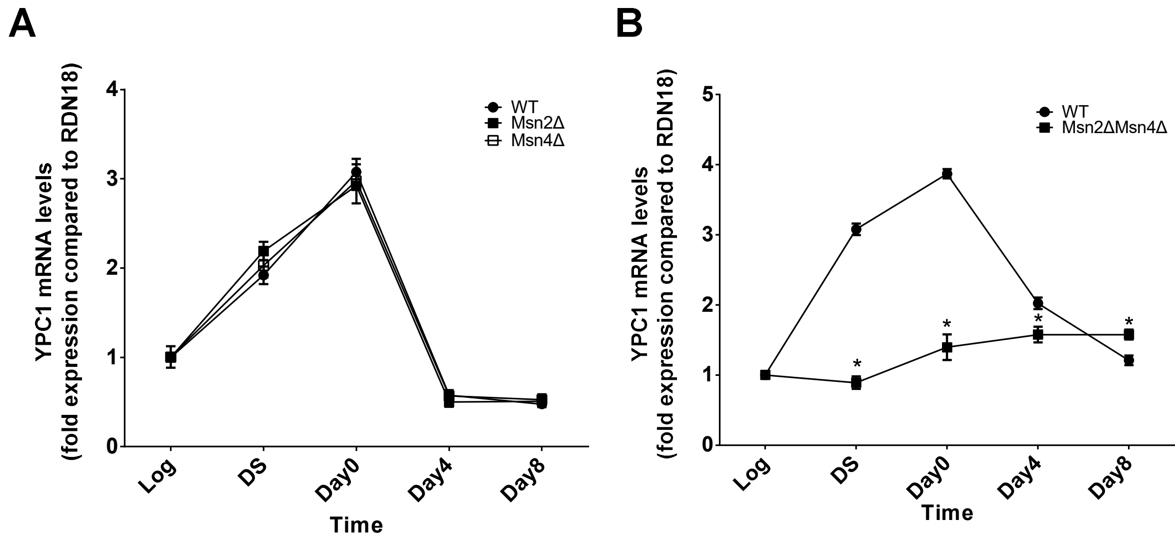


Figure 2.6 MSN2/4 activation upregulates YPC1 in aging yeast cells.

A and **B**, qPCR analyses for YPC1 mRNA levels. WT, Msn2Δ, Msn4Δ, or Msn2ΔMsn4Δ cells were grown in complete SC medium for indicated time durations and YPC1 mRNA levels were determined by qPCR analyses as described in Materials and Methods. Relative YPC1 mRNA levels were analyzed by the 2ΔΔCt method. Data represent mean ± SD; n=3. *p<0.05

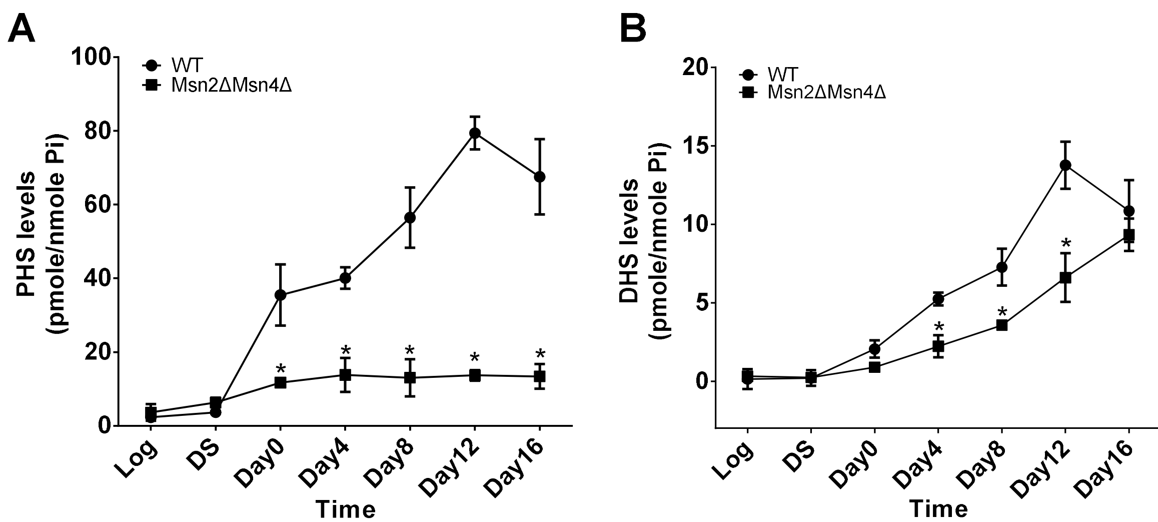


Figure 2.7 LC-MS/MS analyses for SBs.

A and **B**, WT and Msn2ΔMsn4Δ cells were harvested at indicated time points and the levels of PHS (C), and DHS (D) were determined by LC-MS/MS analyses as described in Materials and Methods. Data represent mean ± SD; n=3. *$p<0.05$

Genes	No. of STREs in the promoter region	Distance between two STREs (nt)
YDC1	1	N/A
YPC1	3	85, 185
LAG1	2	462 nt
LAC1	1	N/A
DPL1	1	N/A
YSR2	1	N/A
YSR3	0	N/A
LCB4	2	196
LCB5	0	N/A

Table 2.1 STREs in promoter regions of the genes of yeast sphingolipid-metabolizing enzymes.

Strain	Genotype	Relevant gene or description	Source
JK93d	MATα leu2–3,112 ura3–52 rme1 trp1 his4	Parental strain	This study
Ydc1Δ	Isogenic to JK93d, Δ087::UraBbrP1	Δ087=DHcer	(Mao, Xu *et al.* 2000)
Ypc1Δ	Isogenic to JK93d, Δ183::G418	Δ183=PHcer	(Mao, Xu *et al.* 2000)
Ydc1ΔYpc1Δ	Isogenic to JK93d, Δ183::G418 Δ087::UraBbrP1	Δ183=PHcer Δ087=DHcer	(Mao, Xu *et al.* 2000)
Lcb4ΔLcb5Δ	Isogenic to JK93d, lcb4-1 lcb5-1		(Nagiec, Skrzypek *et al.* 1998)
YAG6B	MATa his3 leu2 met15 ura3 YHR018c::kanMX4 YIR034c::kanMX4	SILAC strain	A kind gift from Dr. Nils Faergeman (Gruhler *et al.* 2005)
Yi9	Isogenic to YAG6B, pYES2::FLAG	Empty vector	This study
Yi10	Isogenic to YAG6B pYES2::YPC1	Ypc1p overexpression	This study
Yi11	Isogenic to YAG6B pYES2::YPC1^{C27F}	Ypc1p-C27F overexpression	This study
W303-1A	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15		A kind gift from Dr. Ales Vancura (Galdieri *et al.* 2010)
Msn2ΔMsn4Δ	Isogenic to W303-1A, msn2::HIS3 msn4::URA3		A kind gift from Dr. Ales Vancura (Galdieri, Mehrotra *et al.* 2010)
BY4742	MATα his3-1 leu2-0 lys2-0 ura3-0		(Winzeler *et al.* 1999)
Msn2Δ	Isogenic to BY4742, msn2::kanMX4		(Winzeler, Shoemaker *et al.* 1999)

Msn4Δ	Isogenic to BY4742, msn4::kanMX4	(Winzeler, Shoemaker *et al.* 1999)

Table 2.2 Strains used in this study

Targets	Primers	Sequences (5'>3')
ACT1	Forward	GTATGTGTAAAGCCGGTTTTG
	Reverse	CATGATACCTTGGTGTCTTGG
COX1	Forward	CTACAGATACAGCATTTCCAAGA
	Reverse	GTGCCTGAATAGATGATAATGGT
YDC1	Forward	GAATGGGGTTCTCGCTGGTT
	Reverse	TGGTCGCATACAGCATTGGT
YPC1	Forward	ATTGGCTTCGGGTACGGTTT
	Reverse	CCTCTTTGGCCTCGCATACT
LAG1	Forward	TTACTCCCTTTGTGTTCGGCTT
	Reverse	TCACGACATGGCGCAGATAG
LAC1	Forward	TGCTAAAAGGCGTATGCAGAGA
	Reverse	TCAAGGGAGCTATCCAGGCA
LCB4	Forward	CTCAGCTACCTTGCGGTTCA
	Reverse	TTTGACAAGGCACAGAGCGG

LCB5	Forward	ATCGGAAACATCGACAACGGA
	Reverse	TCTGGCATCCAGAATTCGTCC
YSR2	Forward	GCCACGCACAAAGAAGGTG
	Reverse	AGGGAAAATAGGACGGGGCT
YSR3	Forward	ATCTGGGTGGTGCATGCTTT
	Reverse	ACAAGCCCCATGCTACACTC
DPL1	Forward	CTGCACATGCTGGGTTTGAC
	Reverse	GAGCGGAACCGACCAGTAAA
N-GFP	Forward	CCCGGGTACCAGATCTATGAGTAAAGGAGAAGAAC
	Reverse	CCGCTCGAGTTGGGCCAATTCCTTTTTAAAGCCTGTAATTC CCACTTTAATTGGGCTAATTCCTTTTTATTAGCTTGTAATTCC TTTTTTAAAGCACCGGATCCAGATCCACCCTTTTGTTTGTCTG CCATGAT
C-GFP	Forward	GGGGTACCGAGCAGTTAGAAAAGAAGTTACAAGCTTTGGAA AAGAAATTGGCACAATTAGAATGGAAGAATCAAGCCTTGGA AAAGAAATTGGCACAAGGTGGATCTGGTAATGGAATCAAAG TTAACTT
	Reverse	CCGCTCGAGGAATTCTTATTTGTATAGTTC

Table 2.3 Primers used in this study

Chapter 3. Accumulation of sphingoid bases leads to yeast aging

3.1 Introduction

In chapter 2, it is established that SBs are accumulated during the aging process. Because SBs are known to inhibit cell growth and induce apoptosis (Park *et al.* 2003) and activate Sch9 protein which can promote aging process through *TOR1* (Liu *et al.* 2013), accumulation of SBs is likely to affect the cellular aging process. For these reasons, several experiments are performed in this chapter to establish that increased SBs reduce yeast chronological lifespan.

3.2 Results

3.2.1 Upregulation of sphingoid bases reduces yeast chronological life span

Following the finding that SBs and their metabolic enzymes are altered during the aging process, I wondered if these alterations contributed to yeast aging. To this end, I determined if YPC1 overexpression or deletion of both LCB4 and LCB5 accelerated aging by increasing SBs whereas blocking the increase in SBs by inhibiting both YPC1 and YDC1 could delay aging.

First, I determined if overexpression of YPC1 affected CLS in yeast cells. Galactose is needed to induce YPC1 overexpression, however, its presence in the medium may interfere with CLS assays because galactose can serve as a carbon source to sustain cell growth. For this reason, we constructed a mutant yeast strain (*GalΔ*) in which galactose cannot be used as a carbon source for growth due to deletion of the Gal1 gene. We transformed Gal1Δ cells with either Ypc1p or Ypc1p-C27F expressing construct to test the effect of YPC1 overexpression on CLS. Western blot

analyses demonstrated that the expression of either Ypc1p or Ypc1p-C27F could be induced by galactose to similar levels in *GalΔ* cells (Fig. 3.1). We also confirmed that GalΔ cells did not grow in the presence of galactose (Fig. 3.1.B). These results suggest that the GalΔ cells expressing Ypc1p and Ypc1p-C27F, respectively, are proper stains for CLS assays. Viability assays showed that GalΔ cells overexpressing WT Ypc1p had a shorter CLS than GalΔ cells overexpressing Ypc1p-C27F, supporting the notion that YPC1 upregulation shortens CLS (Fig. 3.2.A).

Second, we determined if knocking out both LCB4 and LCB5 also shortened CLS of yeast cells by increasing SBs. To this end, we performed CLS assays with WT and Lcb4ΔLcb5Δ stains as described earlier. We found that Lcb4ΔLcb5Δ cells had shortened CLS compared to WT cells (Fig. 3.2.B), suggesting that similar to YPC1 upregulation, knocking out both LCB4 and LCB5 shortens CLS.

Having demonstrated that overexpression of YPC1 or knocking out LCB4/5 shortens CLS, we wondered that increased SBs are involved in the observed effects. To this end, we determined if PHS, when added exogenously, could shorten CLS. The survival rate of cells treated with PHS (10 μM) or its vehicle, ethanol, was measured. The results showed that treatment with PHS reduced CLS compared to treatment with ethanol (Fig. 3.2.C), supporting the notion that the inverse regulation of YPC1 and LCB4/5 contributes to yeast aging by increasing SBs.

To further consolidate the above notion, we determined if the inhibiting aging-related increase in the levels of SBs enhanced CLS. Indeed, we found that Ypc1ΔYdc1Δ cells had a shorter CLS than WT cells (Fig. 3.2.D).

In summary, YPC1 overexpression, deletion of both LCB4 and LCB5, or treatment with PHS decreased the yeast life span whereas knockout of both YDC1 and YPC1 increased CLS. These conjoint results strongly suggest that SBs are negative regulators of the yeast CLS.

3.3 Discussion

Studies from different groups demonstrate that knocking out different sphingolipid-metabolizing enzymes affects yeast replicative or chronological lifespan (Aerts *et al.* 2006, Aerts, Zabrocki *et al.* 2008, Almeida *et al.* 2008, Hernandez-Corbacho, Jenkins *et al.* 2011, Huang *et al.* 2012, Liu, Huang *et al.* 2013, Loiseau *et al.* 2013, Vilaca *et al.* 2014, Voynova *et al.* 2014), suggesting that sphingolipids may regulate yeast aging. However, what exact sphingolipids regulate yeast aging remains largely unclear although previous studies implied but have not proven that SBs may regulate yeast aging (Liu, Huang *et al.* 2013). In this study, I provided strong evidence that SBs produced by the action of YPC1 and YDC1 regulate yeast CLS. First, I demonstrated that SBs but not yeast ceramides were increased during yeast chronological aging. Second, I showed that knocking out both YPC1 and YDC1 inhibited the chronological aging-associated increase in the levels of SBs and prolonged CLS. Third, enforced increases in the levels of SBs in yeast cells due to either YPC1 overexpression or deletion of both LCB4/LCB5 shortened yeast CLS. Finally, treatment with exogenous PHS shortened yeast CLS. Because we previously showed that the mammalian SB (Wang *et al.* 2015), SPH, is also increased in the mouse aging brain, I speculated that this age-associated increase in SPH may contribute to physiological or pathological aging of mammals. This view appears to be supported by recent studies showing that knocking out the S1P lyase gene (SGPL1) markedly increased the levels of free SBs (SPH and DHS) in addition to LCB phosphates (S1P and DHS1P) (Bektas *et al.* 2010), thus shortening the lifespan of mice (Schmahl *et al.* 2007, Vogel *et al.* 2009).

3.4 Materials and Methods

3.4.1 CLS assays

CLS assays were performed in cells grown in liquid synthetic complete medium containing 2% glucose (SDC) supplemented with standard amounts of amino acids. Briefly, yeast strains from frozen stock (-80ºC) were streaked onto either YPD agar plates or SD-ura plates containing 2% glucose and incubated at 30ºC. The following day, cells were inoculated into 5 ml of either SDC medium or SD-ura and grown overnight. The overnight culture was inoculated into 50 ml of either SDC medium or SD-ura medium in a 250-ml flask to an optical density at 600 nm (OD600) of 0.1, and the flask was shaken at 250 rpm at 30ºC. Under these culture conditions, yeast cultures reached a maximum cell density at 48 hours, so Day 3 after inoculation was considered as Day 0 of CLS. Only for the transformed cells, galactose was added to the medium at Day 0 to induce gene overexpression. Subsequently, cellular viability was determined on different days by colony formation unit (CFU) assays. Briefly, cell number was estimated by optical density (OD) for each population and serial dilutions of different cultures were plated onto 3 YPD plates. Plates were incubated at 30ºC for 48 hours before CFU were counted.

3.4.2 Protein expression analysis

Protein expression was assessed by Western blotting analyses using either an anti-FLAG or an anti-Tubulin primary antibody.

3.4.3 Statistical analysis

Data are presented as the mean ± SD and were compared by either Student's t-test, one-way or two-way ANOVA (ANanlysis Of VAriance) with post hoc Tukey HSD (Honestly Significant Difference) using GraphPad Prism (La Jolla, CA). Values of $p<0.05$ were considered significant and marked with an asterisk (*).

3.5 Figures and tables

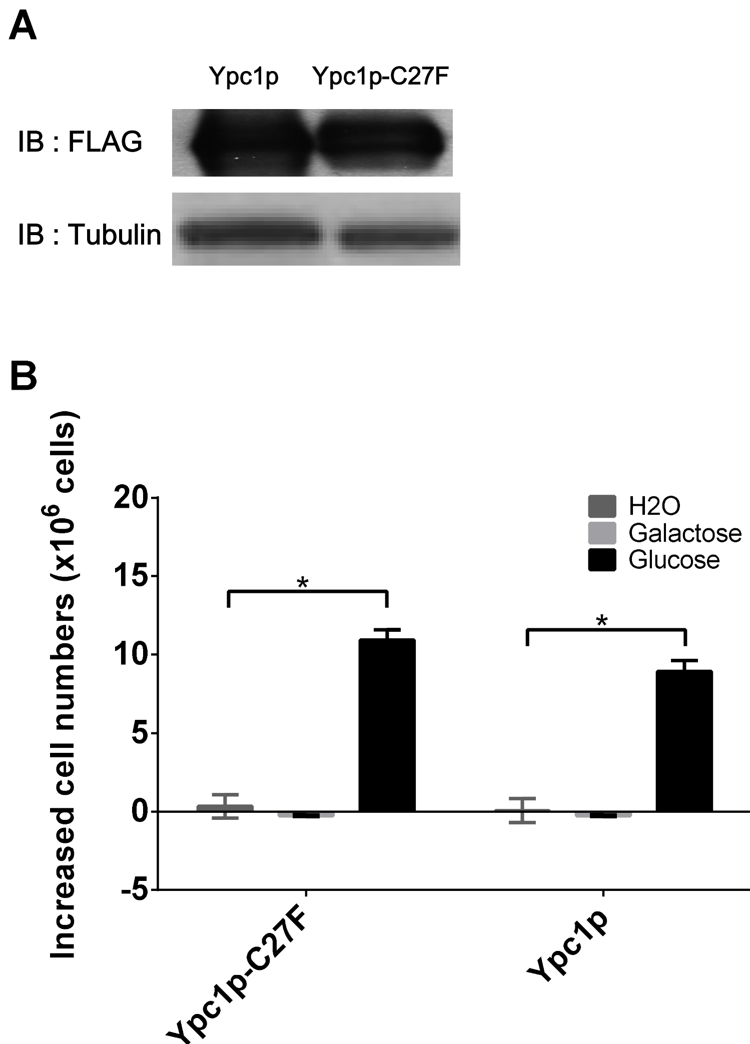


Figure 3.1 Gal1Δ overexpressing either Ypc1p or Ypc1p-C27F.

A, Western blot analyses of Ypc1p and Ypc1p-C27F. Gal1Δ cells expressing the FLAG-tagged Ypc1p or its mutant, Ypc1p-C27F, were analyzed by Western blot analyses using the anti-FLAG antibody as described in Materials and Methods. **B**, Cell numbers were counted 24 hr after addition of either H_2O, glucose or galactose to verify if galactose can induce the cell growth in Gal1 Δ. Data represent mean of ± SD; n=3. *$p<0.05$.

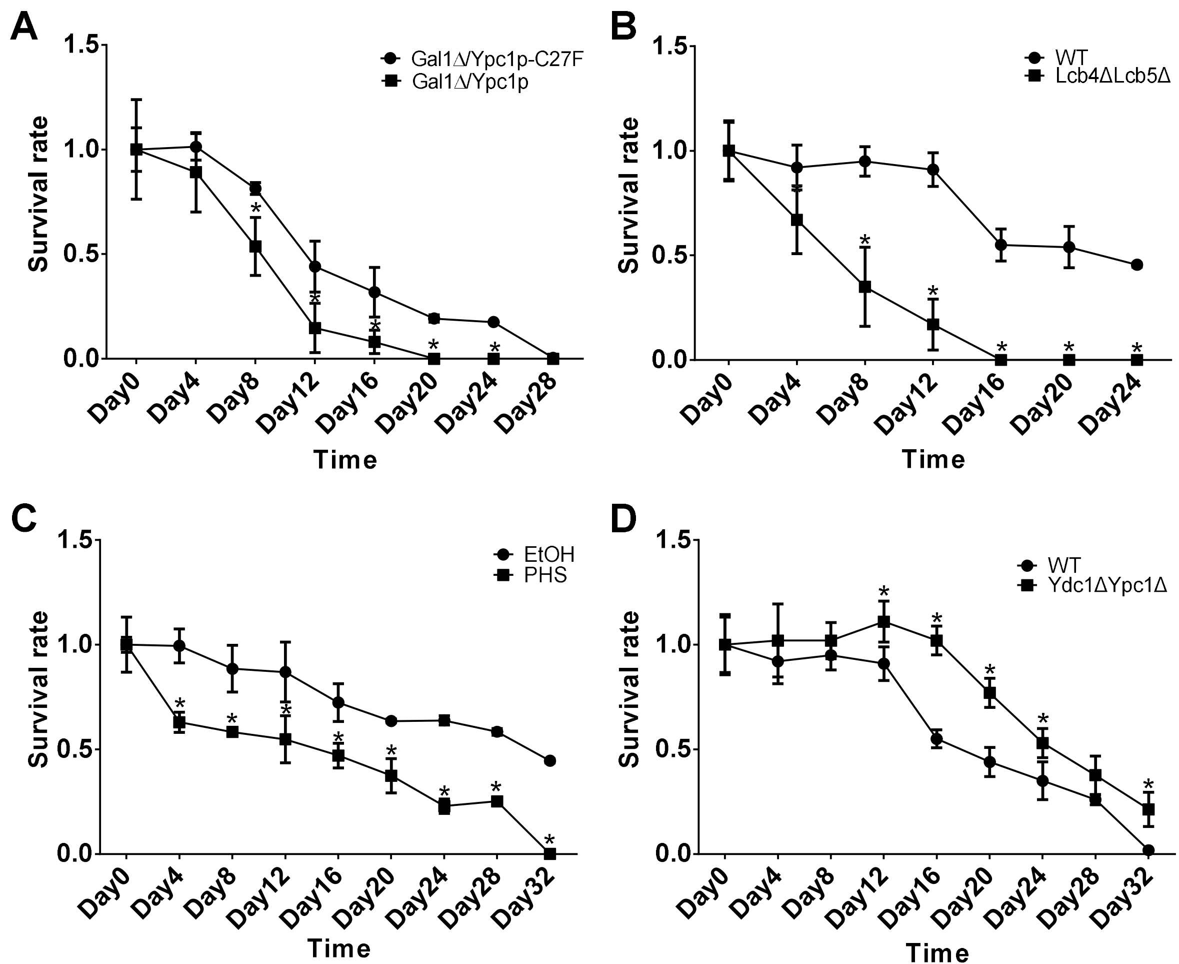


Figure 3.2 Sphingoid bases decrease CLS.

A-D. CLS assays of yeast cells. Gal1Δ cells overexpressing either Ypc1p or Ypc1pC27F (A), WT vs. Lcb4ΔLcb5Δ cells (B), WT cells treated with ethanol or PHS (10uM) (C), and WT vs. Ydc1ΔYpc1Δ (D) were subjected to CLS assays as described in Materials and Methods. Survival rates of yeast cultures were assayed in triplicate. The data represent the mean ± SD of fold changes in survival rates at indicated time points vs. those at the day 0 time point (n=5), *p<0.05.

Chapter 4. Accumulation of sphingoid bases disrupts mitochondrial structure and function

4.1 Introduction

There is evidence showing that sphingolipid metabolism is tightly associated with mitochondrial functions. Especially, one of the research studies showed that the overexpression of *YDC1* induced mitochondrial fragmentation (Aerts, Zabrocki *et al.* 2008). For these reasons, SBs are expected to affect the morphology and general functions of mitochondria. Because declined mitochondrial functions can promote aging process (Hipkiss 2010), there is a possibility that SBs cause premature aging through compromising mitochondrial functions.

To study the effects of increased levels of SBs on mitochondria, I examined if increasing SBs by overexpressing *YPC1* or knocking out both *LCB4* and *LCB5 A)* induces morphological alterations in mitochondria; B) impairs mitochondrial fission or fusion; and C) compromises mitochondrial functions including ATP generation, mitochondrial membrane potential, and respiration. The study in this chapter can help to reveal how SBs affect the cellular aging process by demonstrating that SBs impair mitochondrial structures and functions.

4.2 Results

4.2.1 Increased sphingoid bases alter levels of proteins related to mitochondrial functions

Following the finding that SBs regulate yeast CLS, I wanted to know the underlying mechanism. To this end, I investigated how SBs induced the global changes in proteins through the proteomics-based approach, SILAC as described in Materials and Methods (4.4 Materials and Methods). From the two independent SILAC experiments, I identified 3,570 proteins, among which, 206 proteins showed more than 1.5 folds difference in their expression levels between cells overexpressing Ypc1p and Ypc1p-C27F (Fig. 4.1 and Table 4.1). Proteins showing different expression levels

were categorized regarding their cellular localization and biological functions using the public database (The GO annotation from the *Saccharomyces* Genome Database). The results showed that a cellular component which had the highest cluster frequency was shown to be the mitochondrion (66 out of 206) followed by the nucleus (62 out of 296) and the plasma membrane (32 out of 206), respectively (Fig. 4.2.A). Furthermore, the highest frequency in the biological function was shown to be the oxidoreductase activity (Fig. 4.2.B), suggesting a possibility that SBs affect mitochondrial functions.

4.2.2 Sphingoid bases induce fragmentation of the mitochondrial tubular network

Aging is strongly associated with the alterations in the mitochondrial structures (Lee and Wei 2012, Bratic and Larsson 2013). Because SBs increased by the overexpression of YPC1 altered the levels of many mitochondrial and mitochondrion-related proteins, I postulated that increased SBs might alter the mitochondrial morphology and function in aging cells.

To test this possibility, I first observed the mitochondria morphology by fluorescent microscopy using mitotracker labeling. Cells overexpressing Ypc1p or Ypc1p-C27F were collected 12 h after gene expression induction (late log phase) and stained with the mitotracker before being observed for mitochondrial morphology under a fluorescent microscope. The results showed that cells overexpressing Ypc1p-C27F had an intact mitochondrial tubular network whereas cells overexpressing Ypc1p had a fragmented mitochondrial network (Fig. 4.3.A and 4.3.B), suggesting that increased SBs disrupt the integrity of the mitochondrial network.

To further verify the effect of SBs on the mitochondrial morphology, the morphology of the mitochondrial network was compared between Lcb4ΔLcb5Δ cells and WT cells in the late log phase in SDC medium. The results showed that the mitochondrial network was severely

fragmented in Lcb4ΔLcb5Δ cells whereas this network was intact in WT cells (Fig. 4.3.C and 4.3.D), confirming increased SBs indeed disrupt the integrity of mitochondria in cells.

To further confirm this notion, I investigated if inhibiting the generation of SBs by knocking out both YPC1 and YDC1 can attenuate mitochondrial fragmentation in aging cells. To this end, the integrity of the mitochondrial network was assessed in Ydc1ΔYpc1Δ and WT cells during the aging process. Ydc1ΔYpc1Δ and WT cells were incubated in SDC medium until the late log phase before they were stained with the mitotracker. Fluorescent microscopy revealed that the mitochondrial network was fragmented in WT cells, and this was significantly attenuated in Ydc1ΔYpc1Δ cells (Fig. 4.3.C and 4.3.D), confirming the notion that the aging-related increase in SBs induces the fragmentation of the mitochondrial tubular network in yeast cells.

4.2.3 Sphingoid bases compromise mitochondrial functions

The functions of mitochondria are highly dependent on their structural integrity. Having shown that SBs alter the mitochondrial morphology, I investigated if increased SBs altered the mitochondrial functions, such as mitochondrial respiration, oxygen consumption rates, ATP production, and mitochondrial $\Delta\psi m$.

To establish whether SBs affected mitochondrial respiration, the growth on an oxidative carbon source, glycerol, were determined using WT, Ydc1ΔYpc1Δ, and Lcb4ΔLcb5Δ cells. Compared to WT, the growth of Ydc1ΔYpc1Δ cells was elevated, whereas Lcb4ΔLcb5Δ showed the high sensitivity to oxidative carbon source (Fig 4.4.A), suggesting that SBs induce mitochondrial respiration deficiency. To further verify the effect of SBs on mitochondria respiration, the oxygen consumption rates (OCR) were assessed in different yeast strains by XF96 mitochondrial flux analyzer. I found that cells overexpressing Ypc1p had a lower oxygen consumption rate than cells

overexpressing Ypc1p-C27F (Fig. 4.4.C). Consistently, compared with WT cells, Lcb4ΔLcb5Δ cells showed a significantly lower OCR (Fig. 4.4.D). These results strongly suggest that increased SBs indeed inhibit oxygen consumption, a key mitochondrial function.

Because the respiration is proportional to the ATP generation (Bratic and Trifunovic 2010), I then determined if increased SBs lowered ATP levels in yeast cells. I measured the intracellular ATP levels in different yeast strains. First, I determined if Ypc1p overexpression reduced ATP levels in yeast cells in the log phase. Yeast cells expressing Ypc1p or Ypc1p-C27F were harvested at the late log phase before they were subjected to ATP measurements. As shown in Fig. 4.4.E, cells overexpressing Ypc1p had lower ATP levels than cells overexpressing Ypc1p-C27F. I then determined if knocking out LCB4 and LCB5 had a similar effect on ATP level to Ypc1p overexpression. WT or Lcb4ΔLcb5Δ cells were grown to the late log phase before they were subjected to ATP measurements. As shown in Fig. 4.4.F, Lcb4ΔLcb5Δ cells had lower ATP levels than WT cells, confirming that increased SBs inhibit ATP production.

Because the Δψm is regarded as the main driving force for ATP production (Dimroth *et al.* 2000), I decided to measure Δψm by JC-1 staining to verify the effect of increased SBs on Δψm as well. Mitochondria were isolated from different yeast strains and assayed for Δψm. I found that cells overexpressing Ypc1p showed lower Δψm than cells overexpressing Ypc1p-C27F (Fig. 4.4.G). Consistently, Lcb4ΔLcb5Δ cells showed a significantly lower Δψm than WT cells (Fig. 4.4.H). In contrast, Ydc1ΔYpc1Δ cells had higher Δψm than WT cells (Fig. 4.4.H).

It is also known that yeast cells defective in mitochondrial respiration are resistant to paraquat because paraquat-induced superoxide production required Δψm that was essential for paraquat uptake into mitochondria (Blaszczynski *et al.* 1985, Cocheme and Murphy 2008). In the spot assay, Lcb4ΔLcb5Δ showed the highest resistance to the paraquat among other cells including WT and

Ydc1ΔYpc1Δ. Moreover, the growth of Ydc1ΔYpc1Δ cells in the presence of 4 mM paraquat was almost abolished (Fig. 4.4.B), verifying that the relatively intrinsic Δψm was increased in Ydc1ΔYpc1Δ cells. Taken together, these results suggest that increased SBs indeed compromise mitochondrial functions by decreasing Δψm, intracellular ATP levels, and basal OCR.

4.2.4 Sphingoid base levels are increased in mitochondria during yeast aging

Because increased SBs impair both mitochondrial structure and functions, I wondered if SBs are increased in mitochondria in aging cells. I measured the levels of SBs from mitochondria purified from different yeast strains by the sucrose gradient ultracentrifugation as described (Gregg *et al.* 2009). Immunoblotting was performed to verify the purity of the purified mitochondria using antibodies against porin, Alp1p, Pma1p, Dpm1p, or Vps10p, which are markers for various organelles including mitochondria, vacuoles, plasma membranes, ER or late Golgi, respectively. I found that the mitochondrial marker porin but not the makers for the other organelles was detectable in the mitochondrial preparations, suggesting that the mitochondria were highly purified (Fig. 4.4.A). With LC-MS/MS, I found that the levels of SBs were increased in mitochondria from cells overexpressing Ypc1p compared to cells overexpressing Ypc1p-C27F (Fig. 4.5.B). Consistently, compared with WT cells, Lcb4ΔLcb5Δ cells showed significantly higher SB levels whereas Ydc1ΔYpc1Δ cells showed lower SBs levels in the mitochondria (Fig. 4.5.C). These results suggest that SBs produced in either the ER or Golgi complex by the alkaline ceramidases are transported to mitochondria and act locally to impair mitochondrial morphology and functions.

4.2.5 Sphingoid bases inhibit mitochondrial fusion and decrease mtDNA copy numbers

Because altered SBs are known to affect membrane structure and permeability (Loiseau, Obata *et al.* 2013), I investigated whether an increase in the mitochondrial SBs affected mitochondrial fusion using *in vitro* mitochondrial fusion assays. Mitochondria were isolated from yeast cells expressing N-GFP and C-GFP respectively and combined to initiate mitochondrial fusion in the presence of various concentrations of PHS. I found that PHS inhibited mitochondrial fusion in a concentration-dependent manner (Fig 4.6.A and 4.6.B), suggesting that increased SBs indeed inhibit mitochondrial fusion. To verify the inhibition of mitochondrial fusion is a specific effect of PHS, I also treated mitochondria with $C_{8:0}$-phytoceramide ($C_{8:0}$-PHC). Interestingly, there was no significant inhibition of mitochondrial fusion upon the $C_{8:0}$-PHC treatment (Fig 4.6.A and 4.6.B). This result indicates that mitochondrial fusion is specifically induced by PHS.

Because mitochondrial fusion is strongly associated with mtDNA maintenance (Chen *et al.* 2010, Hori *et al.* 2011), I then sought to determine if inhibition of mitochondrial fusion caused by SBs leads to the decreased number of mitochondria using a qPCR. It was observed that mtDNA copy number was reduced in *YPC1* overexpressing cells by 25% compared with control cells (Fig. 4.6.C). Lcb4ΔLcb5Δ also showed a decrease in mtDNA copy number compared with WT cells (Fig. 4.6.D). These results suggest that increased SBs due to the inverse regulation of YPC1 and LCB4/5 reduce the mtDNA copy number likely by inhibiting mitochondrial fusion.

4.3 Discussion

Although SBs are known to inhibit cell growth and induce apoptosis (Park, Kang *et al.* 2003), the underlying mechanism by which SBs regulate yeast aging remains unclear. Using a proteomic

approach, SILAC, we found that the expression of various proteins important for maintaining the mitochondrial morphology and functions are altered upon YPC1 overexpression (Fig. 4.1 and Fig. 4.2). This suggests to us that SBs may alter the mitochondrial structure and functions. Indeed, we provided strong evidence that SBs produced by the action of YPC1 and YDC1 are transported to the mitochondria and inhibit mitochondrial fusion and mitochondrial functions. First, we showed that YPC1 overexpression increased the levels of PHS and DHS in the yeast mitochondria. Second, either overexpression of YPC1 or deletion of LCB4/5 induced fragmentation of the mitochondria. Consistently, Aerts et al. showed that overexpression of YDC1 (which may increase the levels of DHS in yeast cells by catalyzing the hydrolysis of dihydroceramides) induced mitochondrial fragmentation (Aerts, Zabrocki *et al.* 2008). Third, knocking out both YPC1 and YDC1 inhibited fragmentation of the mitochondria in aging yeast cells. Fourth, our *in vitro* studies showed that PHS inhibited the mitochondrial fusion in the test tube. These results strongly suggest that SBs may directly target on the mitochondrial membrane and alter the structural integrity of the mitochondria. Because the proper structure of the mitochondria is essential for mitochondrial functions, it is conceivable that SBs may also compromise the mitochondrial functions. Indeed, we showed that either YPC1 overexpression or LCB4/5 deletion decreased the intracellular levels of ATP and inhibited mitochondrial respiration. Because numerous studies demonstrated that declined mitochondrial functions can promote aging process (Hipkiss 2010, Kong, Trabucco *et al.* 2014), we postulated that increased SBs due to the inverse regulation of the ceramidases and SB kinases shortens yeast CLS at least in part by compromising both the structural integrity and functions of the mitochondria.

4.4 Materials and Methods

4.4.1 Mitochondria isolation

Mitochondria were isolated from yeast cells by the yeast mitochondria isolation kit (BioVision, CA) according to the manufacturer's instructions. Although the resulting samples are enriched in mitochondria, they may contain other organelles such as the endoplasmic reticulum, Golgi complex, and vacuoles. To obtain pure mitochondria, this crude mitochondrial fraction was subjected to further fractionation as described (Gregg, Kyryakov *et al.* 2009). Briefly, a sucrose gradient was constructed by multiple layers extending from 60% sucrose to 15% sucrose in EM buffer (10 mM MOPS/KOH (pH 7.2), 1 mM EDTA) in a Beckman Ultra-Clear centrifuge tube (Beckman Coulter Life Sciences). Three ml of the crude mitochondrial fraction in SEM buffer (10 mM MOPS/KOH, pH 7.2, 250 mM sucrose, and 1 mM EDTA) was placed on the top of 15% (w/v) sucrose and centrifuged in a Beckman SW44 Ti swinging-bucket rotor for 1 h at 134,000g at 4°C. The intact mitochondria were obtained from a brown band at the 60%/32% sucrose interface, and purified mitochondria were used for sphingolipid analyses, mitochondrial membrane potential assays, and mitochondrial fusion assays.

4.4.2 Stable isotope labeling by amino acids in cell culture (SILAC)

The yeast strain YAG6B, which is auxotrophic to both lysine and arginine (Gruhler, Olsen *et al.* 2005), was transformed with pYES2::YPC1 or pYES2::YPC1^{C27F}. Yeast cells overexpressing YPC1 and YPC1^{C27F} were grown in a medium containing 100 mg/L arginine and lysine or 100 mg/L [$^{13}C^{6}$] arginine and [$^{13}C^{6}$] lysine for more than 10 generations to ensure that the most of the amino acids in cells were replaced with ones from the medium. Proteins were extracted from yeast cells and were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE). The gel was stained with Coomassie Blue and excised into 5−10 small slices. Each gel slice was further cut into 1 mm^3 cubes, in which proteins were digested by trypsin into peptides as described in (Shevchenko *et al.* 2006). Peptides resulting from trypsin digestion were analyzed by liquid chromatography mass spectrometry with very high mass accuracy and sequencing speed. Tryptic peptides were analyzed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, MA). The mass spectrometer was programmed to operate in a data-dependent mode, such that the top 10 most abundant precursors in each MS scan were subjected to CAD (electron multiplier detection, collision energy = 35%, isolation width = 2.8 Da, threshold = 20000). Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 15 seconds. Raw data files were loaded directly into PEAKS 7 software (Bioinformatics Solutions Inc., ON, Canada) where the data were refined and subjected to de novo sequencing and database searching. Search parameters included trypsin specificity, up to 2 missed cleavages, fixed carbamidomethylation (C, +57 Da). Oxidation (M, +16 Da) was further specified as variable modifications. The tolerance values used were 10 ppm for parent ions and 0.6 Da for fragment ions. Since proteins were isotopically labeled, variable modification for lysine (+4.03 and +8.01 for light and heavy label, respectively) and arginine (+6.02 and 10.02 for light and heavy label, respectively) were also considered. The resulting peptide sequences were searched against a forward-reversed UniProt database consisted of *Saccharomyces cerevisiae* sequence (downloaded from UniProt on Jan 31, 2014; 7802 entries) (Ma *et al.* 2003).

4.4.3 ATP assay

Yeast cultures at the late-log phase were pelleted, and the cell pellets were washed once with PBS and resuspended in 100 µl of 5% trichloroacetic acid (TCA). Cells were vortexed and incubated for 15 min at room temperature. Subsequently, cell debris was pelleted and supernatants were

collected and diluted 1:1000 before ATP measurements, which were done using ENLITEN® ATP Assay System Bioluminescence Detection Kits (Promega, WI) according to the manufacturer's instructions.

4.4.4 The fluorescent microscopy

The morphology of mitochondria was investigated by fluorescent microscopy. For the fluorescent microscopic analyses, cells were harvested at the late-log phase and washed with PBS and stained with MitoTracker® Green FM (Life Technology, CA) at 500 nM for 30 min according to the manufacturer's instructions. Ten μl of cell suspension was spotted onto a microscopic slide and observed under a fluorescence microscope (Zeiss Axio Imager Z2; Carl Zeiss, Inc., NY).

4.4.5 Oxygen consumption rate assay

Oxygen consumption rates were measured under a Seahorse instrument (Seahorse Bioscience, MA) according to the manufacturer's instructions. Cells were incubated overnight in either YPD medium for knockout strains or SC-ura medium with 2% galactose for transformed strains and diluted to OD600 0.1 in fresh SC-ura media containing 2% glucose. Diluted cells were used to seed XF96 plates and incubated for 60 min at 30°C. The Seahorse sensor cartridge was rehydrated overnight following the manufacturer's instructions. XF96 culture plates and sensor cartridge were mated and placed in the Seahorse instrument after the initial check-up. Three measurements were taken for the basal reading and averaged. The mean value of the three readings across the 1-min span was calculated for each well, and at least twelve wells were assayed for each strain.

4.4.6 Mitochondrial membrane potential assay

The mitochondrial inner membrane potential ($\Delta\psi m$) was determined by staining with the membrane-permeable lipophilic cationic fluorochrome JC-1 (BD Biosciences, NJ) according to the manufacturer's instructions. One hundred µg of isolated mitochondria from each yeast strain was loaded onto a 96-well plate and were stained JC-1. The fluorescence intensity at the emission wavelength of 530 (green) or 590 nm (red) was measured under the SpectraMax Plus 384 Microplate Reader (Molecular Devices, CA) and the ratio of the red to green fluorescence intensity was calculated.

4.4.7 Mitochondrial fusion assay

The mitochondrial fusion assay was performed as described (Hori, Yoshida *et al.* 2011). Briefly, the sequence (N-GFP) encoding an N-terminal mitochondrion-targeting sequence (MTS), the first 157 amino acids of GFP (amino acids 1–157), and a C-terminal leucine zipper, was PCR-amplified using the pVT100U-mtGFP yeast plasmid (Vernet *et al.* 1987) as a template with the following primers: N-GFP-F (5'-CCCGGGTACCAGATCTATGA GTAAAGGAGAAGAAC-3') and N-GFP-R (5'-CCGCTCGAGTTGGGCCAATTCCTTTTTAAAGCCTGT

AATTCCCACTTTAATTGGGCTAATTCCTTTTTATTAGCTTGTAATTCCTTTTTTAAAGC ACCGGATCCAGATCCACCCTTTTGTTTGTCTGCCATGAT-3') (Table S2). The sequence (C-GFP) encoding the N-terminal MTS, the amino acids 158–238 of GFP, and leucine zipper, was PCR-amplified using the pVT100U-mtGFP plasmid as a template and the following primers: C-GFP-F (5'-GGGGTACCGAGCAGTTAGAAAAGAAGTTACAAGCTTTGGAAAAGAAATTGGCACAA TTAGAATGGAAGAATCAAGCCTTGGAAAAGAAATTGGCACAAGGTGGATCTGGTAA

TGGAATCAAAGTTAACTT-3') and C-GFP-R (5'-CCGCTCGAGGAATTCTTATTTGTATAGTTC-3') (Table S2). The PCR-amplified DNA fragments were subcloned into the KpnI / XhoI sites of ADH promoter-containing pVT100U to create pVT100U-N-GFP and pVT100U-C-GFP, respectively.

Each of the above vectors was transformed into JK93d WT cell and mitochondria were isolated from each strain harboring either pVT100U-N-GFP or pVT100U-C-GFP. *In vitro* mitochondrial fusion was performed as described previously (Meeusen *et al.* 2004). Isolated mitochondria were incubated on ice for 20 min and centrifuged at 10,000 g for 2 min. The pellets were suspended in stage 1 buffer (20 mM PIPES-KOH at pH 6.8, 150 mM KOAc, 5 mM $Mg(OAc)_2$ and 0.6 M sorbitol) and incubated at 22 °C for 20 min. The samples were then centrifuged at 10,000 g for 2 min at 4°C. The pellets were suspended in stage 2 buffer (the stage 1 buffer plus 0.2 mg/mL creatine phosphokinase, 40 mM creatine phosphate, 1 mM ATP and 1 mM GTP) and were incubated at 22°C in the presence of a sphingolipid or FCCP (Sigma, MO) that was used as a mitochondrial fusion inhibitor at a final concentration of 100 μM. The fluorescence intensity at the emission wavelength of 530 (green) was measured under the SpectraMax Plus 384 Microplate Reader (Molecular Devices, CA).

4.4.8 Quantitative levels of mtDNA

The relative copy numbers of mtDNA were determined using an RT-qPCR strategy as described (Stuart *et al.* 2006). Yeast cells were grown in 5 ml of SC-ura medium, and yeast cultures were harvested by centrifugation at late log phase. Total cellular nucleic acids were isolated as described (Hoffman and Winston 1987) and treated with DNase-free RNase A (10 μg/ml) at 37°C for 1.5 h. RNA-free nuclear DNA and mtDNA were subjected to qPCR, which was run in a standard 25 μl

SYBR Green reaction: 12.5 µl of iQ SYBR Green Supermix (Bio-Rad, CA), 10 µl of diluted template, and 1.25 µl of each nuclear gene primer (ACT1 forward and reverse primers, in Table S2) or each mtDNA primer (COX1 forward and reverse primers, in Table S2). The difference [$\Delta C_{t=}$ C_t (*COX1*) – C_t (*ACT1*)] in the average Ct values of COX1 and ACT1 *was* determined to represent the relative mtDNA copy number of each strain.

4.5 Figures and tables

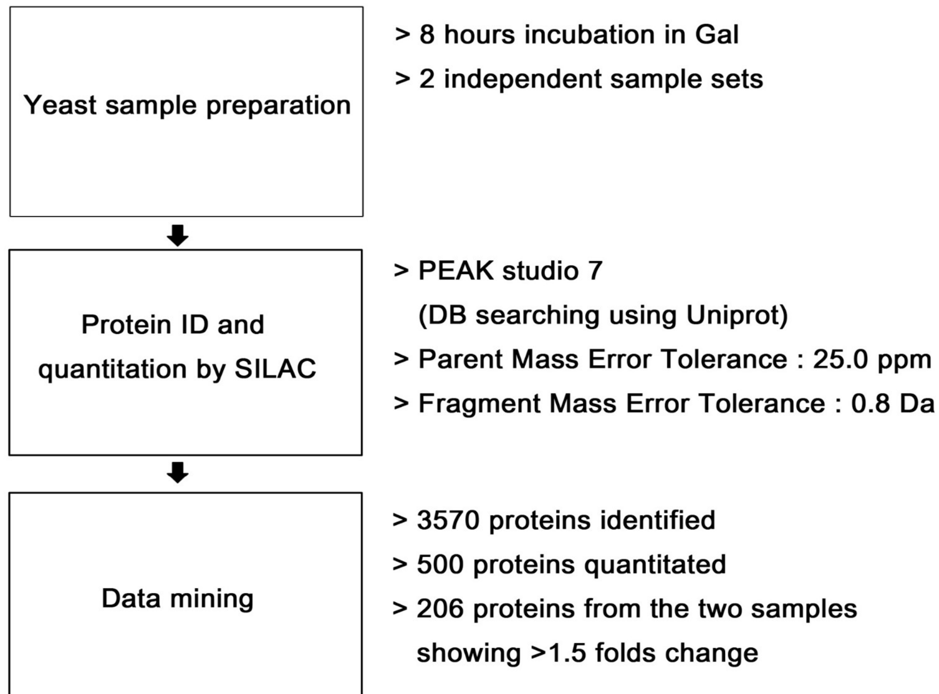


Figure 4.1 Workflow of SILAC

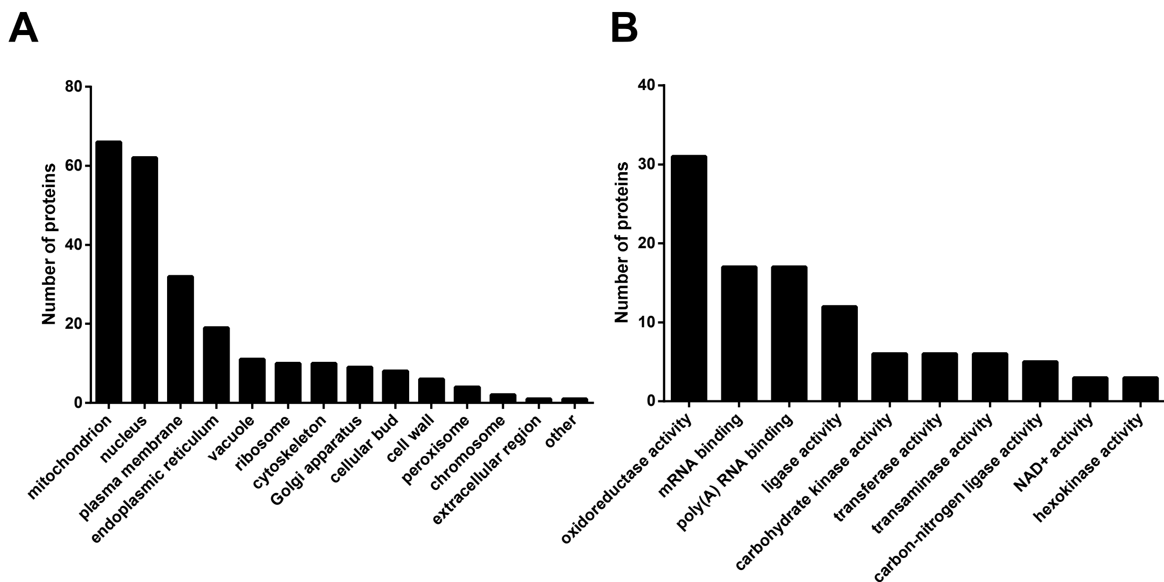


Figure 4.2 Ypc1p overexpression alters the levels of proteins related to mitochondrial functions.

Yeast cells overexpressing Ypc1p or Ypc1p-C27F were subjected to SILAC as described in Materials and Methods. **A**, Bar graph representation of GO annotation results for the cellular components. **B**, Bar graph representation of GO annotation results for the biochemical functions.

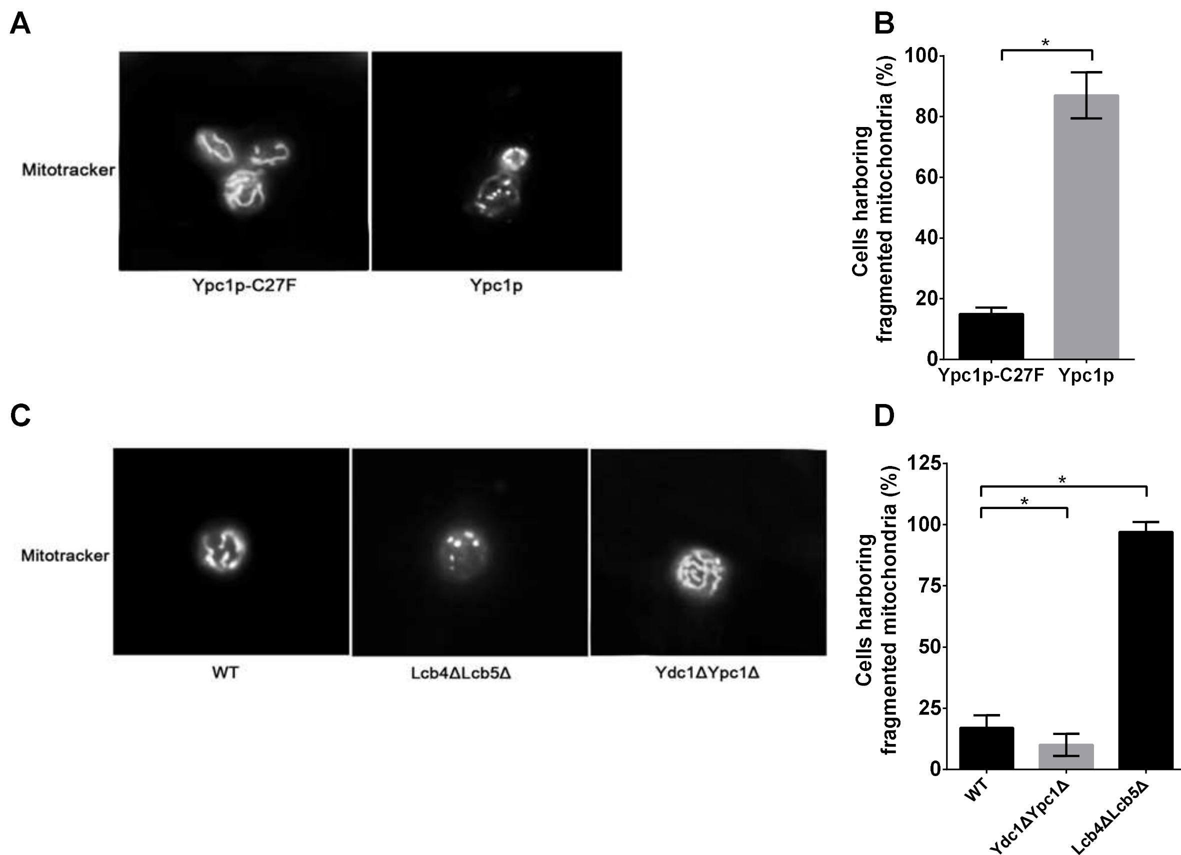


Figure 4.3 Sphingoid bases alter mitochondrial morphology.

A-D, fluorescent microscopic analyses of mitochondrial morphology. Gal1Δ overexpressing either YPC1 or Ypc1p-C27F (A), and WT, Ydc1ΔYpc1Δ, and Lcb4ΔLcb5Δ (C) were labeled with the mitochondrion-specific fluorescent dye, the mitotracker (500 nM), for 30 min before the morphology of mitochondria was observed under a fluorescent microscope as described in

Materials and Methods. The numbers of cells harboring fragmented mitochondria were counted under a fluorescence microscope and expressed as a percentage of the total numbers of cells overexpressing Ypc1p or Ypc1p-C27F (B), WT, Lcb4ΔLcb5Δ, or Ydc1ΔYpc1Δ cells (D). Data represent mean ± SD; 10 random fields with 100x magnification. $*p<0.05$

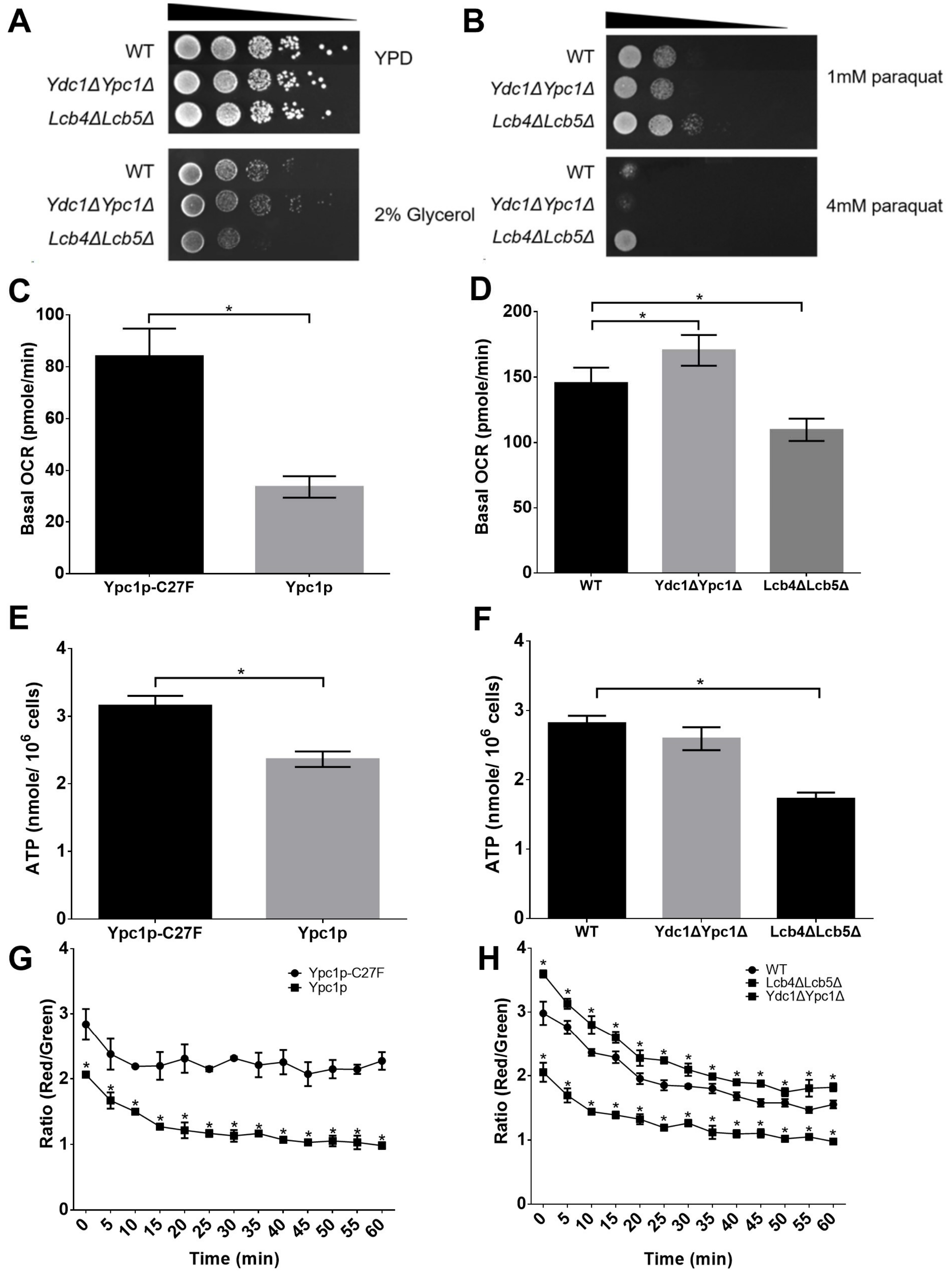

A
WT
Ydc1ΔYpc1Δ
Lcb4ΔLcb5Δ
YPD
2% Glycerol
B
1mM paraquat
4mM paraquat
C
Basal OCR (pmole/min)
Ypc1p-C27F
Ypc1p
D
WT
Ydc1ΔYpc1Δ
Lcb4ΔLcb5Δ
E
ATP (nmole/ 10^6 cells)
F
G
Ratio (Red/Green)
Time (min)
H

Figure 4.4 Sphingoid bases impair mitochondrial functions.

A, Utilization of nonfermentable carbon sources of mutant yeast cells. Serial dilutions of mid-log phase cultures containing 10^6–10^2 cells (WT, Lcb4ΔLcb5Δ, and Ydc1ΔYpc1Δ) were spotted onto YPD and YEP plates containing 2% (wt/vol) glycerol. **B**, Sensitivity to oxidative inducing-agent (paraquat) of yeast cells. Serial dilutions of mid-log phase cultures containing 10^6–10^2 cells (WT, Lcb4ΔLcb5Δ, and Ydc1ΔYpc1Δ) were spotted onto YPD plates containing either 1 mM or 4 mM paraquat. **C** and **D**, Basal oxygen consumption. **E** and **F**, intracellular ATP assays. **G** and **H**, mitochondrial membrane potential (Δψm) assays. Yeast cells overexpressing Ypc1p or Ypc1p-C27F (C, E, and G) were grown overnight (O/N) in SC medium and WT, Ydc1ΔYpc1Δ, or Lcb4ΔLcb5Δ in complete SC medium (D, F, and H) before yeast cultures were subjected to OCR assays (C and D), intracellular ATP assays (E and F), or Δψm assays (G and H) as described in Materials and Methods. Data represent mean ± SD; n=9 for the OCR assays, n=6 for the ATP assay, and n=12 for the Δψm assay. *$p<0.05$

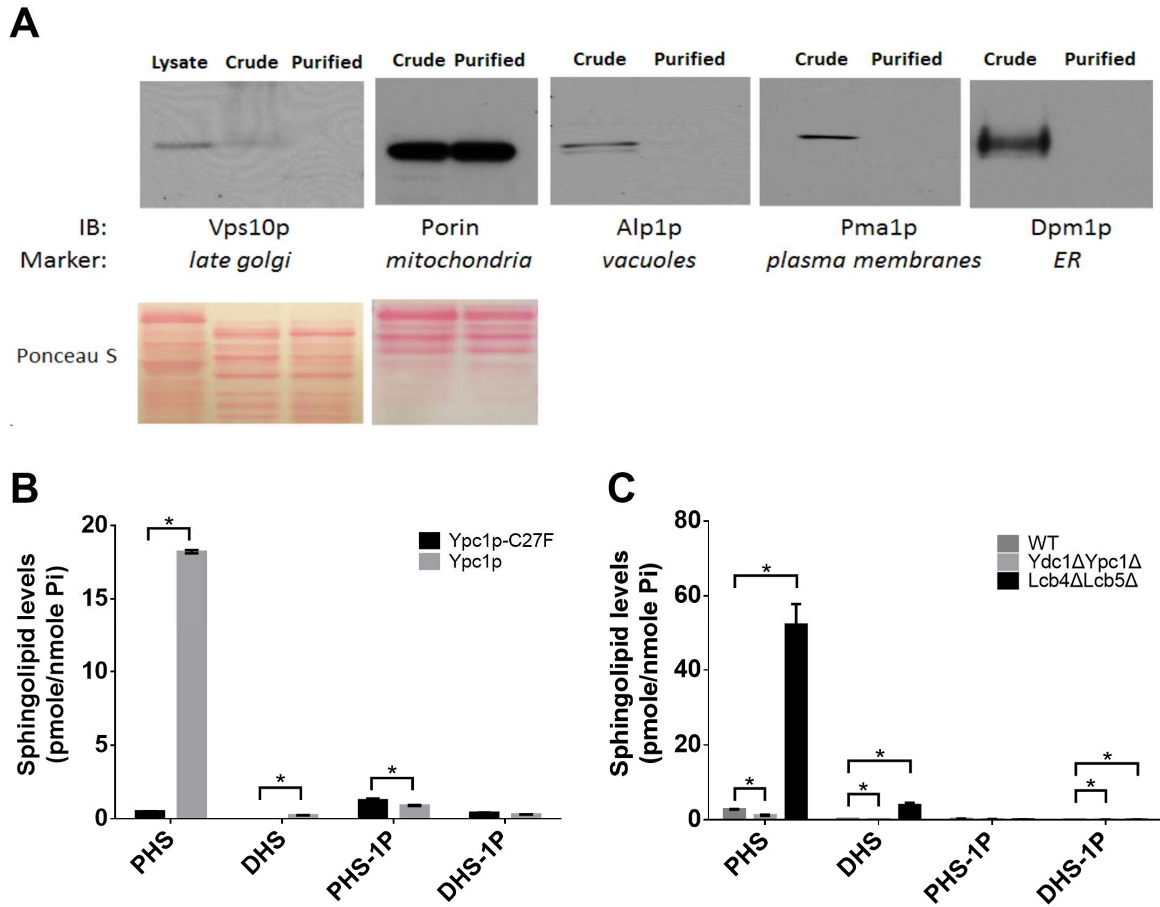


Figure 4.5 Sphingoid base levels are increased in mitochondria in aging yeast cells.

A, Isolation of mitochondria. Mitochondria were isolated from yeast cells and their purity was confirmed by Western blot analyses with antibodies against different organelle-specific proteins as described in Materials and Methods. **B** and **C**, LC-MS/MS analyses of mitochondrial SBs. LC-MS/MS was performed to determine the levels of SBs in mitochondria isolated from cells overexpressing Ypc1p or Ypc1p-C27F (B), WT, Lcb4ΔLcb5Δ, or Ydc1ΔYpc1Δ cells (C) as described in Materials and Methods. Data represent mean ± SD; n=3. *$p<0.05$

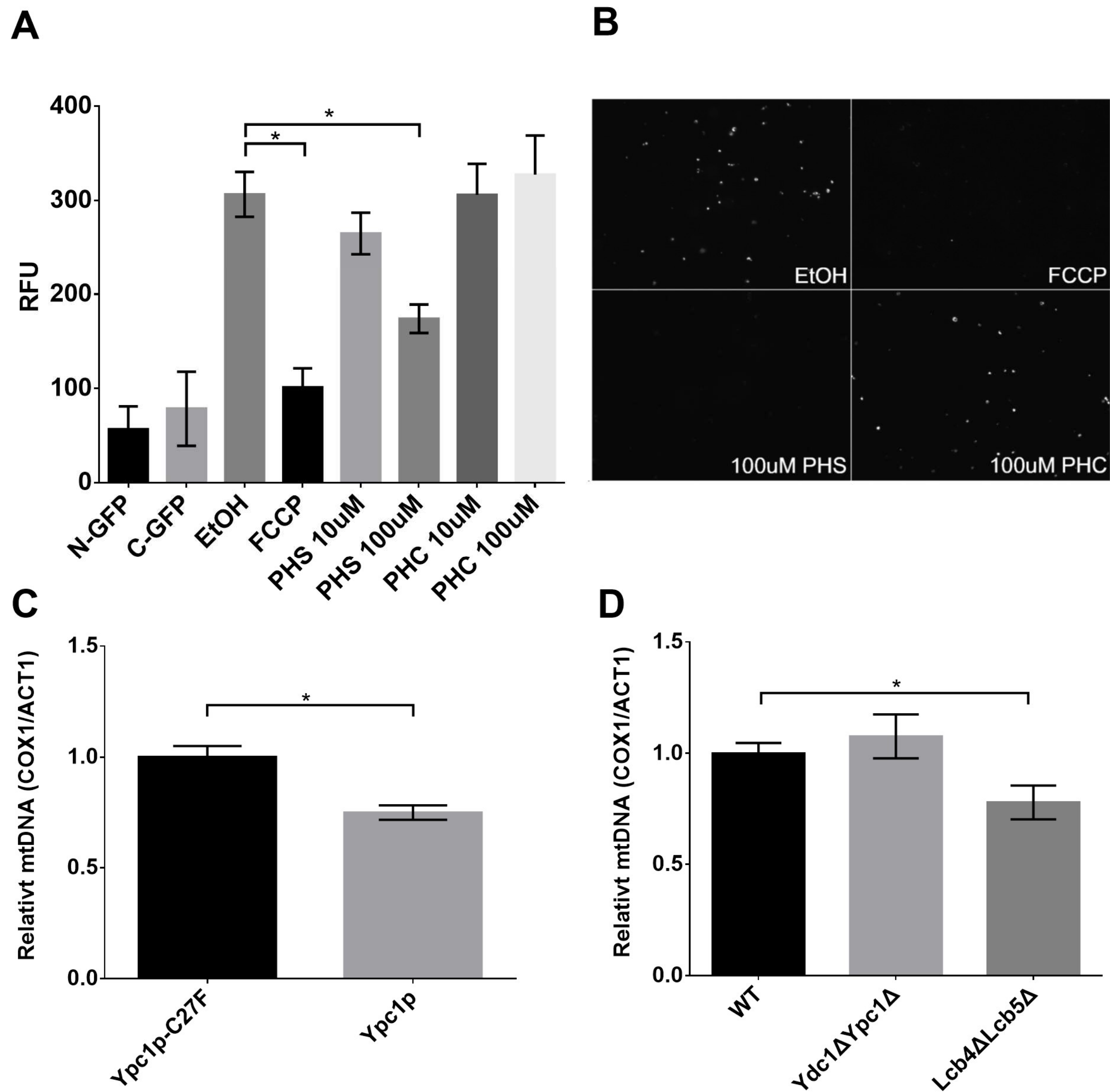


Figure 4.6 Sphingoid bases inhibit mitochondrial fusion and decrease mtDNA copy number. **A**, *in vitro* mitochondrial fusion assay. Isolated mitochondria were treated with different concentrations of PHS, and mitochondrial fusion was assayed by GFP fluorescence measurements as described in Materials and Methods. Data represent mean ± SD; n=6. *p<0.05. **B**, representative

GFP fluorescent images of mitochondrial fusion reactions. **C** and **D**, mtDNA quantification in cells overexpressing Ypc1p or Ypc1p-C27F (C), WT, Lcb4ΔLcb5Δ, or Ydc1ΔYpc1Δ cells (D). Relative mtDNA copy numbers were analyzed by a 2ΔΔCt method using RT–qPCR data. Data represent mean of ± SD; n=3. *p<0.05.

Accession	**Gene**	**Description**	**Average ratio (H:L)**
P15992	HSP26	Heat shock protein 26	5.90
P09733	TUB1	Tubulin alpha-1 chain	4.08
P38298	YPC1	Alkaline ceramidase YPC1	3.94
P53228	NQM1	Transaldolase NQM1	3.90
P32368	SAC1	Phosphoinositide phosphatase SAC1	3.20
P29952	PMI40	Mannose-6-phosphate isomerase	3.15
Q00055	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)] 1	3.14
P00330	ADH1	Alcohol dehydrogenase 1	2.84
Q04432	HSP31	Probable chaperone protein HSP31	2.63
P32602	SEC17	Alpha-soluble NSF attachment protein	2.63
P14832	CPR1	Peptidyl-prolyl cis-trans isomerase	2.59

P37291	SHM2	Serine hydroxymethyltransferase cytosolic	2.55
P07264	LEU1	3-isopropylmalate dehydratase	2.47
P32356	NTH1	Neutral trehalase	2.43
P06780	RHO1	GTP-binding protein RHO1	2.36
Q04728	ARG7	Arginine biosynthesis bifunctional protein ArgJ	2.33
Q12154	GET3	ATPase GET3	2.33
Q06151	DCS1	m7GpppX diphosphatase	2.32
P38067	UGA2	Succinate-semialdehyde dehydrogenase [NADP(+)]	2.31
P38013	AHP1	Peroxiredoxin type-2	2.29
Q12207	NCE102	Non-classical export protein 2	2.26
Q12335	PST2	Protoplast secreted protein 2	2.25
P13586	PMR1	Calcium-transporting ATPase 1	2.25
P54838	DAK1	Dihydroxyacetone kinase 1	2.21
P00950	GPM1	Phosphoglycerate mutase 1	2.16
P06169	PDC1	Pyruvate decarboxylase isozyme 1	2.16
Q12019	MDN1	Midasin	2.14
Q03558	OYE2	NADPH dehydrogenase 2	2.06

P14904	LAP4	Vacuolar aminopeptidase 1	2.05
P00358	TDH2	Glyceraldehyde-3-phosphate dehydrogenase 2	2.04
Q01976	YSA1	ADP-ribose pyrophosphatase	2.03
P25293	NAP1	Nucleosome assembly protein	1.99
Q12458	YPR1	Putative reductase 1	1.97
P38079	YRO2	Protein YRO2	1.97
P06208	LEU4	2-isopropylmalate synthase	1.95
P04385	GAL1	Galactokinase	1.94
P07267	PEP4	Saccharopepsin	1.93
P06738	GPH1	Glycogen phosphorylase	1.91
P38715	GRE3	NADPH-dependent aldose reductase GRE3	1.90
P54783	ALO1	D-arabinono-1 4-lactone oxidase	1.89
P46367	ALD4	Potassium-activated aldehyde dehydrogenase	1.87
P47018	MTC1	Maintenance of telomere capping protein 1	1.87
P12709	PGI1	Glucose-6-phosphate isomerase	1.86
P37012	PGM2	Phosphoglucomutase-2	1.85
P47143	ADO1	Adenosine kinase	1.85

P80210	ADE12	Adenylosuccinate synthetase	1.84
P41338	ERG10	Acetyl-CoA acetyltransferase	1.84
P38972	ADE6	Phosphoribosylformylglycinamidine synthase	1.83
P17255	VMA1	V-type proton ATPase catalytic subunit A	1.81
P39958	GDI1	Rab GDP-dissociation inhibitor	1.81
Q06142	KAP95	Importin subunit beta-1	1.81
P00549	CDC19	Pyruvate kinase 1	1.81
P38427	TSL1	Trehalose synthase complex regulatory subunit TSL1	1.81
Q07551	YDL124W	NADPH-dependent alpha-keto amide reductase G	1.81
P22147	XRN1	5'-3' exoribonuclease 1	1.81
P16862	PFK2	6-phosphofructokinase subunit beta	1.80
P04802	DPS1	Aspartate--tRNA ligase cytoplasmic	1.79
P00942	TPI1	Triosephosphate isomerase	1.77
P08524	ERG20	Farnesyl pyrophosphate synthase	1.77
P31539	HSP104	Heat shock protein 104	1.77
P38911	FPR3	FK506-binding nuclear protein	1.76
P41921	GLR1	Glutathione reductase	1.74

P11154	PYC1	Pyruvate carboxylase 1	1.74
Q12074	SPE3	Spermidine synthase	1.73
P14540	FBA1	Fructose-bisphosphate aldolase	1.73
P49089	ASN1	Asparagine synthetase [glutamine-hydrolyzing] 1	1.73
P25694	CDC48	Cell division control protein 48	1.71
P38625	GUA1	GMP synthase [glutamine-hydrolyzing]	1.71
P32379	PUP2	Proteasome subunit alpha type-5	1.71
P34227	PRX1	Mitochondrial peroxiredoxin PRX1	1.70
P00729	PRC1	Carboxypeptidase Y	1.69
P14742	GFA1	Glutamine--fructose-6-phosphate aminotransferase	1.68
P15496	IDI1	Isopentenyl-diphosphate Delta-isomerase	1.68
Q99383	HRP1	Nuclear polyadenylated RNA-binding protein 4	1.67
P32589	SSE1	Heat shock protein homolog SSE1	1.66
Q05016	YMR226C	Uncharacterized oxidoreductase YMR226C G	1.66
P38993	FET3	Iron transport multicopper oxidase FET3	1.65
P23724	PRE7	Proteasome subunit beta type-6	1.64
P36114	YKR018C	Mitochondrial outer membrane protein YKR018C G	1.64

P15891	ABP1	Actin-binding protein	1.64
P04840	POR1	Mitochondrial outer membrane protein porin 1	1.64
P32527	ZUO1	Zuotin	1.62
P40303	PRE6	Proteasome subunit alpha type-4	1.61
P31412	VMA5	V-type proton ATPase subunit C	1.60
P15703	BGL2	Glucan 1 3-beta-glucosidase	1.60
Q12118	SGT2	Small glutamine-rich tetratricopeptide repeat-containing protein 2	1.60
P40150	SSB2	Heat shock protein SSB2	1.60
P46956	PHO86	Inorganic phosphate transporter PHO86	1.59
P53173	ERV14	ER-derived vesicles protein ERV14	1.58
P32486	KRE6	Beta-glucan synthesis-associated protein KRE6	1.58
P53278	YGR130C	Uncharacterized protein YGR130C G	1.58
P14906	SEC63	Protein translocation protein SEC63	1.57
Q05359	ERP1	Protein ERP1	1.57
P29547	CAM1	Elongation factor 1-gamma 1	1.56
P40010	NUG1	Nuclear GTP-binding protein NUG1	1.55

P41811	SEC27	Coatomer subunit beta'	1.55
P05030	PMA1	Plasma membrane ATPase 1	1.55
Q12754	RRP12	Ribosomal RNA-processing protein 12	1.55
Q03048	COF1	Cofilin	1.54
P39730	FUN12	Eukaryotic translation initiation factor 5B	1.54
P16861	PFK1	6-phosphofructokinase subunit alpha	1.54
P39076	CCT2	T-complex protein 1 subunit beta	1.53
P04807	HXK2	Hexokinase-2	1.53
Q12532	TAE2	Translation-associated element 2	1.53
P08431	GAL7	Galactose-1-phosphate uridylyltransferase	1.52
Q08972	NEW1	[NU+] prion formation protein 1	1.51
Q01939	RPT6	26S protease regulatory subunit 8 homolog	1.51
P54113	ADE16	Bifunctional purine biosynthesis protein ADE16	1.51
P15705	STI1	Heat shock protein STI1	1.51
P17709	GLK1	Glucokinase-1	1.50
Q05567	DPL1	Sphingosine-1-phosphate lyase	1.50
P22515	UBA1	Ubiquitin-activating enzyme E1 1	1.50

P32497	NIP1	Eukaryotic translation initiation factor 3 subunit C	1.50
P07170	ADK1	Adenylate kinase	1.50
P22023	KRE5	Killer toxin-resistance protein 5	1.49
P54885	PRO2	Gamma-glutamyl phosphate reductase	1.49
P32835	GSP1	GTP-binding nuclear protein GSP1/CNR1	1.49
P32836	GSP2	GTP-binding nuclear protein GSP2/CNR2	1.49
P39993	GEA2	ARF guanine-nucleotide exchange factor 2	1.48
P22137	CHC1	Clathrin heavy chain	1.47
Q07478	SUB2	ATP-dependent RNA helicase SUB2	1.47
P25491	YDJ1	Mitochondrial protein import protein MAS5	1.47
P00445	SOD1	Superoxide dismutase [Cu-Zn]	1.46
P35732	DEF1	RNA polymerase II degradation factor 1	1.45
P24031	PHO3	Constitutive acid phosphatase	1.44
P31383	TPD3	Protein phosphatase PP2A regulatory subunit A	1.44
P19146	ARF2	ADP-ribosylation factor 2	1.44
P39683	NPT1	Nicotinate phosphoribosyltransferase	1.43
P40024	ARB1	ABC transporter ATP-binding protein ARB1	1.43

P34760	TSA1	Peroxiredoxin TSA1	1.43
Q02932	KAP120	Importin beta-like protein KAP120	1.43
P15303	SEC23	Protein transport protein SEC23	1.43
P22146	GAS1	1 3-beta-glucanosyltransferase GAS1	1.43
P38707	DED81	Asparagine--tRNA ligase cytoplasmic	1.43
P20606	SAR1	Small COPII coat GTPase SAR1	1.43
P16603	NCP1	NADPH--cytochrome P450 reductase	1.43
P47176	BAT2	Branched-chain-amino-acid aminotransferase cytosolic	1.43
P40474	QDR2	Quinidine resistance protein 2	1.42
P47037	SMC3	Structural maintenance of chromosomes protein 3	1.42
Q03940	RVB1	RuvB-like protein 1	1.42
P32352	ERG2	C-8 sterol isomerase	1.42
P36008	TEF4	Elongation factor 1-gamma 2	1.41
P40302	PRE5	Proteasome subunit alpha type-6	1.41
P27692	SPT5	Transcription elongation factor SPT5	1.41
P19524	MYO2	Myosin-2	1.41

P52593	NUP188	Nucleoporin NUP188	1.40
P25294	SIS1	Protein SIS1	1.40
Q03103	ERO1	Endoplasmic oxidoreductin-1	1.40
P32471	EFB1	Elongation factor 1-beta	1.40
P38623	RCK2	Serine/threonine-protein kinase RCK2	1.40
P40069	KAP123	Importin subunit beta-4	1.40
P19097	FAS2	Fatty acid synthase subunit alpha	1.39
P46680	AIP1	Actin-interacting protein 1	1.38
P07342	ILV2	Acetolactate synthase catalytic subunit mitochondrial	1.38
P23615	SPT6	Transcription elongation factor SPT6	1.38
P0CX25	RPL43A	60S ribosomal protein L43-A	1.37
P33401	PGM1	Phosphoglucomutase-1	1.37
P07703	RPC40	DNA-directed RNA polymerases I and III subunit RPAC1	1.37
P17555	SRV2	Adenylyl cyclase-associated protein	1.37
P32565	RPN2	26S proteasome regulatory subunit RPN2	1.36
P32337	PSE1	Importin subunit beta-3	1.36

P0CX55	RPS18A	40S ribosomal protein S18-A	1.36
Q99190	TSC13	Very-long-chain enoyl-CoA reductase	1.36
P23254	TKL1	Transketolase 1	1.36
P10081	TIF1	ATP-dependent RNA helicase eIF4A	1.36
Q02642	EGD1	Nascent polypeptide-associated complex subunit beta-1	1.35
P53691	CPR6	Peptidyl-prolyl cis-trans isomerase CPR6	1.35
P10080	SBP1	Single-stranded nucleic acid-binding protein	1.35
P28272	URA1	Dihydroorotate dehydrogenase (fumarate)	1.35
Q00955	ACC1	Acetyl-CoA carboxylase	1.35
P53914	KRE33	UPF0202 protein KRE33	1.34
Q02486	ABF2	ARS-binding factor 2 mitochondrial	1.34
Q04951	SCW10	Probable family 17 glucosidase SCW10	1.34
P36047	SDS22	Protein phosphatase 1 regulatory subunit SDS22	1.33
Q07896	NOC3	Nucleolar complex-associated protein 3	1.33
Q05905	HRI1	Protein HRI1	1.33
P41940	PSA1	Mannose-1-phosphate guanyltransferase	1.32
P33307	CSE1	Importin alpha re-exporter	1.32

P02829	HSP82	ATP-dependent molecular chaperone HSP82	1.32
P26637	CDC60	Leucine--tRNA ligase cytoplasmic	1.31
P29704	ERG9	Squalene synthase	1.31
Q03161	YMR099C	Glucose-6-phosphate 1-epimerase G	1.31
P07263	HTS1	Histidine--tRNA ligase mitochondrial	1.31
P27614	CPS1	Carboxypeptidase S	1.30
P25375	PRD1	Saccharolysin	1.30
Q12250	RPN5	26S proteasome regulatory subunit RPN5	1.30
P10592	SSA2	Heat shock protein SSA2	1.30
P15454	GUK1	Guanylate kinase	1.29
P11745	RNA1	Ran GTPase-activating protein 1	1.29
Q03690	CLU1	Clustered mitochondria protein 1	1.29
P15108	HSC82	ATP-dependent molecular chaperone HSC82	1.28
P04397	GAL10	Bifunctional protein GAL10	1.28
P40495	LYS12	Homoisocitrate dehydrogenase mitochondrial	1.28
P16521	YEF3	Elongation factor 3A	1.28
P00817	IPP1	Inorganic pyrophosphatase	1.28

P06105	SCP160	Protein SCP160	1.27
P26321	RPL5	60S ribosomal protein L5	1.27
P00925	ENO2	Enolase 2	1.27
P25087	ERG6	Sterol 24-C-methyltransferase	1.27
P02994	TEF1	Elongation factor 1-alpha	1.27
P02407	RPS17A	40S ribosomal protein S17-A	1.26
P38934	BFR1	Nuclear segregation protein BFR1	1.26
P15646	NOP1	rRNA 2'-O-methyltransferase fibrillarin	1.26
P35691	TMA19	Translationally-controlled tumor protein homolog	1.26
P36015	YKT6	Synaptobrevin homolog YKT6	1.26
P07260	CDC33	Eukaryotic translation initiation factor 4E	1.25
P36016	LHS1	Heat shock protein 70 homolog LHS1	1.25
P32324	EFT1	Elongation factor 2	1.25
P46654	RPS0B	40S ribosomal protein S0-B	1.25
P11484	SSB1	Heat shock protein SSB1	1.25
P41807	VMA13	V-type proton ATPase subunit H	1.25
P53731	ARC35	Actin-related protein 2/3 complex subunit 2	1.24

P33775	PMT1	Dolichyl-phosphate-mannose--protein mannosyltransferase 1	1.23
P32473	PDB1	Pyruvate dehydrogenase E1 component subunit beta	1.23
P15180	KRS1	Lysine--tRNA ligase cytoplasmic	1.23
P09436	ILS1	Isoleucine--tRNA ligase cytoplasmic	1.22
P47079	CCT8	T-complex protein 1 subunit theta	1.22
Q12117	MRH1	Protein MRH1	1.22
P0CX36	RPS4B	40S ribosomal protein S4-B	1.22
P05759	RPS31	Ubiquitin-40S ribosomal protein S31	1.22
P53064	RTF1	RNA polymerase-associated protein RTF1	1.21
P29509	TRR1	Thioredoxin reductase 1	1.21
Q12466	TCB1	Tricalbin-1	1.20
P0CX39	RPS8A	40S ribosomal protein S8-A	1.20
Q03532	HAS1	ATP-dependent RNA helicase HAS1	1.19
P34160	STO1	Nuclear cap-binding protein complex subunit 1	1.19
Q08179	MDM38	Mitochondrial distribution and morphology protein 38	1.19
P38817	GGA2	ADP-ribosylation factor-binding protein GGA2	1.19

P05737	RPL7A	60S ribosomal protein L7-A	1.18
P36060	MCR1	NADH-cytochrome b5 reductase 2	1.18
P52918	MSN5	Protein MSN5	1.18
P07284	SES1	Serine--tRNA ligase cytoplasmic	1.18
P0CX54	RPL12B	60S ribosomal protein L12-B	1.18
P14020	DPM1	Dolichol-phosphate mannosyltransferase	1.17
P31373	CYS3	Cystathionine gamma-lyase	1.17
P20967	KGD1	2-oxoglutarate dehydrogenase mitochondrial	1.17
Q12447	PAA1	Polyamine N-acetyltransferase 1	1.17
P27476	NSR1	Nuclear localization sequence-binding protein	1.17
Q08977	YPL260W	UPF0662 protein YPL260W G	1.17
P0CX83	RPL19B	60S ribosomal protein L19-B	1.17
P41805	RPL10	60S ribosomal protein L10	1.16
P39077	CCT3	T-complex protein 1 subunit gamma	1.15
P23248	RPS1B	40S ribosomal protein S1-B	1.15
P0CX48	RPS11B	40S ribosomal protein S11-B	1.15
P26785	RPL16B	60S ribosomal protein L16-B	1.14

P0CX42	RPL23B	60S ribosomal protein L23-B	1.13
P28777	ARO2	Chorismate synthase	1.12
P39078	CCT4	T-complex protein 1 subunit delta	1.12
P05317	RPP0	60S acidic ribosomal protein P0	1.12
P09457	ATP5	ATP synthase subunit 5 mitochondrial	1.12
P15019	TAL1	Transaldolase	1.11
P0CX52	RPS16B	40S ribosomal protein S16-B	1.11
P33442	RPS1A	40S ribosomal protein S1-A	1.10
Q02753	PL21A	60S ribosomal protein L21-A	1.09
P33322	CBF5	H/ACA ribonucleoprotein complex subunit 4	1.09
P05626	ATP4	ATP synthase subunit 4 mitochondrial	1.09
P40212	RPL13B	60S ribosomal protein L13-B	1.08
P38328	ARC40	Actin-related protein 2/3 complex subunit 1	1.07
P0CX46	RPL2B	60S ribosomal protein L2-B	1.06
P38687	SRP68	Signal recognition particle subunit SRP68	1.06
Q06252	YLR179C	Uncharacterized protein YLR179C G	1.06

P28241	IDH2	Isocitrate dehydrogenase [NAD] subunit 2 mitochondrial	1.06
P0CX49	RPL18A	60S ribosomal protein L18-A	1.05
P07280	RPS19A	40S ribosomal protein S19-A	1.05
P26783	RPS5	40S ribosomal protein S5	1.03
P0CS90	SSC1	Heat shock protein SSC1 mitochondrial	0.96
P14126	RPL3	60S ribosomal protein L3	0.96
P25443	RPS2	40S ribosomal protein S2	0.96
P0CX29	RPS23A	40S ribosomal protein S23-A	0.94
P15873	POL30	Proliferating cell nuclear antigen	0.93
P18239	PET9	ADP ATP carrier protein 2	0.93
P02406	RPL28	60S ribosomal protein L28	0.92
P05373	HEM2	Delta-aminolevulinic acid dehydratase	0.92
P42943	CCT7	T-complex protein 1 subunit eta	0.89
P0C0W1	RPS22A	40S ribosomal protein S22-A	0.87
Q3E7Y3	RPS22B	40S ribosomal protein S22-B	0.87
P00128	QCR7	Cytochrome b-c1 complex subunit 7	0.87

P32191	GUT2	Glycerol-3-phosphate dehydrogenase mitochondrial	0.86
P19414	ACO1	Aconitate hydratase mitochondrial	0.86
Q00711	SDH1	Succinate dehydrogenase flavoprotein subunit mitochondrial	0.85
Q12680	GLT1	Glutamate synthase [NADH]	0.84
P40215	NDE1	External NADH-ubiquinone oxidoreductase 1 mitochondrial	0.83
P36010	YNK1	Nucleoside diphosphate kinase	0.83
P05375	OPI3	Phosphatidyl-N-methylethanolamine N-methyltransferase	0.80
P07143	CYT1	Cytochrome c1 heme protein mitochondrial	0.78
P23776	EXG1	Glucan 1 3-beta-glucosidase I/II	0.78
P37303	GLY1	Low specificity L-threonine aldolase	0.76
P47120	LIA1	Deoxyhypusine hydroxylase	0.76
P10659	SAM1	S-adenosylmethionine synthase 1	0.69
P25555	GBP2	Single-strand telomeric DNA-binding protein GBP2	0.65
P21801	SDH2	Succinate dehydrogenase iron-sulfur subunit mitochondrial	0.58

P39105	PLB1	Lysophospholipase 1	0.51
P32340	NDI1	Rotenone-insensitive NADH-ubiquinone oxidoreductase	0.50
P39676	YHB1	Flavohemoprotein	0.45
Q12512	ZPS1	Protein ZPS1	0.45
P32468	CDC12	Cell division control protein 12	0.26
P06106	MET17	Protein MET17	0.18
P32804	ZRT1	Zinc-regulated transporter 1	0.11

Table 4.1 Expression ratio of proteins in yeast cells in response to YPC1 overexpression (SILAC).

(H, Ypc1p-overexpression. L, Ypc1p-C27F overexpression.)

Chapter 5. Discussion and future direction

5.1 Introduction

Sphingolipid metabolites, such as ceramides and SBs, have shown to act as bioactive lipids to accelerate aging. However, much remains unclear about how these sphingolipids and their metabolizing enzymes are regulated during aging processes. In this study, I for the first time demonstrate that SBs are markedly increased in yeast cells during the course of nutrient deprivation, a stress that induces aging, due to an inverse regulation of their producing enzymes (the alkaline ceramidases, YPC1 and YDC1) and their degrading enzymes (the SB kinases, LCB4 and LCB5). More importantly, this study reveals that increased SBs shorten yeast CLS by impairing both the structural integrity and functions of mitochondria, thus, playing a key role in mediating yeast aging.

5.2 Models for the aging study

Aging is an inherently complex process that is manifested by an organism at genetic, molecular, cellular, organ, and system levels. There are three important aspects of the aging process: 1) a continuous decline in biological functions over time, 2) a decreased resistance to multiple forms of stress, and 3) an increased susceptibility to numerous diseases (Harman 1956). For example, aging is known to promote various physiological phenomena related to a reduction in the number of cellular tissues as well as body fluid, a decrease of metabolic rate, and loss of biological adaptability (Kregel and Zhang 2007). Although a number of studies have been carried out to determine the causes of aging, much remains unclear about the mechanisms that cause aging.

The budding yeast, *Saccharomyces cerevisiae,* has been used as a model system to study the aging process because of its short lifespan, the ease of culture, its susceptibility to genetic manipulation, and limited numbers of budding (dividing) (Gershon and Gershon 2000). The metabolism of sphingolipids and their roles in regulating biological processes are well conserved between yeast and mammals (Mao, Xu *et al.* 2000).

Furthermore, increasing studies suggest that regulation of the metabolism of sphingolipids may affect aging in the yeast system. LAG1 (longevity assurance gene) was the first yeast longevity gene cloned in 1994 (D'Mello N *et al.* 1994). This study found that the transcriptional level of LAG1 decreased with the replicative age of yeast cells and that deletion of LAG1 extended replicative life span, suggesting that LAG1 may limit yeast longevity. However, the biochemical function of LAG1 was elusive until Brandwagt *et al*. revealed that LAG1 is a ceramide synthase that synthesizes ceramides from fatty acyl-CoAs and LCBs (DHS or PHS) (Brandwagt *et al.* 2000). These observations indicate that ceramides or their derivatives may have a role in regulating aging process in yeast cells. The yeast ISC1 gene was also known to regulate the yeast life span. ISC1 encodes an inositol phosphosphingolipid phospholipase C, an ortholog of mammalian neutral sphingomyelinase-2 that hydrolyzes complex sphingolipids into ceramides (Sawai *et al.* 2000). Cells deficient in ISC1 showed a dramatic decline in CLS (Almeida, Marques *et al.* 2008). Furthermore, it was recently demonstrated that downregulation of LCB1, an essential subunit of the yeast serine palmitoyltransferase (SPT) catalyzing the first step of sphingolipid biosynthesis, can increase the yeast lifespan (Liu, Huang *et al.* 2013). Aerts et al. demonstrated that overexpression of Ydc1, which catalyzes the hydrolysis of dihydroceramide into DHS, shortened CLS (Aerts, Zabrocki *et al.* 2008). These results suggest that sphingolipids may play an important role in regulating yeast aging.

In the current study, the yeast system was utilized to test the effect of SBs on the aging process. Although the sphingolipid metabolism is well conserved between human and yeast and yeast is a well-established aging study model, it is still required to verify the result using higher models such as mammalian cells and animal models.

However, there are a couple of issues to address to use other models to study the aging process. First, there are no standards, to my knowledge, of cell culture system to study the aging process. It has been shown that ceramides are increased and promote the histochemical senescence marker in aging human fibroblasts (Mouton and Venable 2000). In addition, long-chain hexosylceramides and lactosylceramides were also accumulated in cultured human cells and their accumulation was prevented by the caloric restriction (Hernandez-Corbacho, Jenkins *et al.* 2011). However, the cell culture system might be closer to a model of cell mechanisms that shows only age-associated changes. Second, animal models are good but not enough to study human aging. The discovery that fruit flies, roundworms, and rodent models carry genes that affect their longevity is exciting, particularly because many of those genes have human counterparts (Mitchell *et al.* 2015). However, the fact remains that the complexity of human physiology cannot be replicated in simpler organisms because aging is a complex process that may consist of both environmentally stimulated and inherently programmed components.

5.3 Accumulation of sphingoid bases during the aging process

In the present study, I show that SBs are persistently accumulated in yeast cells during aging process due to an inverse regulation of Ypc1p and Lcb4p, the enzymes responsible for the generation and degradation of SBs, respectively. More importantly, we further demonstrate that increased SBs shorten CLS by compromising the structural integrity and functions of mitochondria.

This study strongly suggests that SBs and their metabolizing enzymes are important regulators of aging of yeast.

SBs are well known to be increased in response to various stress including the carbon source depletion (Alvarez-Vasquez, Sims *et al.* 2007) and heat stress (Meier, Deloche *et al.* 2006). In the current study, SBs were genetically increased by using either YPC1 overexpression or LCB4/5 double deletion. However, the amount of increased SBs by the genetic modification was much higher than physiologic SB levels up-regulated by various stresses (Alvarez-Vasquez, Sims *et al.* 2007). Although my study definitely indicates that SBs shortens the yeast CLS, the usage of SB levels adjusted to the physiological level is still required to better understand how SBs affect yeast aging.

5.4 mRNA levels of sphingolipid enzymes

Furthermore, the activity of limited numbers of sphingolipid enzymes was tested in the current study. For other sphingolipid enzymes, only mRNA levels were measured to determine if they affect SBs levels during the aging process. A possible way to improve is a quantification of protein levels of various sphingolipid enzymes in yeast strains. Using a TAP (Tandem affinity purification) tagging method (Ghaemmaghami *et al.* 2003), it will be possible for me to detect the expression levels of sphingolipid enzymes by Western blot using TAG antibody. Then, the expression levels of each enzyme would be compared to the expression of a control protein such as tubulin and actin.

5.5 Two lifespans of yeast cells

There are two concepts to test the lifespan of yeast cells, CLS, and RLS. Both were designed to provide amenable systems to develop hypotheses about aging and, at least at the level of identifying conserved genes between human and yeast. Interestingly, aging gene identification has been successfully linked to the counterparts of mammals. However, mechanisms driving aging has proven more difficult (Smart 2002, Longo *et al.* 2012).

In the current study, CLS was chosen to study the yeast aging because the CLS methods are relatively simple and easier to be applied to the neuron cells which are not dividing. There are at least three established methods to measure CLS: 1) monitoring survival of cells grown in 2% glucose medium and maintained in the medium modified by the cells during the growth, and post-diauxic phases, 2) monitoring survival of cells grown and maintained in the medium described above but switched to water during the post-diauxic phase, and 3) monitoring survival on 2% glucose agar plates. I think multiple methods should be used to confirm results because there are a number of potential artifacts that can affect the results obtained by CLS including media acidification, and cell re-growth.

Another important aspect is that many genes and components of pathways affect both RLS and CLS. The genes have conserved orthologues or analogs in higher eukaryotes, such as Tor/Sch9, and Ras/PKA as well as many downstream factors (Yan *et al.* 2007, Enns *et al.* 2010). Many studies also showed the strong correlation between sphingolipid and RLS (Guillas *et al.* 2001, Schorling, Vallee *et al.* 2001, Jiang *et al.* 2004). For the reasons, testing RLS is still necessary to verify the results presented in this study. The concept and method of both CLS and RLS are described below.

CLS (chronological lifespan):

CLS is the length of time that a non-dividing yeast cell survives. CLS is typically measured by growing a culture of yeast cells into the post-diauxic state, following which most cells exit the cell cycle. The post-diauxic phase is the period that begins approximately 24 hours after initial inoculation when cells deplete extracellular glucose, dramatically reduce growth and switch to a mitochondrial respiratory mode of metabolism dependent on the ethanol generated during fermentation (WernerWashburne *et al.* 1996). Because changes in the medium, conditions, or techniques can have effects on chronological survival of cells, it is important to choose the appropriate methods to measure CLS. There are at least three established methods to measure CLS including cell incubation in 2% glucose SDC, in water and in buffered media. Following incubation in the medium, the cell cultures will be plated on YPD plates to count colony forming units (CFU), which represents the cell survival rate.

RLS (replicative lifespan):

The assay takes advantage of the fact that yeast cells divide by asymmetric budding, with the daughter cell that is produced being smaller than the mother from which it is derived. The question to be addressed by this method is "how many times can one cell divide". RLS analysis is typically performed by a manual separation of daughter cells from their mother cells by using a standard tetrad dissection microscope equipped with a micromanipulator. Additionally, there is another option to do RLS analysis. The budding of each daughter cell usually leaves a ring-shaped deposit, named the bud scar, on the cell wall of the mother cell because the neck of buds forms them. The rings can be stained with calcofluor, a fluorescent dye. The exact number of times an individual

mother cell has undergone division can thus be determined by counting the number of bud scars present.

5.6 Effects of sphingoid bases on mitochondria

Mitochondrial functions and morphology are associated with aging. Growing evidence suggests that SBs affect the mitochondrial structural integrity and function. In mammalian cells, SPH was shown to increase the permeability of mitochondria, resulting in the release of cytochrome C from mitochondria in the breast cancer cell line, which in turn activates the intrinsic pathway of apoptosis (Cuvillier, Nava *et al.* 2001). In plant cells, treatment with DHS reduced the number of mitochondrial cristae while increasing ROS levels, resulting in apoptosis (Saucedo-Garcia *et al.* 2011). It was also demonstrated that a peptide, OSIP108, has a preventive role from an interruption of mitochondrial ultrastructure by ROS and its preventive role was abolished by exogenous DHS (Spincemaille *et al.* 2014).

In the present study, it was also demonstrated that SB levels elevated by either YPC1 overexpression or LCB4/5 double deletion inhibited mitochondrial fusion and caused fragmentation of the mitochondrial tubular network, resulting in decreases in mtDNA copy numbers, ATP synthesis, mitochondrial membrane potential, and oxygen consumption rates. The result indicates SBs compromise the mitochondrial functions. In addition, mitochondrial fusion plays critical roles in maintaining functional mitochondria (Youle and van der Bliek 2012). Because the present study showed both mitochondrial dysfunction and mitochondrial fusion inhibition, it is still required to determine the causality between them.

Interestingly, the mitochondrial fusion assay in the current study showed that only PHS inhibits the mitochondrial fusion, not PHC. Ceramides are known to promote the formation of channels in the outer membrane of the mitochondria to initiate the release of cytochrome c (Siskind, Kolesnick *et al.* 2002). C16:0-PHC was reported to have a 2-fold higher ability to permeabilize the human mitochondrial membrane as compared to ceramide (Perera *et al.* 2012). There are two possible explanations for the observation. First, mitochondrial membrane permeabilization may facilitate the fusion process because membrane fusion events typically utilize pore-forming domains to initiate the melding of lipid bilayers (Cleland and Youle 2011). Another explanation comes from an observation that mitochondria isolated from yeast cells are significantly enriched in PHC (21.7 fold) relative to the whole cell. Mitochondria, on the other hand, were significantly depleted in SBs (Kitagaki *et al.* 2007). For the reason, highly enriched PHC might attenuate the effect of exogenous PHC on the mitochondrial fusion whereas depleted SBs could amplify the SB effect.

5.7 Another mechanism by which sphingoid bases affect the aging process

Based on the SILAC experiment, levels of the mRNA binding proteins are found to be altered upon the YPC1 overexpression. In many cases, mRNA binding proteins regulate mRNA turnover and/or translation (TTR-RBPs), which subsequently affects the aging process (Masuda *et al.* 2009). Interestingly, it was reported that mRNA binding proteins that are regulators of gene expression at the posttranscriptional level affect the yeast lifespan (Orozco *et al.* 2013). Although there are few studies on the proteins which were identified by SILAC, it would be intriguing to study the association between those proteins and the yeast aging process.

Additionally, several proteins identified by SILAC are related to autophagy. In yeast, autophagy is critical for survival during nutrient deprivation, as it enables recycling of macromolecules to

provide new nutrients and energy (Martinez-Lopez *et al.* 2015). Because yeast has LC3 homolog, Atg8p (Ichimura *et al.* 2004), the autophagy study might be started with checking Atg8p localizations upon the increase of SBs.

5.8 Conclusion

In conclusion, the data presented in this study strongly suggests that increased SBs due to the inverse regulation of their producing enzymes YPC1 and their degrading enzymes LCB4 and LCB5 shortens yeast CLS by compromising the mitochondrial fusion and functions. This important finding may facilitate our understanding of the role and mechanism of the action of sphingolipids in physiological and pathological aging of humans and other mammals.

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