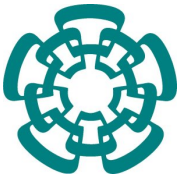

**CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL**

**UNIDAD DE GENÓMICA AVANZADA**

# Systematic analysis of mitochondrial-autophagy activity in yeast chronological-life span genetic factors

Tesis que presenta

**IBT. Dávila Alemán Francisco Daniel**

Para obtener el grado de

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En la especialidad de

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**Director de tesis**
**Dr. Alexander de Luna Fors**

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## Table of Contents

**Abbreviations**

| | |
|---|---|
| 8-oxodG | 7,8-dihydro-8-oxo-2´-deoxyguanosine |
| ADP | Adenosine diphosphate |
| ALS | Amyotrophic lateral sclerosis |
| ATG | Autophagy related |
| ATP | Adenosine triphosphate |
| CAT | Catalase |
| CCCP | Carbonyl cyanide m-chlorophenyl hydrazone |
| CLR | Chronological life span |
| CMA | Chaperone mediated autophagy |
| CuSOD | Copper superoxide dismutase |
| ER | Endoplasmic reticulum |
| ETC | Electron transport chain |
| FAD | Flavin adenine dinucleotide |
| FQR | Ferredoxin quinone reductase |
| GFP | Green fluorescent protein |
| GPDH | Glycerol-3-phosphate dehydrogenase |
| GPX | Glutathione peroxidase |
| GR | Glutathione reductase |
| GTP | Guanosine triphosphate |
| $H_2O_2$ | Hydrogen peroxide |
| MA | Mitochondrial autophagy |
| MAPK | Mitogen-activated protein kinase |
| Mbp | Mega base pair |
| MDV | Mitochondrial derived vesicles |
| MnSOD | Manganese superoxide dismutase |
| mtDNA | Mitochondrial deoxyribonucleic acid |
| mtUPR | Mitochondrial unfolded protein response |
| NAD | Nicotinamide adenine dinucleotide |
| NO | Nitric oxide |
| $O_2$.- | Superoxide anion |
| OGDH | 2-oxoglutarate dehydrogenase |
| ONOOO- | Peroxynitrite |
| $^{o}$OH | Hydroxyl radical |
| OXPHOS | Oxidative phosphorylation |
| PAS | Phagophore assembly site |
| PDH | Pyruvate dehydrogenase |
| PKA | Protein kinase A |

| | |
|---|---|
| QC | Quality control |
| RLS | Replicative life span |
| ROS | Reactive oxygen species |
| SCG | Synthetic complete glucose |
| SCL | Synthetic complete lactate |
| SEP | Superecliptic pHluorin |
| SGA | Synthetic genetic array |
| TCA | Tricarboxylic acid |
| TOR | Target of rapamycin |
| WT | Wild type |
| YAFC | Yeast aging factors collection |
| ZnSOD | Zinc superoxide dismutase |

**Abstract**

Aging is a multifactorial process caused by the progressive accumulation of molecular damage. Mitochondrial dysfunction is one of the hallmarks that promote such cellular damage in aged cells, underlying the development of several age-related diseases. Cells can selectively degrade dysfunctional mitochondria through autophagy for promoting cellular homeostasis and longevity. However, the genetic connection between longevity and mitochondrial autophagy is poorly understood. In order to identify the genetic factors involved in life span determination that affect mitochondrial autophagy, a dual autophagy biosensor was generated and used for measuring selective mitochondrial degradation by implementing a novel high-throughput flow cytometry approach. In this manner, over ~500 single-knockout yeast strains with altered life span phenotype and the genes *UBC4* and *SWC5* were identified as possible novel molecular factors involved in selective mitochondrial autophagy. This suggests that the maintenance of mitochondrial homeostasis is controlled by the ubiquitin-proteasome system and the Swr1 chromatin-remodeling complex. Using this novel high-throughput approach, we can elucidate the genetics behind the quality control systems that protect mitochondria during the aging process.

**Resumen**

El proceso de envejecimiento se caracteriza por el acumulamiento progresivo de daño celular a través del tiempo de vida de un organismo. La pérdida de la función mitocondrial es una de las principales causas que promueven el daño celular durante el proceso de envejecimiento, dando paso al desarrollo de enfermedades degenerativas. Las células pueden degradar las mitocondrias disfuncionales a través del proceso de autofagia, promoviendo la homeostasis celular y la longevidad. Sin embargo, se conoce muy poco acerca de la conexión genética entre la determinación de la longevidad y la degradación de mitocondrias por autofagia. Con este propósito, generé un biosensor de autofagia para la medición de la degradación mitocondrial en más de 500 mutantes de levadura con esperanza de vida alterada, implementando una nueva metodología en base a citometría de flujo a gran escala. Se logró identificar a *UBC4* y *SWC5* como posibles factores genéticos involucrados en la actividad de autofagia de mitocondrias, sugiriendo que el mantenimiento de la homeostasis mitocondrial se encuentra controlado por el sistema de degradación por ubiquitinación-proteasoma y el complejo de remodelación de cromatina Swr1. La implementación de esta nueva metodología desarrollada podrá contribuir al completo entendimiento de los mecanismos moleculares involucrados en el proceso de control de calidad que protegen a la mitocondria a través del proceso de envejecimiento celular.

## Introduction

*Mitochondria: A multifaceted organelle of the cell.*

Mitochondria is a fundamental cellular organelle that plays an essential role in numerous cellular processes such as apoptosis signaling cascade (Parsons & Green, 2010), intracellular homeostasis sensing, viral infection response (Galluzi et al., 2012), $Ca^{2+}$ and copper homeostasis, biosynthesis of iron-sulfur clusters, heme, lipids, steroid hormones and amino acid neurotransmitters, and is implicated in ß-oxidations of fatty acids (Nunnari & Suomalainen, 2012). However, cellular energy production, in the form of adenosine triphosphate (ATP), is one of the primary mitochondrial function (Sazanov, 2015; Lasserre et al., 2015). Importantly, mitochondria possess their own genetic material (mtDNA) that encodes structural mitochondrial proteins necessary for ATP production and RNA molecules required for protein translation (Cui et al., 2011).

The cellular energy production comprises three key metabolic pathways: glycolysis, the tricarboxylic acid (TCA) cycle (also knows as citric-acid cycle or Kreb´s cycle), and oxidative phosphorylation (OXPHOS) (Bierly, 2013). The energy metabolism starts via glycolysis which breaks down glucose into pyruvate, with the generation of two molecules of ATP and NADH (Cooper, 2000). Under aerobic conditions, pyruvate is transported to the mitochondrial matrix, where it is oxidatively decarboxylated, reducing one molecule of NAD+ to NADH and producing Acetyl-CoA (Berg, 2002).

Acetyl-CoA enters the TCA cycle and generates one molecule of GTP, three of NADH and one $FADH_2$, energy shuttles that transport and deliver high-energy electrons into the electron transport chain (Cooper, 2000). Finally, during OXPHOS, the electrons of $FADH_2$ and NADH are transferred to the Electron Transport Chain (ETC), producing the reduction of the final acceptor $O^2$ to $H_2O$. The electrons move through the inner mitochondrial membrane components of the ETC (Complexes I, III and IV), generating a proton gradient resulting in a chemiosmotic potential, which leads the production of 30+ ATP molecules by ADP phosphorylation via ATP synthase (Cooper, 2000; Bratic & Trifunovic, 2010).

*Cellular damage by mitochondria: Reactive Oxygen species production.*

Although the energy metabolism process through OXPHOS is proficient, electrons can escape from the ETC pathway. As consequence, electrons can reduce oxygen ($O_2$) to generate highly reactive free radicals named reactive oxygen species (ROS) (e.g. superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (OH.), and hydrogen peroxide ($H_2O_2$)). These molecules eventually induce a systematic oxidative stress, capable of damaging different cellular components such as DNA, Proteins, and Lipids (Bratic & Trifunovic, 2010).

Mitochondrial ROS production is considered an intrinsic side-effect of the standard cell energy metabolism. Mitochondria is then a fundamental organelle, essential for life, but is also involved in the degenerative process, promoting aging and leading the development of age-related diseases (Venditti et al., 2013). Nevertheless, even if ROS is primarily related to cellular damage; these molecules are also important in redox signaling and cellular homeostasis (Murphy, 2009).

The electrons escaping from the Complexes I and III of the ETC represent the major sites of ROS generation. This is due to the significant changes in the redox potential of these reactive complexes, which are determinant for the reduction of Oxygen and the formation of the highly reactive free radical superoxide anion ($O_2^{\cdot-}$) (Balaban et al., 2005) (Figure 1).

Even though the mitochondrial ETC is the main ROS production site (~90% of the total ROS present in the cell) (Bratic & Trifunovic, 2010), ROS can also be generated in other cellular organelles (e.g. ER, as part of the misfolded protein response; and peroxisomes, during the metabolism of long-chain fatty acids) and as products of several metabolic enzyme reactions (e.g. NADPH oxidase, lipoxygenase, Nitric oxide synthase, OGDH, PDH, GPDM and FQR) (Holmström & Finkel, 2014).

To cope with the continuous generation of ROS, cells are well equipped with multiple molecular pathways that counteract the deleterious effects of ROS accumulation. Manganese Superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX), are the major cytosolic and mitochondrial enzymatic

defense mechanisms to transform ROS in water. Besides, molecules such as pyruvate, flavonoids, ascorbate, glutathione, and carotenoids can also protect the cell against the damaging effects of ROS in non-enzymatic defense mechanisms (Cui et al., 2011).

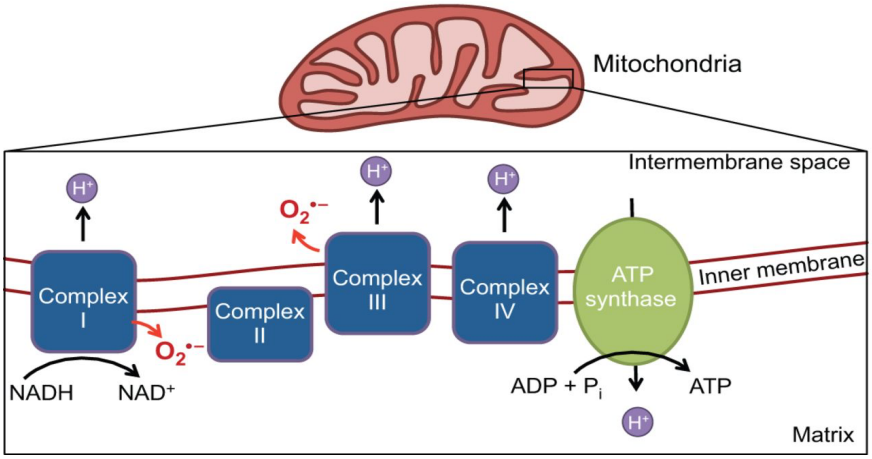


Figure 1 - OXPHOS simplified scheme and ROS generation. NADH and $FADH_2$ transfer electrons to the ETC to pump protons via Complexes I, III, and IV to generate ATP via ATP synthase. Complexes I and III could release electrons and generate Superoxide ($O_2$-) that could damage mtDNA, proteins, and lipids (reproduced from Bigarella et al., 2014).

*Mitochondrial function declines with age: The damage of mtDNA by ROS*

A report by Lu et al. (1999) demonstrated that even though cells possess a broad system to struggle with the oxidative damage produced by ROS, the activity of this enzymatic protecting system declines progressively during the aging process. Thus, producing an imbalance between ROS production and ROS elimination, causing the accumulation of ROS and triggering a systematic oxidative damage in the cell (Wei & Lee, 2002; Wei & Lee, 2007).

Without the proper control of ROS levels inside the cell, these free radicals can react with nucleic-acid molecules causing severe oxidative damage (oxidized DNA bases, abasic sites and, more critical, break of the DNA strands) which leads to deregulation in transcriptional profiles, gene silencing, and genomic instability (Dizdaroglu & Jaruga, 2011).

One of the main oxidative damage caused by ROS in DNA is the purine product 7,8-dihydro-8-oxo-2´-deoxyguanosine (8-oxodG). 8-oxodG can cause G:T transversions during replication of DNA. Interestingly, a previous report revealed that 8-oxdG is highly detected in mtDNA, meaning that mtDNA is highly affected by oxidative damage by ROS (Hamilton et al., 2001). The hypothesis behind this data, suggest that mtDNA is more affected by oxidative damage due to: i) the proximity of the ETC, the primary source of ROS; ii) the lack protective barrier (histones) and iii) the inefficiency of repair oxidized mtDNA. However, these potential causes of mtDNA damage have controversial results and are not well understood (Alexeyev, 2009).

DNA lesions caused by free radical molecules can be repaired by different pathways including base excision, nucleotide excision, mismatch repair, tandem lesions, and double-strand break repair (Cui et al., 2011). The unrepair of oxidatively damaged mtDNA leads to the accumulation of mutations and deletions across time, impairing mitochondrial functionality and generating imbalance in cellular homeostasis, programmed cell death, and diverse signaling pathways (Krishman et al., 2008). In fact, mtDNA mutations have been extensively reported as characteristic of the normal organismal aging process and during the development of age-related neurodegenerative diseases such as Alzheimer's, Parkinson´s, Huntington´s, and Amyotrophic lateral sclerosis (Payne & Chinnery, 2015; Federico et al., 2012).

The direct experimental evidence that proves that mutations in mtDNA drive the aging process was the generation of a "mtDNA mutator mouse" by Trifunovic et al., (2004). The mtDNA mutator mice expresses a defective mtDNA polymerase that causes the accumulation of mitochondrial mutations across time, which in turn generate mitochondrial dysfunction. The phenotype of the mtDNA mutator mice displays several aging hallmarks such as kyphosis (exaggerated rounding of the back), anemia, alopecia, grey hair, cardiomyopathy, reduced fertility, osteoporosis, sarcopenia (loss of skeletal muscle mass), and a strongly-reduced life span (Bratic & Larsson, 2013) (Figure 2). Therefore, mitochondrial dysfunction is one of the well-established hallmarks of the aging process (Lopez-Otín et al., 2013).

Figure 2 - The mtDNA mutator mice. (Left) mtDNA mutator mice shows clear aged phenotype in comparison with WT mice (right) (reproduced from Trifunovic et al., 2004)

*Mitochondria possess quality-control mechanisms to maintain organelle and cellular homeostasis.*

Mitochondria need extensive protecting control pathways that promote their efficient and appropriate function. Therefore, mitochondria possess different mitochondrial maintenance pathways to preserve mitochondrial function and preventing the detrimental effects that mitochondrial dysfunction caused in the cell (Osiewacz & Bernhardt, 2013).

These mitochondrial maintenance pathways constitute an efficient quality-control (QC) network, acting at the molecular, organelle, and cellular levels (Fisher et al., 2012) (Figure 3).

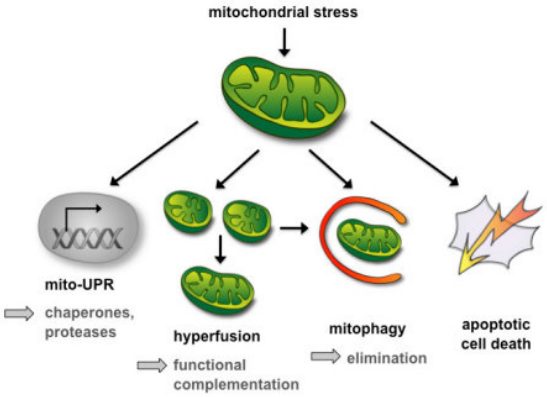


Figure 3 – Molecular, organelle and cellular mitochondrial quality-control (QC) pathways (reproduced from Winklhofer, 2017). The molecular QC pathways are the first barrier to restore the organelle homeostasis (e.g., Mito-UPR). If molecular QC is

overwhelmed, the dynamic organelle QC pathways are activated (e.g., fission/fusion, mitophagy). Organelle QC is characterized by excluding the damaged mitochondria from the healthy population. Lastly, the cellular QC is activated if all QC pathways fail. Cell-death pathway is activated, preventing mitochondrial damage to affect other cellular compartments.

The first molecular QC system centers on the maintenance of redox homeostasis. This QC system is formed by ROS scavengers, molecules that act as antioxidant barriers which maintain the balance of ROS levels inside the cell, preventing mitochondrial oxidative stress and cellular damage. Importantly, the loss of ROS homeostasis is associated with Vitamin E deficiency disease, ataxia (loss of the coordination of muscle movements and reflexes) and amyotrophic lateral sclerosis (ALS) (Scheibye-Knudsen et al., 2015).

The second molecular QC system counteracts the adverse effects of mtDNA mutations by DNA repair mechanisms. The main mtDNA modification is produced by ROS, generating the formation of an oxidative derivative of guanine (8-oxodG). The base excision repair pathway is the primary mitochondrial DNA repair mechanism involved in to eliminate and replace the oxidize base (Kazak et al., 2012).

The last molecular QC mitochondrial system involves the refolding or removal of damaged mitochondrial proteins. This pathway, called the mitochondrial unfolded protein response (mtUPR), activates the transcription of mitochondrial chaperones and proteases, encoded in the nucleus, to repair, refold or remove misfolded or oxidized mitochondrial proteins (Lehmann & Martins, 2013).

When the molecular mitochondrial QC pathways are not enough to counteract mitochondrial damage, cells can activate the next level of mitochondrial maintenance pathway, the organelle QC, to reestablish mitochondrial and cellular homeostasis. These organellar QC pathways underlying dynamic mechanisms to sort, isolate, remove, transport, and degrade through the lytic lysosome/vacuole organelle, dysfunctional or damaged mitochondrial areas from the mitochondrial network. (Lehmann & Martins, 2013).

One of the primary organellar QC strategies consists in the isolation of damaged mitochondria via a fission process of the mitochondrial network, restricting the fusion of the dysfunctional organelle with the healthy mitochondrial network and

degraded by the degradative pathway mitophagy. Mitophagy is one of the most important and last organellar QC mechanism strategy of the cell. This "self-eating" process involves the complete and highly-specific degradation of the damaged organelle through the delivery of the selected cargo to the lysosome/vacuole of the cell, where the degradation process begins by the action of hydrolytic enzymes (Youle & Narendra, 2011). Remarkably, mitophagy malfunction is related to aging and several age-related diseases such as Parkinson's, Huntington's, Alzheimer's, and immune-related diseases, as well as metabolic pathologies, ALS, and different types of cancer (Kroemer, 2015).

Additionally, Mitochondrial-derived vesicles (MDV), a second organelle maintenance strategy, are small mitochondrial derived buds that contain oxidized or damaged mitochondrial membrane proteins, which are present under oxidative stress conditions and in aged cells (Roberts et al., 2016; Hughes et al., 2016). The MDV is subsequently fused with the lysosome/vacuole of the cell where damaged proteins are degraded.

Finally, the cellular QC level will activate if both the molecular and organellar QC pathways cannot restore cell homeostasis. This QC strategy is triggered by the release of mitochondrial signaling proteins (e.g. Cytochrome c) to the cytoplasm, producing the activation of the cell-death signaling cascade, preventing the proliferation of cells with damaged mitochondria (Lehmann & Martins, 2013).

As we age, the functionality of these mitochondrial quality control mechanisms decreases progressively, producing the accumulation of molecular damage inside the cell and cause the organelle dysfunction (Held and Houtkooper, 2015).

*Mitochondrial degradation by specific autophagy.*

Autophagy is an evolutionarily conserved cellular process which has a critical role in maintaining cellular homeostasis (Feng et al., 2014). The main physiological roles of autophagy are i) degradation and recycling of cellular component under stress and starvation conditions in order to maintain cellular viability (e.g., during the early neonatal starvation period) (Kuma et al., 2004); ii) eliminating superfluous organelles for cellular adaptation to nutrient conditions or to avoid the maintenance of

unnecessary cellular organelles; iii) mediating cellular differentiation and maternal mtDNA inheritance in most multicellular organisms (Ashrafi & Schwarz, 2012); and iv) a quality-control pathway that removes damaged or dysfunctional organelles (mitochondria, peroxisomes, ribosomes, endoplasmic reticulum) that can have a detrimental effect on cellular homeostasis (Reggiori & Klionsky, 2013).

To accomplish all the physiological roles in which autophagy is involved, cells have developed different kinds of autophagy. Three major types of autophagy have been identified: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (Awan & Deng, 2014) (Figure 4).

CMA is a mammalian degradation pathway in which damaged and dysfunctional proteins and subunits of multi-protein complexes are recognized by chaperones, targeted, and translocated through the lysosome, the lytic cellular organelle that contains degradative enzymes (Cuervo & Wong, 2014).

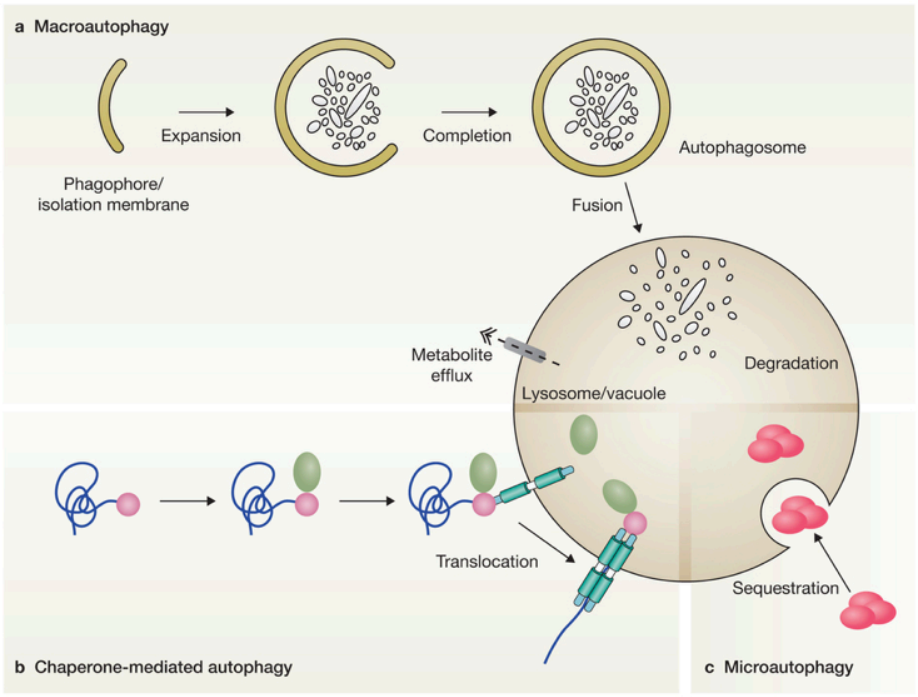


Figure 4 – Different types of autophagy (reproduced from Baros et al., 2013). (A) Macroautophagy is the main type of autophagy described to be involved bulk and selective degradation of cytoplasmic components. (B)Chaperone-mediated autophagy, only described in mammalian cells, is a merge degradative pathway that combines the chaperone recognition for translocation of the damaged proteins through the lysosome. (C) Microautophagy is the less studied type of autophagy due to the recent discovery and difficult to differentiate between macroautophagy. This type of autophagy directly sequestrates the cargo in the boundaries of the lytic organelle.

The second type of autophagy is microautophagy, which has the characteristic of degrading cytoplasmic cargos in a selective (degradation of damaged organelles) or non-selective manner (bulk-cytoplasm components), by direct engulfment through invagination of the lysosome/vacuole membrane boundaries. This type of degradation pathway has been involved in different cellular homeostasis mechanisms such as maintenance of organelle size, membrane composition, cell survival under nitrogen starvation, and transition of the cell cycle, from arrest to exponential growth, when nutrient starvation conditions are over. However, microautophagy has been proposed as a compensatory mechanism for the main autophagy type, macroautophagy, when the latter is compromised or overwhelmed (Li et al., 2012).

Finally, the major and best-described type of autophagy type is macroautophagy. Macroautophagy starts by labeling and recognition of the cargo (selective and non-selective cargo) by a double-membrane vesicle called autophagosome, which isolates and engulfs the cytoplasmic component to be degraded. Finally, the autophagosome fuses with the vacuole/lysosome of the cell (Reggiori & Klionsky, 2013).

One of the most determinant aspects of macroautophagy in cellular homeostasis is the selective degradation of dysfunctional mitochondria, a process called mitophagy. Mitophagy preserves the population of healthy mitochondria inside the cell. In fact, mitochondrial degradation through autophagy is considered the most important and specialized component of mitochondrial and organelle homeostasis (Kanki et al., 2015). Importantly, mitophagy is the only QC pathway that can eliminate damaged mtDNA, preventing the segregation of mtDNA mutations that lead to the development of human diseases, and represents the last organellar QC barrier for restoring the cellular homeostasis before the appearance of cell-death signals. Moreover, mitophagy deficiency has been closely related to the development of human diseases such as cancer, Parkinson's, Huntington's, Alzheimer's, ALS, muscle atrophy, diabetes, metabolic diseases and aging (Springer & Macleod, 2016).

*Mitochondrial autophagy signaling: A non-well understood selective degradation pathway.*

More than 30 **a**u**t**opha**g**y-related (*ATG*) genes that are part of the macroautophagy machinery have been described. These genes are responsible for autophagosome formation, cargo recognition, lysosome-vacuole fusion, and/or vacuolar degradation

and recycling. Interestingly, the core *ATG* genes are highly conserved from yeast to humans (Ashrafi & Schmarz, 2012). Most of the *ATG* core genes are also involved in the selective type of autophagy, since all the steps controlled by them are also needed for specific degradations. In contrast, only a specialized group of genes is involved in the dysfunctional or superfluous mitochondria recognition, engulfment, and degradation (Tan et al., 2016).

The activation of the mitochondrial autophagy pathway is directly controlled by the **t**arget **o**f **r**apamycin (TOR) kinase, which is a master regulator of nutrient sensing and a direct repressor of autophagy. When nutrients are limiting, TOR is in an inactivated form, allowing autophagosome formation through dephosphorylation of the Atg13 protein, a core autophagy protein that starts the signaling pathway for recruiting Atg1, a key kinase involved in autophagosome biogenesis. In addition, the protein kinase A/Ras/cAMP signaling pathway (PKA) has also been implicated as an autophagy repressor by phosphorylated Atg13, independently of TOR, inhibiting the formation of the Atg1 complex (Müller et al., 2014; Kanki et al., 2014) (Figure 5). However, even though mitophagy employs the core autophagy machinery, the exact molecular mechanisms that control the selective recognition, sequestration by the autophagosomes and degradation of mitochondria by autophagy remains to be fully elucidated.

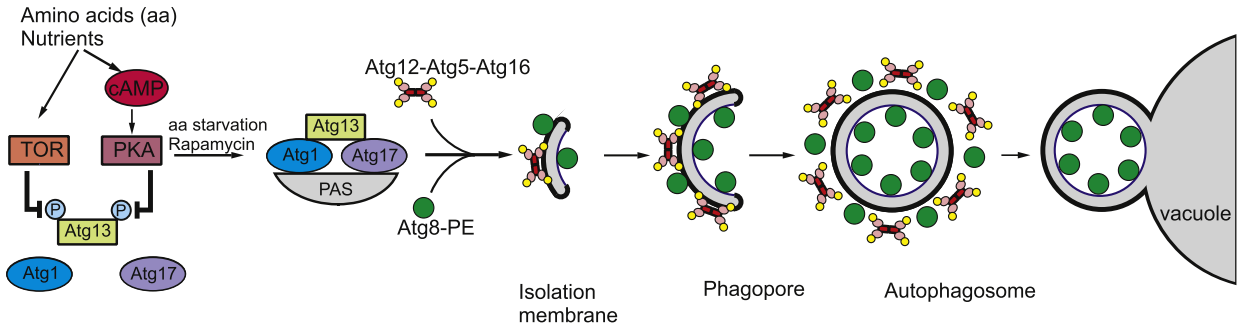


Figure 5 - Simplified autophagy signaling pathway (reproduced from Müller et al., 2015) - The two main pathways that negatively control autophagy are TOR and cAMP/PKA/RAS pathways. During rich nutrient conditions both signaling pathways are activated and phosphorylate Atg13. Phosphorylated Atg13 does not recruit the autophagy machinery for the autophagosome formation (Atg1-Atg17). During nutrients depletion or by rapamycin induction, Atg13 can bind and activates the autophagy machinery. This machinery is recruited to the Phagophore Assembly Site (PAS) and form the autophagosome by interacting with structural Atg proteins (Atg12-Atg5-Atg16-Atg8-PE). Then, the autophagosome recognizes the cytoplasmic cargo to be degraded and fuses with the vacuole of the cell.

Open questions to fully understand the selective mitochondrial degradation by mitophagy remain. For instance, how do the different types of autophagy interact and communicate to maintain mitochondrial homeostasis? What kind of mitochondrial damage activates mitophagy? Which pathway signals activate mitophagy? and how do cells efficiently select and distinguish dysfunctional mitochondria from healthy organelles during the aging process? All these questions will help us to fully understand the biology behind mitochondrial degradation during the aging process.

To address these type of questions, mitochondrial degradation by autophagy can be studied in model organisms by activating mitophagy under different laboratory conditions. As an example, in the yeast model *Saccharomyces cerevisiae*, mitophagy can be induced by i) culturing cells under non-fermentable carbon source (e.g., glycerol or lactate) and then switching to a medium without nitrogen with a fermentable carbon source (e.g., glucose) (Kanki et al., 2009); or by ii) allowing the cell culture to grow in a non-fermentable carbon source until post-log phase (Abeliovich, 2011). On the other hand, in flies, worms, and mammalian models, mitochondrial degradation can be activated under different environmental conditions (e.g. high fat diet, hypoxia, normal aging) (Sun et al., 2015), chemical-induction conditions (promoting the rise of dysfunctional and depolarized mitochondria by uncoupling ETC using carbonyl cyanide m-chlorophenylhydrazone (CCCP) or paraquat) (Kanki et al., 2014), and by genetic perturbations (Huntingtin transgene expression, POLGγ mutation) (Joseph AM et al., 2013; Sun et al., 2015).

*Mitochondrial QC activity by autophagy declines with age.*

Most of the identified genes involved in the maintenance of mitochondrial function and autophagy process have an impact on organismal life span determination (Sutphin et al., 2011). The decline in mitochondrial autophagy with age leads the reduction of the life- and health-span of the organism, promoting mitochondrial dysfunction which has been established as one of the hallmarks of the aging process (López-Otín et al., 2013). This evidence, suggest that mitochondrial autophagy possesses a specific regulation network that degenerates during aging, indicating that keeping an efficient signaling for removal of damaged organelles across time, is fundamental for delaying deleterious effects of the aging process (Palikaras et al.,

2017; Diot et al., 2016).

Supporting this hypothesis, Rubinsztein et al. (2011) described that *ATG* genes and *ATG* gene-activators possess a reduced expression and diminished activity in aged-tissues. In addition, Rana et al. (2013) showed that the overexpression of Parkin, an E3 ubiquitin ligase involved in mammalian signaling of mitochondrial autophagy, in the fly *Drosophila melanogaster*, increased their maximum life span by reducing the levels of protein aggregates and activating the turnover of dysfunctional mitochondria via mitophagy during aging (Rana et al., 2013). However, the exact reasons for why mitochondrial autophagy declines with age are not well understood (Martinéz-Lopez et al., 2015).

Interestingly, mitochondrial dysfunction has been recognized as a molecular factor that cause this decrease in the regulation of autophagy machinery. A recent report demonstrated that yeast cells under amino-acid starvation completely block autophagy via PKA signaling caused by defective mitochondria (Graef & Nunnari, 2011). This evidence suggests a crosstalk between mitochondrial dysfunction and autophagy regulation (Okamoto, 2011). To support this result, Sullivan et al. (2016) reported that mitochondrial dysfunction disrupts the structure and morphology of the lysosomes in mice neuronal cells, preventing the degradation of the damaged organelles. Importantly, these results correlate with the characteristic features presented in some neurodegenerative diseases, where neural cells with a mitochondrial dysfunction in the ETC promote the aggregation of misfolded proteins and accumulate dysfunctional organelles (Parker et al., 2008).

ROS are also emerging as important players in mitochondrial autophagy regulation. Hydrogen peroxide $H_2O_2$ can interact with nitric oxide (NO) to generate highly reactive molecules, such as peroxynitrite (ONOOO-), which could modify mitochondrial and Atg proteins, affecting their functionality and signaling for degradation. In addition, $H_2O_2$ can produce hydroxyl radical by Fenton reaction that in turn provoke mtDNA and nDNA mutations that could influence the regulation of *ATG* genes (Lee et al., 2012). However, the answer to the question of why mitochondrial autophagy decreases with age remains unclear. Some authors suggest that due to the complexity of signaling and regulation of mitochondrial autophagy, combining with all the cellular processes that change during aging, the

inhibition of this selective degradation pathway is "complex and multifactorial" (Martinéz-Lopez et al., 2015).

*S. cerevisiae as a model organism for aging research.*

There are many different and attractive model organisms used in aging and autophagy research, each one with its advantages and certain pitfalls. One of these advantageous models is the budding yeast *S. cerevisiae*. This unicellular eukaryotic organism is a widely-used model for studying complex biological processes, including aging, autophagy, and mitochondrial function (Breitenbach et al., 2012).

*S. cerevisiae* has a number of advantageous features, in particular: i) a short life cycle (2~3 days); ii) a small genome size (12 Mbp) comprising 6,000 genes (4,800 nonessential and 1,200 essential); iii) whole-genome sequenced and functional annotation; iv) a stable haploid phase; v) orthologous genes in the human genome; vi) it is cheap to grow in small and large scales; vii) a single knockout-deletion collection is available, allowing whole-genome screening analysis; viii) a stablished high-throughput method for measuring yeast life span (Garay et al., 2014); ix) efficient homologous recombination system, allowing genetic engineering; and x) two different aging models: a replicative life span (RLS) and a chronological life span (CLS) (Gershon & Gershon, 2000). Both aging models have contributed to have a better understand in the molecular pathways implicated in the aging process and how age-related diseases are developed (Kaeberlein., 2010; Longo et al., 2012) (Figure 6).

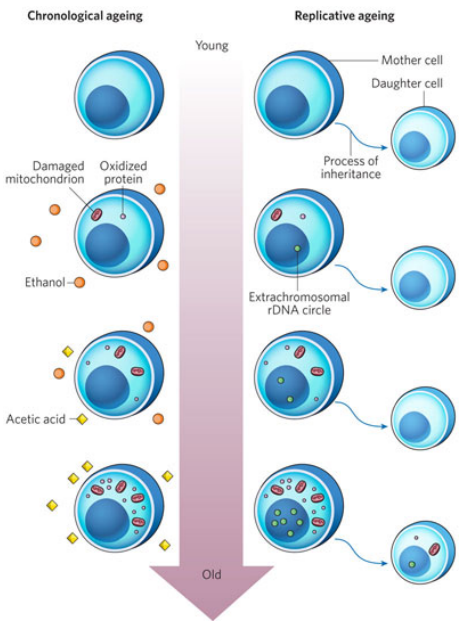


Figure 6 - CLS and RLS aging models in yeast (reproduced from Kaeberlein, 2010). Different Ageing-factors affect both models that determine their life span phenotype. In CLS, Damaged mitochondria and oxidative stress are well-established to play a role in life span determination.

RLS is defined as the number of times that an individual cell divides before senescence (usually around 20-25 cell divisions) (Kaeberlein et al., 2007). The RLS approach provides a reliable aging model of mitotically-active cells. The RLS analysis is typically determined by counting and removing the daughter cells, which are easily distinguishable by size differences, from the mother cell by micro manipulation approaches, limiting the scale of RLS analysis at the genome-wide scale (Piper, 2006). However, recent promising high-throughput approaches have been developed to achieve less laborious and complicated measurement of RLS by using genetically modified strains with daughter cell inviability (Jarolim S et al., 2004) or by automated microfluidic systems (Chan-Jo et al., 2015). Using this pproach, nearly 100 genes have been identified to play a role in yeast replicative aging (e.g. *SIR2, FOB1*) (Kaeberlein et al., 2005). Importantly, most of these genes represent potential aging modulators, possessing a conserved role during the normal aging of mitotic or stem cell population in higher eukaryotes (Longo et al., 2012).

On the other hand, the CLS aging model is defined by the ability to reactivate the cell cycle after a prolonged stationary phase period (Kaeberlein, 2010). The CLS is typically assayed by growing yeast cells in rich liquid media. Once cells deplete all

nutrients available in the media, they enter a quiescence phase. CLS is determined by measuring the viability of the culture by taking a sample of the aged-culture, re-growing in fresh medium and monitoring the time until the culture reaches the exponential phase (Murakami et al., 2008).

Yeast CLS analyses have allowed a comprehensive description of the central genetic factors and regulatory pathways implicated in the aging process of non-dividing cell types (e.g. neurons, myocytes) (Fabrizio et al. 2010; Garay et al., 2014; Gresham et al. 2011; Matecic et al. 2010), providing relevant knowledge of how aging-associated diseases (e.g. neurodegenerative diseases, cardiomyopathy, metabolic diseases) develop during the normal aging process (Parrella & Longo, 2008).

Two nutrient-sensing signaling pathways, TOR and RAS/PKA, have been recognized as the main molecular modulators of chronological aging. Strikingly, both molecular pathways are highly conserved in higher organisms such as worms, flies, and mammals. Notably, TOR and RAS/PKA sensing pathways are well-established autophagy regulators, confirming the closer linking between this cytoplasm degradation pathway and life span determination (Longo et al., 2012).

Interestingly, several studies describe and strongly remark the importance and direct influence of mitochondrial functionality (e.g. efficiency of mitochondrial respiration, decreasing in mitochondrial-gene expression) as a key regulator of CLS determination (Aerts et al., 2009; Bonawitz & Shadel, 2007; Ocampo et al., 2012). Additionally, it has been demonstrated that during the aging process, oxidative damage generated by mitochondrial dysfunction, accumulates with cellular age, supporting the role of mitochondrial function in CLS determination (Pan, 2011). It is noteworthy that other mitochondrial functions, besides energy production, such as mitochondrial biogenesis, mitochondrial autophagy, mitochondria-nuclear signaling, redox state and mitochondrial DNA integrity, have also been found to be involved in yeast longevity (Barros et al., 2010).

*The baker's yeast as a model for mitochondrial autophagy studies*

During the first efforts to unravel the molecular factors responsible for the control of the autophagy pathway, the lack of a model organism and standardized methods,

limited our understanding of this degradation pathway (Karanasios & Ktistakis, 2016). It was until the ground-braking work performed by Yoshinori Ohsumi's research group demonstrating that the yeast *S. cerevisiae* possesses the same degradation process under starvation conditions, allowing the performance of the first genetic screen analysis that identified the first genetic factors (*ATG* genes) involved in this degradative pathway (Takeshige et al., 1992; Yang & Klionsky, 2010). This remarkable study, revolutionized our understanding of autophagy, discovering the key cellular role that autophagy plays in the organismal homeostasis and life span determination (Ohsumi, 2014; Yang & Klionsky, 2010) (Figure 7).

The implementation of the baker's yeast as a proficient autophagy model organism led to the identification of the core genetic factors (the autophagy-related genes) and the different signaling pathways that allow the correct performance of this degradative process. It is noteworthy that most of the autophagy-related genes identified in yeast have a homolog in mammalian systems (Yang & Klionsky, 2010). Until now, 41 autophagy-related genes have been described using this model organism (Galluzzi et al., 2017).

*S. cerevisiae* has been established as one of the most widely used autophagy model systems to unravel the molecular mechanisms and signaling pathways compromised in the autophagy degradation pathway. Some of the valuable characteristics that *S. cerevisiae* provides as an autophagy model system are: i) it has a degradative organelle (the vacuole) that can be easily monitored through the complete autophagy process by microscopy (Karanasios & Ktistakis, 2016); ii) it provides the opportunity to analyze the molecular basis of the different types of autophagy by altering the growth and induction conditions (Müller et al., 2015); and iii) a number of different, simple, and state of the art approaches and assays have been established to monitor the different steps and types of autophagy (Delorme-Axford et al., 2015).

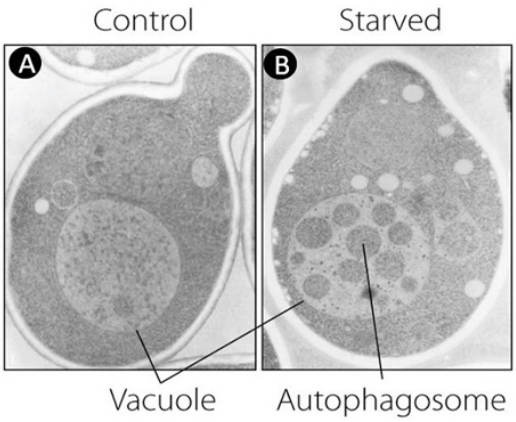


Figure 7 – Yeast allows the easy monitoring of autophagy by microscopy (reproduced from Takeshige et al., 1992). Electron microscopy visualization of yeast cells and its vacuole under (A) nutrient-rich conditions and (b) starvation conditions.

The molecular machinery of the autophagic process in yeast is complex. The autophagy degradation process begins with the activation of the Atg1 kinase complex (Atg1, Atg13. Atg17, Atg29 and Atg31) and the Atg9 complex (Atg2, Atg18, Atg23 and Atg27), which recruit the entire autophagy machinery for the formation of the autophagosome at the phagophore assembly site (PAS) (Reggiori & Klionsky, 2013). Then, membrane expansion of the double-membrane vesicle, that will surround and engulf the selective cargo for degradation, requires the activity of the PI3K complex (Atg6, Atg14, Atg38, Vps34, Vps15), the ubiquitin-like conjugation system (Atg3, Atg4, Atg7, Atg10, Atg16 and Atg12-Atg5 complex) and the Atg8-PE complex (Farré & Subramani, 2016). Finally, after the autophagosome formation is complete, the fusion of the autophagosome with the vacuole depends on the machinery complex (Ypt7, Mon1-Ccz1, Vam3, Vam7, Atg17, Atg29, Atg31 and Atg11) (Liu & Klionsky, 2016) (Figure 8).

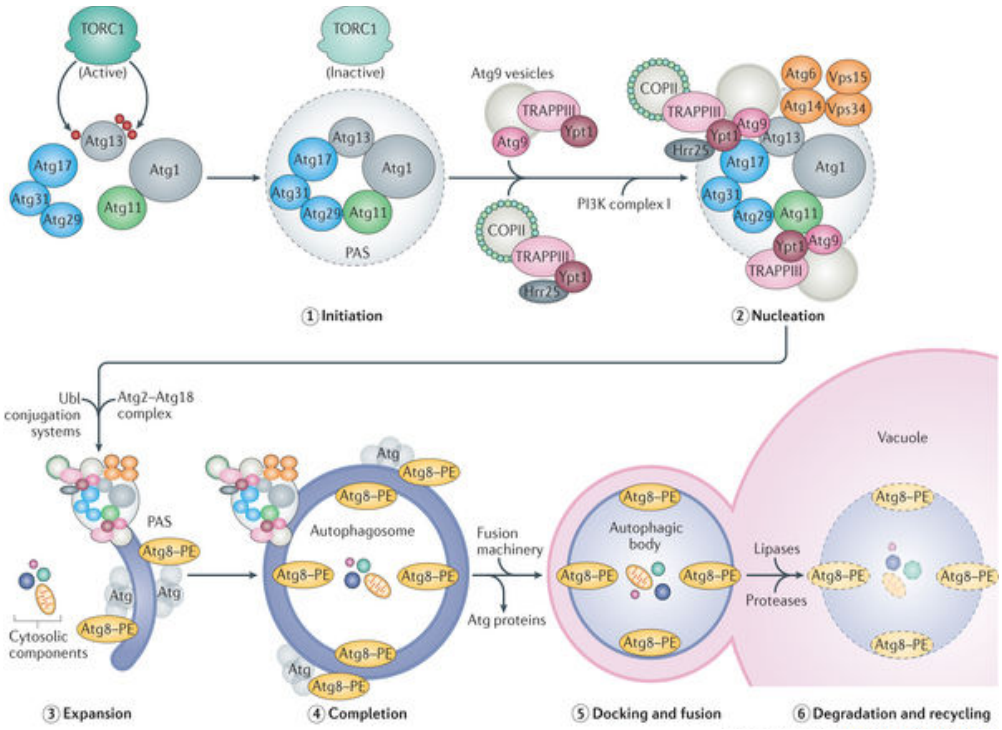


Figure 8 Molecular machinery of macroautophagy in yeast (reproduced from Farré & Subramani, 2016). The autophagy possesses 6 primary step: 1) the activation and initiation of the formation of the phagophore by TORC1 inactivation; 2) the establishing of the PAS and recruitment of the autophagy machinery (nucleation); 3) the expansion of the double-membrane vesicle; 4) the maturation of the autophagosome and isolation of the cytoplasmic cargo (completion); 5) the transport to and fusion of the autophagosome to the vacuole (docking and fusion); and finally 6) the degradation process by hydrolytic enzymes and recycling of the sub products.

*Yeast analyses to identify essential genes in the mitochondrial autophagy signaling pathway*

The degradation of mitochondria via autophagy was first reported by Takeshige et al. (1992) through the observation of the presence of mitochondria inside of an autophagosome. However, the selective sorting and degradation of mitochondria were first demonstrated and characterized by Kanki & Kliosky (2008). They observed that the degradation of mitochondria is selectively regulated by nutrient conditions, determining the number of mitochondria depending on the growth conditions. It is noteworthy that they described that *ATG11, ATG20,* and *ATG24* which are essential genes for other selective types of autophagy, are also required for efficient mitochondrial degradation.

Kanki & Kliosky revealing study (2008) demonstrated that mitochondria possess a unique molecular and signaling pathway for selecting, isolating and degradation of

the superfluous and damaged organelle. Nevertheless, those recognition pathways need to be revealed. In this regard, two leading groups in autophagy research performed genome-wide screens to determine how the molecular mechanisms of mitochondrial autophagy take place. Using mitochondrially-targeted fluorescent proteins, fluorescence and electron microscopy approaches, and immunoblotting assays, both groups identified *ATG32* as a novel key gene involved in mitochondrial signaling and degradation by autophagy (Kanki et al., 2009; Okamoto et al., 2009).

Atg32 (Bcl-2-like protein 13 homologous in mammals) (Otsu et al., 2015) is a mitochondrial outer membrane protein which acts as a "signaling marker" for the organelle degradation. When Atg32 is phosphorylated (signaling in this process is not well understood), it recruits an adaptor protein, Atg11, activating the autophagy machinery. This leads to sequestration of the organelle by the double-membrane autophagosome and its degradation in the vacuole (Aoki et al., 2011). However, the signaling pathway that activates this organelle degradation remains to be characterized.

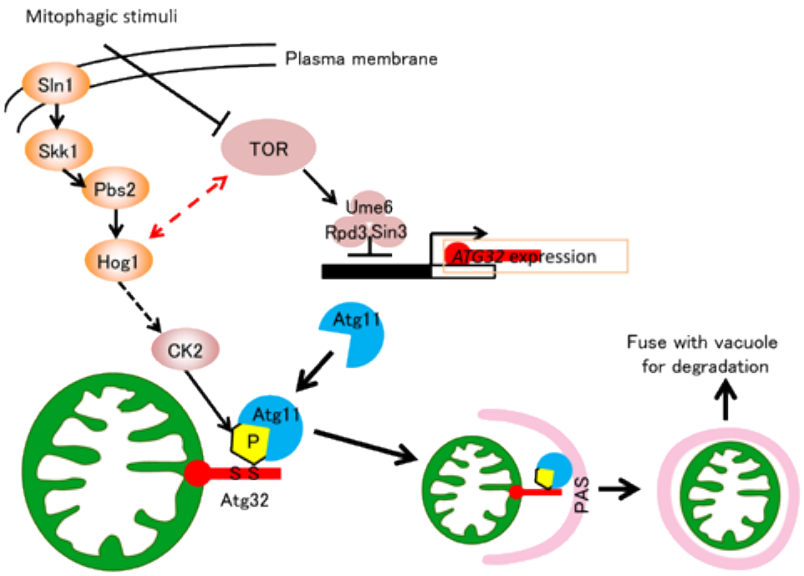


Figure 9 – Mitochondrial autophagy signaling pathway in yeast (reproduced from Kanki et al., 2015). Mitochondrial dysfunction is the primary autophagic stimuli for the phosphorylation of Atg32 by Atg11, triggering the signaling of the macroautophagy machinery. The phosphorylation of Atg32 is carried out by the casein kinase 2 through Hog1 phosphorylation cascade. Under rich nutrients conditions, TOR promotes the inhibition of the *ATG32* expression by activated the autophagy repressors Ume6, Rpd3 and Sin3. However, the complete signaling pathway for mitochondrial degradation remains incomplete.

Interestingly, Kanki et al. (2013) and Mao et al. (2011) performed two genetic screen analyses of yeast knockout mutants in kinase genes and MAPK-signaling pathways, respectively. These studies identified that Casein kinase 2 and two MAPK-signaling pathways (Hog1 & Slt2) are involved in the correct signaling of mitochondria for autophagy degradation. Although, the complete understanding of the signaling pathway for mitochondrial degradation is still incomplete and further studies are required (Figure 9).

*Yeast biosensors to monitor mitochondrial autophagy in vivo*

Different approaches have been developed to monitor mitochondrial autophagy in yeast (Klionsky et al., 2016). One of the most common assays for monitoring the delivery of the mitochondria into the vacuole involves the use of a chimeric protein marker fusing a fluorescent protein (e.g., GFP) to a mitochondrial protein (e.g., Om45 – a mitochondrial-outer membrane protein; or Idh1 – a mitochondrial-matrix protein) (Kanki & Klionsky, 2008). The principle of this technique is simple: When Om45-GFP/Idh1-GFP-mitochondria are delivered to the vacuole by activating mitochondrial autophagy, the biomarker is processed, releasing intact GFP. The amount of free GFP signal inside the vacuole reflects the activity of mitochondrial autophagy, and this can be measured semi-quantitatively using fluorescent microscopy or immunoblotting assays (Kanki et al., 2009).

An alternative assay for precisely monitoring mitochondrial autophagy in yeast is the use of the dual fluorescent protein biosensor Rosella (Rosado et al., 2008). The principle of this assay is ingenious. The dual biosensor results from the fusion of two fluorescent proteins, a pH-stable and rapid-maturing red fluorescent protein variant (DsRed.T3) fused with a pH-sensitive green fluorescent protein variant (pHluorin). When mitochondria are tagged with the Rosella biosensor and mitochondrial degradation is activated, we can observe the stable DsRed.T3 signal inside the vacuole of the cell, while the pH-sensitive GFP signal is not detectable (Figure 10).

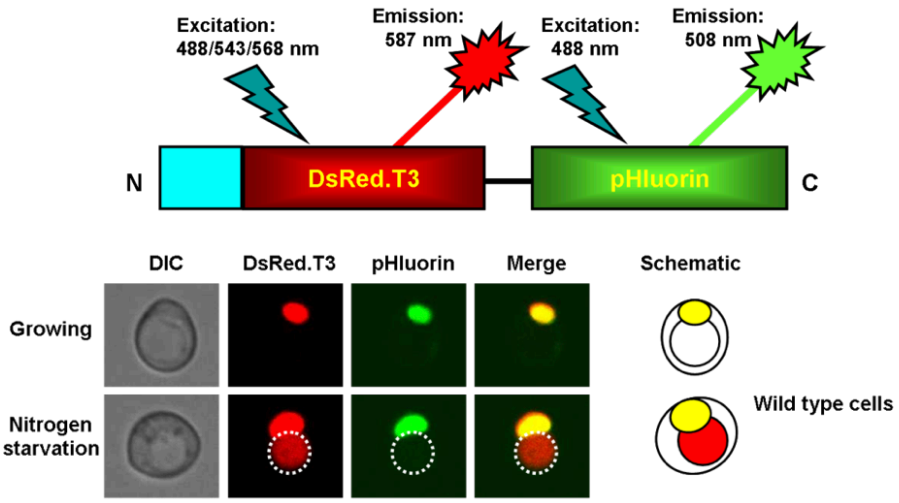


Figure 10 - Rosella biosensor for monitoring autophagy (reproduced from Rosado et al., 2008). The Rosella biosensor is composed of two fluorescent proteins, a stable Red Fluorescent protein (DsRed.T3) and a pH-sensitive Green Fluorescent Protein (pHluorin). Under growing conditions, both fluorescent protein signals are detected (nucleus tagged) in the cellular-tagged compartment, but under nitrogen starvation conditions, autophagy is activated, and the cytoplasmic components start to be degraded inside the vacuole of the cell. Due to the low pH conditions inside the vacuole,only the Red fluorescent protein remains stable and therefore is the only one that produces a signal.

This biosensor has three advantages: i) yeast cells expressing this biosensor can be easily monitored and quantify different types of autophagy by confocal microscopy and flow cytometry approaches; ii) it can be genetically manipulated to monitor the degradation process of different types of cellular compartment (cytosol, mitochondria, nucleus) (Mijaljica et al., 2011); and iii) it can be used for monitoring autophagy in mammalian cells, allowing the comparison between model systems (Sargsyan et al., 2015). In addition, the Rosella biosensor allows the monitoring of selective mitochondrial autophagy in a high-throughput manner. A recent high-throughput microscopy study using respiratory deficient yeast strains tagged with this biosensor allowed to determine that the ERMES complex, a complex that connects mitochondria with the endoplasmic reticulum, is a critical factor involved in selective mitochondrial degradation (Böckler & Westermann, 2014). Strikingly, the Rosella biosensor enables the identification of novel mitophagy factors that would not be possible to identify using conventional autophagy monitoring assays. Further, less laborious, time-consuming, and precise quantitative high-throughput approaches are required for monitoring mitochondrial autophagy and to identify novel genetic factors necessary for the effective maintenance of mitochondrial homeostasis through the aging process.

*Regulation of mitochondrial autophagy during CLS: The biological question of my Masters project.*

Due to the biological relevance of the selective degradation of the complete dysfunctional mitochondria and their physiological relevance to human health, aging, and age-related diseases, the present research project focuses on understanding the regulation of mitochondrial autophagy during the aging process.

Although mitochondrial autophagy signaling pathways have been widely studied, open key questions remain unexplored. Previous reports have revealed that mitochondrial function and efficiency of organelle QC pathways have a significant impact in life span determination. However, characterization of the regulation of mitochondrial autophagy during the aging process remains incomplete. Therefore, my Masters project was focused on identifying which of the genetic factors that determine longevity in yeast are involved in the regulation of mitochondrial autophagy. For this purpose, the main objective was to develop a high-throughput assay to precisely quantify the mitochondrial autophagy in a mutant lacking each genetic CLS factor identified by Garay et al. (2014) (621 single-knockout yeast strains) using a modified version of the selective-autophagy biosensor Rosella.

## Hypothesis

Autophagy of dysfunctional mitochondria possesses a closed relation in chronological life span determination. Therefore, the alteration of any longevity genetic factor will affect the mitochondrial degradation capacity by autophagy.

## Aim

To quantify mitochondrial autophagy in over 600 single-knockout yeast strains affected in its chronological life span phenotype.

## Objectives

1. To develop and characterize a novel version of the mitochondrial autophagy biosensor Rosella.
2. To select a single-knockout yeast collection based on their respiratory capacity and altered chronological life span (named yeast aging factors collection (YAFC)).
3. To tag the YAFC with the modified version of the mitochondrial autophagy biosensor.
4. To develop a high-throughput flow cytometry-based method to quantify yeast mitochondrial degradation in a precise manner.
5. To quantify the mitochondrial autophagy of each YAFC strain.
6. To correlate the capacity of mitochondrial degradation with the CLS of the longevity yeast collection.
7. To validate possible novel autophagy factors using confocal microscopy.

## Materials and Methods

*Strains and media*

The laboratory yeast strain Y8205 (Genotype: *MATα can1Δ:: STE2pr-Sp_his5 lyp1Δ:: STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0*) was used as parental strain and for the RV2-mt plasmid construction. The short and long-lived deletion yeast strains (Garay et al., 2014) were taken from the yeast deletion collection in the BY4742 genetic background (*MATa xxxΔ::kanMX4 his Δ1 ura3Δ0 leu2Δ0 met15Δ0*). A specific bacterial host strain (BUN20 [*Δlac-169 rpoS(Am) robA1 creC510 hsdR514 ΔuidA (MluI):pir-116 endA(Bt333) recA1 F′(lac+ pro+ ΔoriT:tet*)] ) was implemented for the recovery of the yeast recombinant plasmid RV2-mt to further isolation and sequence analysis (Li and Elledge, 2005).

The growing media used for determination of respiratory capacity in the YAFC was the YPL medium with 1% yeast extract, 2% peptone and 2% lactic acid, buffered to pH 5.5 with 10N NaOH and sterilized by autoclave. For mitochondrial autophagy induction, synthetic complete glucose (SCG) medium (2% glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1% L-glutamic acid monosodium, and 1.93% dropout (-Ura)); synthetic complete lactate (SCL) medium (2% lactic acid, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1% L-glutamic acid monosodium and 1.93% dropout (-Ura)) and synthetic minimal medium lacking nitrogen (SD-N; 0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose) were used (Kanki et al., 2009). All media were buffered to pH 6 with 10N NaOH and sterilized by filtering. Nourseothricin (cloNAT; Werner Bioagents) and geneticin (G418; Invitrogen) were used for selection at final concentrations of 100µg/mL and 200µg/mL, respectively.

*Mitochondrial Autophagy Biosensor Construction*

The mitochondrial autophagy biosensor (RV2-mt) was based on a previous report by Rosado et al. (2008) named Rosella. The coding sequence of the dual-biosensor was completely synthesized by GenScript, using the reported sequence by the authors. RV2-mt plasmid was constructed based on yeast homologous

recombination efficiency and in vivo ligation protocol reported by Finnigan & Thorner (2015).

The amplification of the desired DNA fragments to ligate were the MOBY-TDH3 plasmid backbone, the mitochondrial targeting sequence (52 amino acids) of citrate synthase 1 gene of *S. cerevisiae*, and the modified version of Rosella biosensor (mCherry protein instead of DsRedT.3), were amplified by PCR using the primers in Supplementary Table No.2. These primers have 40 overlapping base pairs that allow the efficient recombination of the fragments. After amplification of the desired DNA fragments, the complete PCR products were used for yeast transformation following the Finnigan & Thorner protocol (2015). The resulting RV2-mt plasmid is a centromere-based vector, without recombination sites for the yeast genome (Ho et al., 2009) (Figure 12).

After transformation, the yeast colonies growing on selecting media were visualized by fluorescence stereo microscopy (ZEISS SteREO Lumar.V12) and 5 GFP-fluorescent positive colonies were picked. Each yeast plasmid was isolated and transformed into bacteria (Finnigan & Thorner, 2015). Each plasmid was analyzed by restriction enzymes, PCR-analysis, and sequencing. The confirmed RV2-mt plasmid was transformed in the parental strain Y8205 and analyzed by confocal microscopy (ZEISS LSM 800) and flow cytometry (LSRFortessa X-20).

*Generation of the "yeast aging factors collection."*

After the selection of those single-knockout yeast strains, derived from the yeast deletion collection, with an altered life span phenotype, the respiratory capacity of each yeast strains was measured. The individual respiratory capacity was determined by calculating the growth rate of each single mutant under the respiratory medium YPL. A total number of 622 yeast mutant strains with altered CLS (Garay et al., 2014) were arranged in seven 96-well plates and grown in YPAD medium for 48 h at 30º C without shaking to mid-log phase. Using the automatic robotic station Tecan Freedom EVO200, 10 mL of saturated culture were taken into 140 mL of fresh YPL medium (OD600 ~ 0.1). The plates were incubated at 30ºC, 1050 rpm and OD600 was measured every hour until all cell cultures reached the stationary phase (16 – 18 hours; OD600 = 0.4 to 0.65).

The yeast mutant strains that fulfilled the selection criteria: altered CLS and efficient respiratory capacity were selected for mitochondrial degradation analysis (total of 553 single knockout yeast strains; Supplementary Table 1).

Each yeast strain was tagged with the generated biosensor RV2-mt using the synthetic genetic array (SGA) methodology (Boone et al., 2007). The parental strain carrying the generated biosensor (Y8205-RV2-mt) was mated with the 553 single-knockout strains selected, followed by one diploid selection step, one sporulation step and three haploid selection steps (Garay et al., 2014).

*Induction of mitochondrial autophagy in yeast cells*

Mitochondrial degradation by autophagy was induced following the protocol previously reported by Kanki et al. (2009) with several modifications due to the optimization to quantify mitochondrial autophagy using high-throughput flow cytometry approach. Briefly, yeast cells expressing the biosensor were grown in 150 µL of SCG medium, with cloNAT and G418 as selection markers, in 96-well plates (Corning 3585) for 48 hours without shaking at 30°C. Then, 10µL were used to inoculate 140µL of SCL to reach OD600 = 0.1 using the automatic robotic station Tecan Freedom EVO200. The plate was inoculated at 30°C at 1050 rpm on an orbital plate shaker for 14-16 h to allow the cells to grow to log phase (OD600 = 0.3 to 0.4). Then the plate was centrifuged, and cells were washed with sterile distilled water three times. Finally, the cells were suspended in 150 µL of SD-N medium and incubated at 30ºC at 1050 rpm for 4.5, 8, and 12 hours before measuring mitochondrial degradation by flow cytometry.

Top hit candidates were grown on 3 mL of SGD medium, with cloNAT and G418 as selection markers, in a test tube at 30 °C at 180 rpm for 48 hours. Then, 0.05 mL of the saturated SGD medium was inoculated into 2.95 mL of SCL to reach an OD600=0.1 and grown at 30 °C at 195 rpm for 16 hours until log phase (OD600 = 0.3 to 0.6). The cells were starved in a 2.0 mL microcentrifuge tube and washed three times with 1.5 mL of sterile distilled water. Finally, mitochondrial autophagy was induced by resuspending the cells in 3 mL of SD-N medium and incubating cells at 30°C at 180 rpm for 24 hours before analyzing by confocal microscopy (Rosado

et al., 2008).

*Monitoring mitochondrial autophagy by confocal microscopy*

A sample of 1 mL of autophagy-induced yeast cells was taken from SD-N test tube and centrifuged at maximum speed for 1 minute to collect the cells. After removing most of the supernatant, cells were resuspended in the remaining volume (~20µL). 2.0 µL of the cell suspension was taken and added 1.5 µL of glycerol 50% on a microscope slide and covered with a coverslip, sealing all the borders. Images were taken with the confocal laser scanning microscope ZEISS LSM 800 using the software ZEN Imaging V. 2.3 blue edition.

*Genome-wide screen analysis of mitochondrial autophagy by flow cytometry*

After the 553 mutant strains of the yeast aging factors collection were tagged with the RV2-mt plasmid, the entire collection was rearranged in eight 96-well plates, including 61 WT - RV2-mt and 33 *atg1*Δ - RV2-mt mutants as references of standard/positive and null autophagy control, respectively. In each well the green and red fluorescent signal were quantified using the LSR Fortessa X-20 coupled to the cell analyzer HTS. For the entire yeast populations, both green and red fluorescent protein signals of, at least, 20,000 cells for each yeast mutant were measured after 4.5, 8, and 12 hours of mitochondrial autophagy induction. Significantly sub-induced and hyper-induced mutants were identified with a Z score assigned to each single-knockout mutant [Z score= (GFP/mCherry rate xxxΔ - GFP/mCherry rate µ WT) / standard deviation WT]. Two-tailed test with an $\alpha = 0.01$ was established. Significant mitochondrial autophagy (MAA) values were settled using a $Z<-2.57$ ($p<0.005$) for sub-induced mutants and $Z>2.57$ ($p<0.005$) for hyper-induced mutants.

*Image analysis*

To validate the possible novel players in the mitochondrial autophagy, the absolute values of the red fluorescent protein signal inside the vacuole of a yeast strain population were quantified. We analyzed at least 300 yeast cells of each strain

population using the image processing software ImageJ Version 2.0, using the "cookbook plugins" analysis software.

## Results

*The generated autophagy biosensor is a reliable tool for monitoring mitochondrial autophagy in yeast cells*

To monitor mitochondrial degradation by autophagy and to easily identify those longevity genetic factors involved in this degradation pathway, we developed a version of the autophagy biosensor Rosella (Rosado et al., 2008). This biosensor is composed of two fluorescent proteins, a pH-sensitive GFP variant (SEP - 488 nm excitation / 508 nm emission) and a pH-stable red fluorescent protein (DsRedT.3 – 543 nm excitation / 587 nm emission). Based on previous work in our research group with both fluorescent proteins DsRedT.3 and mCherry, we decided to modify the original autophagy biosensor and replace the fluorescent signal of the DsRedT.3 with the red fluorescent protein variant mCherry (587 nm excitation / 610 nm emission), due to its brightness and advantages for detection and flow-cytometry analysis under our laboratory setting (Figure 11).

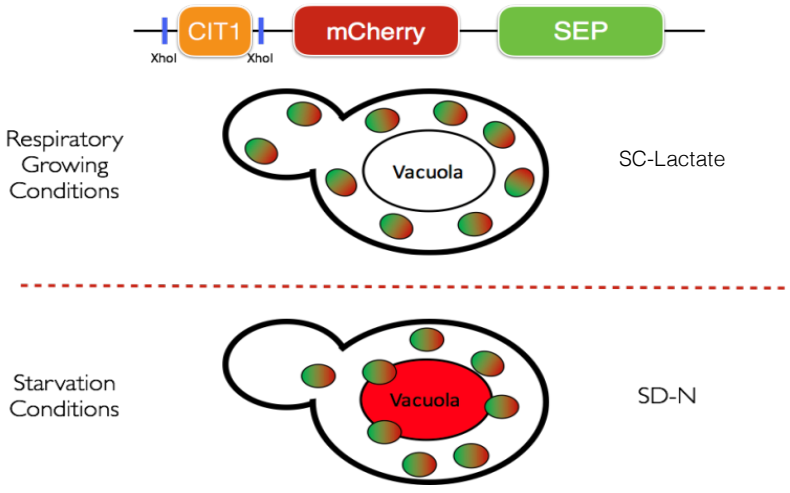


Figure 11 - Schematic representation of the generated RV2-mt biosensor. The Cit1 presequence specifically targets the protein to mitochondria. Under respiratory growing conditions, both fluorescent proteins inside the mitochondria are detected. However, when mitochondrial degrades, they are transferred to the vacuole of the cell by autophagy. Inside this lytic organelle, only the red fluorescent protein remains stable. A visualization of a red vacuole under confocal microscopy will indicate that mitochondrial degradation pathway by autophagy is completely activated.

The complete biosensor sequence was chemically synthesized (Genscript Biotech Corporation) based on the sequence reported for the biosensor by Rosado et al. (2008). The synthesized biosensor composed of 1,459 bp was cloned in the commonly used cloning vector pUC57 (Yanisch-Perron et al., 1985).

For the construction of the yeast vector that expresses the autophagy biosensor, we generated a simple plasmid construction based on the efficient homologous recombination and in vivo DNA-ligation properties of the budding yeast *S. cerevisiae*. This method allowed the efficient and cost-effective assembly of multiple DNA fragments to create a single plasmid molecule. The principle of this approach is to use the PCR fragments that correspond to the plasmid with 30-40 homologous base pairs endings, mix them, and use them to transform yeast in a single step (Finnigan & Thorner, 2015; Van Leeuwen et al., 2015) (Figure 12).

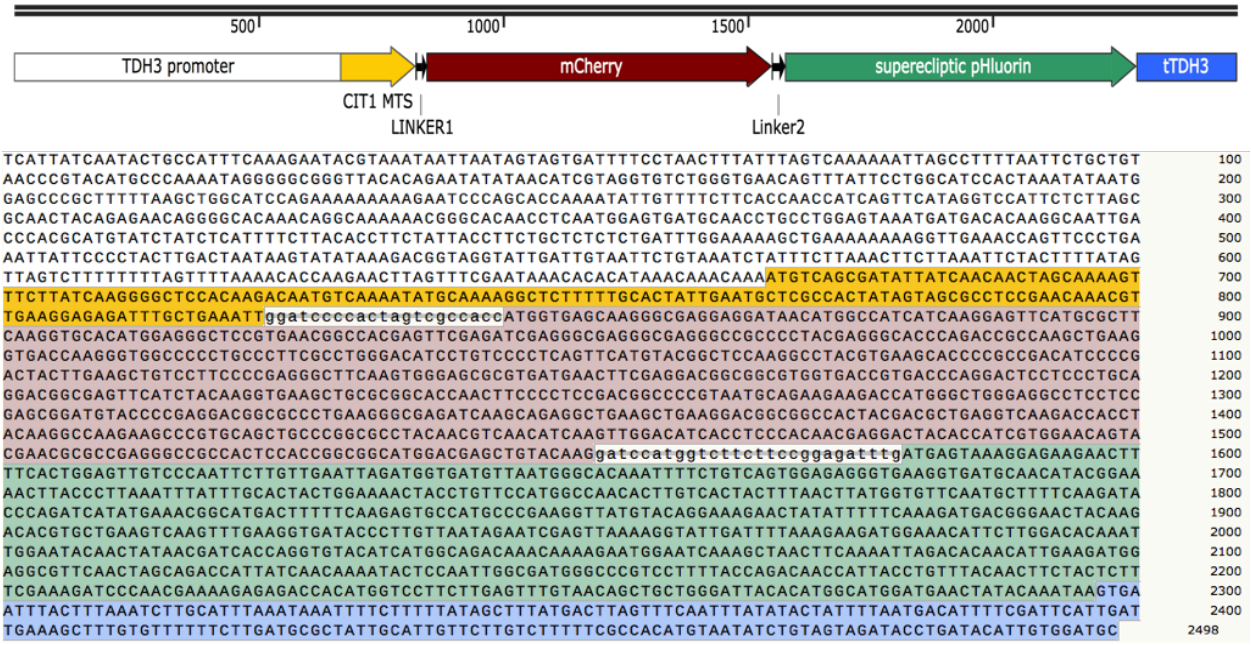


Figure 12 - Biosensor RV2-mt map and sequence. The biosensor cassette is expressed under the constitutive TDH3 promoter. 52 amino acids of the CIT1 gene is used for target the biosensor to the yeast mitochondria. The terminator of TDH3 is used to efficeintly stop the transcription of the biosensor.

For the recovery of the constructs generated, yeast lysis digestion and bacteria plasmid transformation were implemented (Finnigan & Thorner, 2015). The

resulting plasmids were analyzed by enzymatic digestion, PCR, and sequencing. The RV2-mt plasmid was efficiently assembled in all bacteria colonies tested (5 colonies). To validate that the generated plasmid would allow us to discriminate between an autophagy-active WT strain and a mutant with no autophagy activity, the plasmid was transformed in the parental WT yeast strain Y8205 and in a defective autophagy mutant strain, *atg1*Δ. Mitochondrial autophagy was induced in the resulting strains and analyzed by confocal microscopy. These experiments showed that the SEP and mCherry fluorescent signals were observed in mitochondria under respiratory conditions (SCL at 30 ºC and 195 rpm). Importantly, the exclusive presence of mCherry signal inside the vacuole was observed only in autophagy-active WT cells under mitochondrial degradation conditions (SD-N at 30ºC and 180 rpm) (Figure 13).

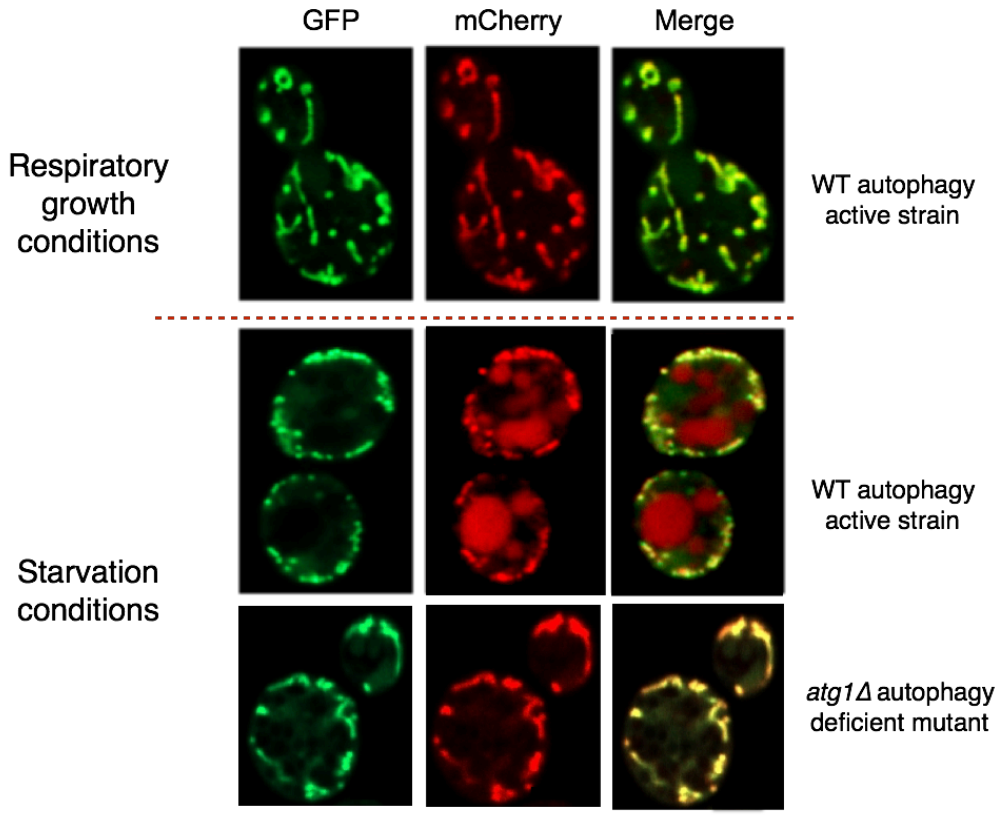


Figure 13 - WT and autophagy-deficient mutant *atg1Δ* yeast cells expressing RV2-mt biosensor. Under respiratory growth conditions, the biosensor is strictly localized in mitochondria (upper panel). Under starvation conditions, WT strain can efficiently activate mitophagy, and the biosensor is transferred to the vacuole losing the GFP signal. *atg1Δ* autophagy-deficient is incapable of delivering the biosensor to the vacuole.

Our results indicate that the plasmid generated is a reliable and straightforward tool for monitoring autophagy activity in yeast cells.

*Most yeast mutants altered in chronological life span have standard respiratory growth capacity.*

The next step was the implementation of a high-throughput methodology for precisely quantifying the loss of SEP fluorescent signal under stress conditions for the entire yeast aging factors collection. To efficiently activate mitochondrial degradation by autophagy in yeast cells, we implemented the mitophagy (selective mitochondrial autophagy) induction protocol reported by Kanki et al. (2009). This protocol starts by growing the yeast cells on medium with a fermentable carbon source (e.g. glucose) and then transferring the cell culture to medium with a non-fermentable carbon source (e.g. lactate). Such mitophagy induction protocol requires cells with respiratory growth capacity. For this reason, we evaluated the respiratory capacity of all single mutant yeast strains with altered CLS phenotype identified by Garay et al. (2014).

We found that ~4% of the yeast mutant strains were not able to grow under respiratory conditions ($p<0.05$). Remarkably, most of the deficient respiratory capacity yeast mutants possess a short-lived phenotype, supporting the notion of the direct influence of mitochondrial functionality in longevity determination (Ocampo et al., 2012) (Figure 14).

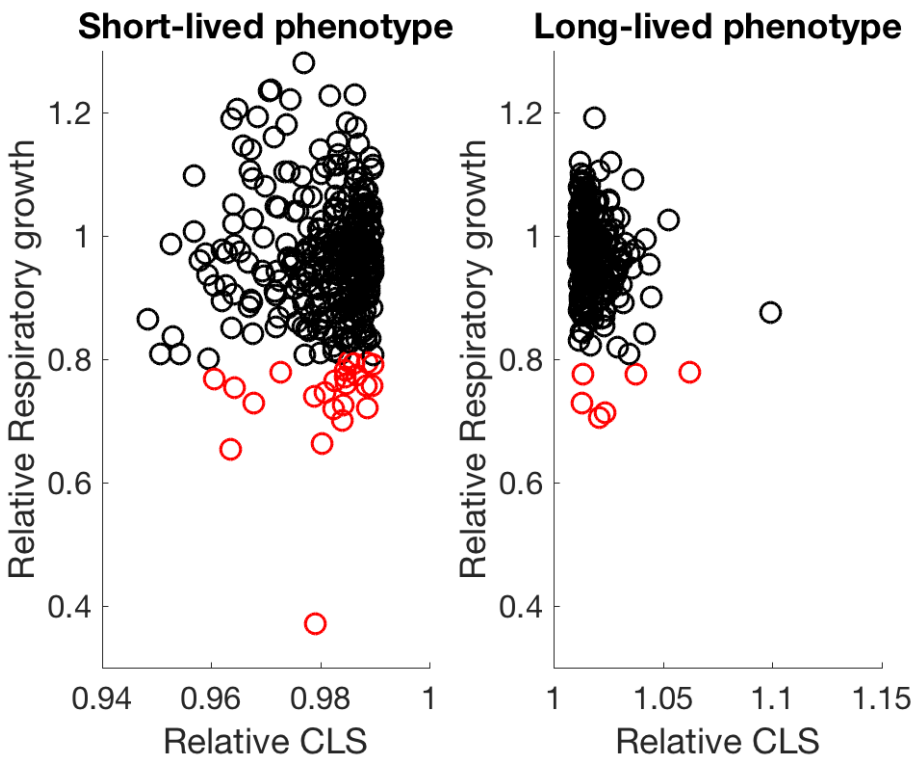


Figure 14 – The respiratory capacity of the whole longevity yeast collection. To eliminate the yeast mutants deficient in non-fermentative respiratory capacity for the mitochondrial autophagy screening, we measured the respiratory growth rate (y-axis) of the complete longevity yeast collection. Those strains with a relative respiratory growth below 0.8 were removed (red dots). A) short-lived mutants; B) long-lived mutants.

Based on these results, a total of 553 yeast mutants were selected and arranged in 96-well plate format, in addition to eight WT and four null-autophagy mutants (*atg1*Δ) as controls in each plate. The resultant yeast library (eight 96-well plates) was named the yeast aging factors collection (YAFC, Supplemental table 1).

*Mitochondrial autophagy can be efficiently quantified by flow cytometry.*

The following step was to transform each of the 553 yeast mutants of the YAFC with the mitochondrial autophagy biosensor, using the synthetic genetic array (SGA) methodology (Boone et al., 2007). 4% of the YAFC did not sporulate and therefore was not further analyzed.

To precisely quantify the capacity of mitochondrial degradation of each one of the yeast mutants in the YAFC, we decided to develop a novel quantitative high-

throughput methodology for monitoring mitochondrial degradation by flow cytometry. The principle of this methodology is the use of two fluorescent proteins with contrasting characteristics in the biosensor. This biosensor characteristic combined with the high-throughput quantification of the degradation of the GFP signal of each yeast strain population allows the precise quantification of the mitochondrial degradation by rating both fluorescent proteins. The mitochondrial autophagy was estimated by determining the ratio of SEP to mCherry signal (SEP signal /mCherry Signal).

To test our method, we estimated the mitochondrial autophagy in two control yeast strains, a positive-autophagy activity strain (WT) and a deficient-autophagy activity strain (*atg1*Δ) (Figure 13). We induced mitochondrial autophagy in the control yeast strains by changing the log-phase cell culture growing under respiratory conditions, to a nitrogen starvation and fermentable growth conditions (Kanki et al., 2009). Then both fluorescent signals, SEP and mCherry, we quantified in at least 30,000 cells for each strain at different times after autophagy induction, to define an optimum and informative time for the identification of autophagy-positive- and autophagy-null mutants. As expected, we observed a reduction in the GFP/mCherry rate in the WT strain but a stable GFP/mCherry rate in the *atg1*Δ strain (Figure 15). Unexpectedly, the GFP/mCherry rate in the *atg1*Δ yeast population increase after 24 hours of autophagy induction. We hypothesized that one possible explanation of this increase in the GFP/mCherry rate could be by the accumulation of the fluorescent molecules lipofuscins, insoluble aggregates of proteins, lipids and iron which accumulate in aged and proteasome/autophagy deficient cells (Matetic et al., 2010; Höhn & Grune, 2013).

Our results indicated that the protocol employed and the analysis of the outcome data is a reliable methodology for the quantification of mitochondrial degradation and allows the discerning of autophagy-positive- and autophagy-null mutants.

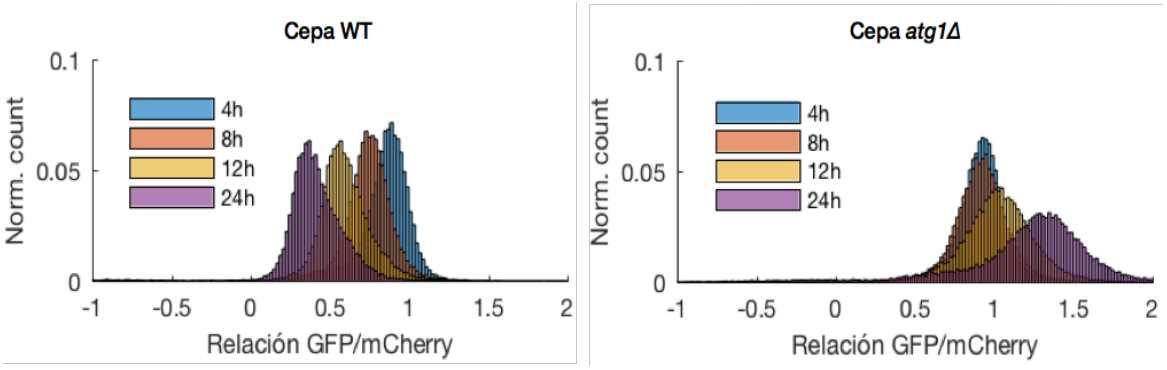


Figure 15 – Histograms of the flow cytometry results of the quantification of mitochondrial degradation in control strains using RV2-mt biosensor. At least 30,000 cells were measured of WT and *atg1Δ* for different times after autophagy induction. A lower GFP/mCherry rate indicate a decreased GFP signal by autophagy. Our results indicate that after 24 h atg1Δ possessed the same null-autophagy activity, while WT strain progressively lost the GFP signal, indicating an active autophagy degradation process.

*The high-throughput analysis of the YAFC suggests that ~10% of the longevity factors are involved in mitochondrial degradation by autophagy*

To identify which longevity genetic factors play a role in mitochondrial degradation by autophagy, we implemented our methodology, in a high-throughput manner, to characterize the capacity of mitochondrial degradation of the YAFC under mitophagy-induction conditions. Our results indicated that most of the longevity genetic factors possess a standard degradation values (MA=~0; 477 mutants). We also observed a small group of mutants with hyper-induced autophagy (29 mutants with significant higher value of MA; $p<0.005$) and a slightly smaller group with reduced-autophagy (23 mutants with significant lower value of MA; $p<0.005$). Using this new high-throughput approach, we identified yeast mutants involved in mitochondrial degradation that had previously not been reported to play a role in this QC pathway (Fig. 16) (Table 1).

| Hyper-induced autophagy strains | |
|---|---|
| **PMT2** | Protein O-mannosyltransferase of the ER membrane / Involved in ER quality control |
| **RPS30B** | Protein component of the small (40S) ribosomal subunit |
| **FYV12** | Protein of unknown function / Function required for Yeast Viability |
| **BRE2** | Subunit of COMPASS (Set1C) complex / functions in silencing at telomeres |

| | |
|---|---|
| **MAK31** | Non-catalytic subunit of N-terminal acetyltransferase of the NatC type |
| **LST4** | Subunit of the Lst4p-Lst7p GTPase activating protein complex for Gtr2p |
| **UBC8** | Ubiquitin-conjugating enzyme that regulates gluconeogenesis |
| **YML084W** | Dubious open reading frame / unlikely to encode a functional protein |
| **SWD1** | Subunit of the COMPASS (Set1C) complex / Required in transcriptional silencing near telomeres |
| **RCF1** | Cytochrome c oxidase subunit / Required for assembly of the Complex III-Complex IV supercomplex |
| **YML083C** | Protein of unknown function |
| **SKI7** | GTP-binding protein / Nuclear transcribed mRNA catabolic process |
| **PUN1** | Plasma membrane protein with a role in cell wall integrity |
| **SUB1** | Transcriptional regulator / Role in nonhomologous end-joining (NHEJ) of ds breaks in plasmid DNA, but not chromosomal DNA |
| **VAC17** | Phosphoprotein involved in vacuole inheritance / Involved in regulation of asymmetric inheritance of aggregated/misfolded proteins and age reset |
| **YLR374C** | Dubious open reading frame; unlikely to encode a functional protein |
| **IZH4** | Membrane protein involved in zinc ion homeostasis |
| **YML082W** | Putative protein predicted to have carbon-sulfur lyase activity |
| **MXR2** | Methionine-R-sulfoxide reductase / Involved in the response to oxidative stress |
| **MAK32** | Protein necessary for stability of L-A dsRNA-containing particles |
| **YNL109W** | Dubious open reading frame; unlikely to encode a functional protein |
| **YAK1** | Sr-Thr protein kinase / Negative regulation of RAS/PKA signaling pathway |
| **SDC1** | Subunit of the COMPASS (Set1C) complex / Required in transcriptional silencing near telomeres |
| **SPG5** | Protein required for proteasome assembly during quiescence |
| **DUS1** | Dihydrouridine synthase |
| **PIF1** | DNA helicase / Possesses strand annealing activity |
| **LGE1** | Protein of unknown function / Null mutant forms abnormally large cells |
| **ENV11** | Protein proposed to be involved in vacuolar functions |
| **LST7** | Subunit of the Lst4p-Lst7p GTPase activating protein complex for Gtr2p |
| **Reduced-autophagy strains** | |
| **RPL23A** | Ribosomal 60S subunit protein L23A |
| **ENV9** | Protein proposed to be involved in vacuolar functions |

| | |
|---|---|
| **OPI3** | Methylene-fatty-acyl-phospholipid synthase / Catalyzes the last two steps in phosphatidylcholine biosynthesis; |
| **MDM31** | Mitochondrial protein that may have a role in phospholipid metabolism |
| **RPN4** | Transcription factor that stimulates the expression of proteasome genes |
| **SWR1** | Structural component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A |
| **CKB1** | Beta regulatory subunit of casein kinase 2 (CK2) |
| **SGF11** | Integral subunit of SAGA histone acetyltransferase complex |
| **MED1** | Subunit of the RNA polymerase II mediator complex |
| **RCY1** | F-box protein involved in recycling endocytosed proteins |
| **ATG10** | Conserved E2-like conjugating enzyme; mediates formation of the Atg12p-Atg5p conjugate, which is a critical step in autophagy |
| **OPI7** | Dubious open reading frame; unlikely to encode a functional protein |
| **SWC5** | Component of the SWR1 complex; complex exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A |
| **MON1** | Subunit of a heterodimeric guanine nucleotide exchange factor (GEF) / Required in Autophagy, Cvt pathway and mitophagy |
| **RPN10** | Non-ATPase base subunit of the 19S RP of the 26S proteasome |
| **RAV2** | Regulator of (H+)-ATPase in Vacuolar membrane / Vacuolar Acidification |
| **TRS85** | Required for membrane expansion during autophagy and the CVT pathway |
| **UPS3** | Mitochondrial protein of unknown function / Involved in regulation of mitochondrial cardiolipin levels |
| **SPT2** | Protein involved in negative regulation of transcription |
| **ATG5** | Conserved protein involved in autophagy and the Cvt pathway; undergoes conjugation with Atg12p to form a complex involved in Atg8p lipidation |
| **UBC4** | Ubiquitin-conjugating enzyme (E2) / mediates degradation of abnormal or excess proteins, including calmodulin and histone H3 |
| **ATG1** | Protein serine/threonine kinase; required for vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway; structurally required for phagophore assembly site formation |
| **VPS30** | Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I and II; Complex I is essential in autophagy |

Importantly, our method allowed us to identify longevity genetic factors previously reported as associated with the reduction (*ATG10, ATG5, VPS30, MON1, MDM31* and *OPI3*) and hyper-activation (*LST4, LST7,* and *KSP1*) of the mitochondrial autophagy (Madeo et al., 2010; Kakua et al., 2012; Sakakibara et al., 2015; Peli-Guli et al., 2015; Umakawa & Klionsky, 2012; Müller et al., 2015). For example, Mon1 had previously been recognized as an essential factor in nonselective and selective

mitochondrial macroautophagy. Mon1 is required for the correct fusion of the autophagosome with the vacuole (Meiling-Wesse et al., 2002). Opi3, a methyltransferase implicated in phospholipid biosynthesis, had been shown to be involved in the formation of autophagosome and is implicated in the upregulation of *ATG32*, the gene encoding the mitochondrial outer membrane protein crucial for mitophagy in yeast (Sakakibara et al., 2015). Finally, Ksp1, a serine/threonine protein kinase, which is a negative regulator of autophagy. Yeast null-mutants in *KSP1* possess an increased autophagy activity, which correlates with our mitochondrial autophagy data (Umakewa & Klionsky, 2012).

These results suggest that the flow cytometry-based methodology developed in this work is a reliable, systematic approach to identify longevity genetic factors as possible new mitochondrial autophagy genetic factors that could participate in the efficient signaling of the damaged organelle (Figure 17).

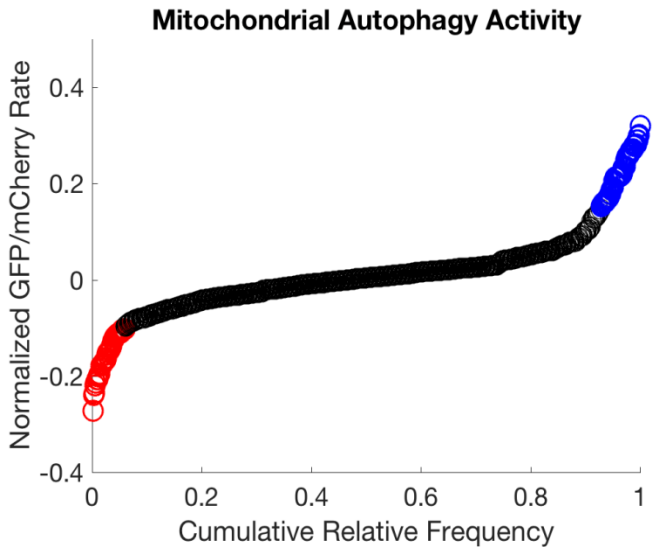


Figure 16 - Mitochondrial autophagy of each longevity genetic factor. In this plot, the graph shows the relative mitochondrial degradation of each yeast knockout mutant of the YAFC (Cumulative relative frecuency). 29 mutants (blue circles) possess a hyper-activation of mitochondrial degradation. On the other hand, 23 mutants (red circles; excluding atg1Δ control strain) possess a null-mutant phenotype.

We noticed that the longevity genetic factors with reduced mitochondrial degradation are implicated in vacuolar function and morphology, mitochondrial dynamics, and phospholipid biosynthesis. On the other hand, hyper-activation of

mitochondrial autophagy is associated to mutant strains altered in mtDNA maintenance, REDOX homeostasis, mitochondrial morphology and chromatin silencing at the telomere. Remarkably, mutants altered in protein kinase and signaling cascades, chromatin remodeling processes, transcriptional regulation, and ubiquitination are represented in both opposite autophagy activity scenarios.

Interestingly, we were able to identify some longevity genetic factors, without previously reported crosstalk with the mitochondrial degradation process. These yeast mutants display a clearly deregulated-autophagy phenotype. Swc5 and Swr1 are essential parts of the Swr1 complex involved in chromatin remodeling process. Depletion of these structural components results in an extended CLS (Garay et al., 2014). However, they showed a significant reduction in mitochondrial autophagy. Another interesting result was the E2 ubiquitin-conjugating enzyme, Ubc4, which mediates the degradation of excessive abnormal proteins. Ubc4 shows a long-lived phenotype and reduced autophagy when this gene is mutated. Finally, Spg5, an essential component of the ubiquitin-proteasome system, had been previously identified as an anti-aging factor (Garay et al., 2014). Interestingly, the yeast deletion mutant of *SPG5*, displayed increased mitochondrial autophagy, suggesting a compensatory effect in the absence of one protein of the QC pathway to restore cellular homeostasis.

*There is no direct causal correlation between the capacity of mitochondrial degradation and chronological life span phenotype.*

Maintenance of mitochondrial homeostasis by efficient mitochondrial autophagy QC pathway had been identified as one of the most important anti-aging pathways that mediate the organism life span (Madeo et al., 2015; Diot et al., 2016). Hence, we decided to look for an association of the mitochondrial autophagy with the chronological life span phenotype in each mutant of the YAFC. Such correlation would indicate the extent to which an extension or reduction of the CLS phenotype could be explained by the mitochondrial degradation capacity.

Interestingly, our results revealed no direct correlation between the mitochondrial degradation capacity and the life span phenotypes. However, we identified a number of specific mutant strains in which this association could be observed. In agreement

with a previous report high-lighting the key role of autophagy in CLS life span extension (Madeo et al., 2010), *lst4*Δ, *lst7*Δ, *spg5*Δ, *yak1*Δ and *pmt2*Δ mutant strains exhibited increased mitochondrial autophagy and a long-lived phenotype. On the other hand, *mon1*Δ, *rav2*Δ, *vps30*Δ, *atg5*Δ, and *atg10*Δ null-autophagy yeast strains showed a short-lived phenotype (Figure 17).

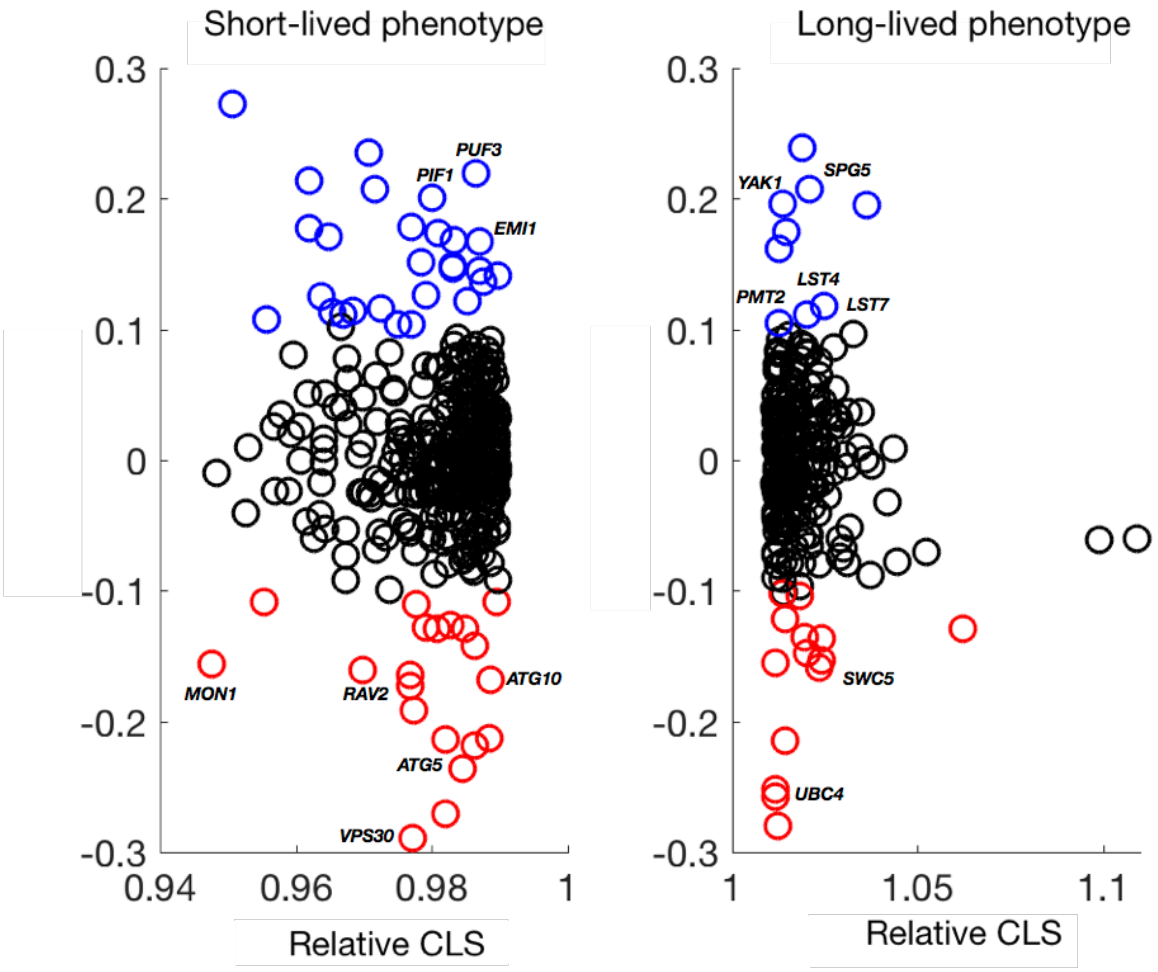


Figure 17 - Correlation between mitochondrial autophagy and relative CLS. Scatter plots showing a not direct correlation between both phenotypes. Strikingly, short- and long-lived yeast strains possess reduced- and hyper-activated mitochondrial autophagy. All autophagy null mutants possess a short-lived phenotype as previously reported (*mon1Δ, rav2Δ, atg5Δ, atg10Δ, vps30Δ*) and those with mutations in signaling pathways which repress autophagy, increase mitochondrial autophagy and extend CLS.

In contrast to the expected results, long-lived strains *ubc4*Δ and *swc5*Δ showed reduced mitochondrial autophagy. Additionally, *puf3*Δ, *pif1*Δ and *emi1*Δ yeast strains, exhibited abnormal mitochondrial morphology and organization, with an increase in the mitochondrial-degradation rate without a beneficial impact on CLS.

*UBC4 and SWC5 are potential novel genetic factors involved in mitochondrial autophagy.*

To validate and characterize the top candidate mutants as probable autophagy factors from our flow cytometry-based screen analysis, we tested 14 reduced-autophagy single-knockout strains by confocal microscopy under standard test-tube conditions (see Materials and Methods). We expected a complete absence of autophagy-activity in these knockout strains, given that our data analysis suggested that they display an *atg1*Δ-like phenotype. Strikingly, *ubc4*Δ and *swc5*Δ displayed a reduced presence of the red fluorescent protein inside the vacuole after 24 hours of nitrogen depletion conditions indicating a reduction in mitochondrial autophagy (Figure 18 c & d). However, *rpn10*Δ, *sgf11*Δ, *swr1*Δ, *rpn4*Δ, *med1*Δ, *spt2*Δ, *mdm31*Δ, *env9*Δ and *ckb1*Δ knockout strains showed a WT-like autophagy phenotype (Figure18e-l).

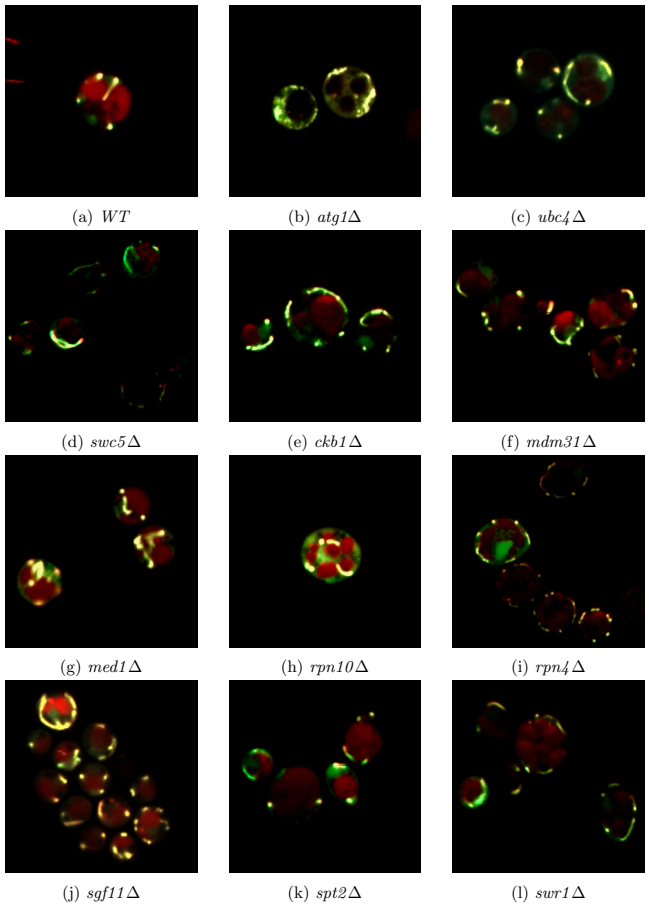


Figure 18 - Validation of top hit yeast candidates. The WT (a) and *atg10*Δ (b) yeast strains possess an expected mitochondrial autophagy phenotype. *ubc4*Δ (c) & *swc5*Δ (d) display a lower presence of red fluorescent protein inside the vacuole, indicating a reduction in mitochondrial autophagy. *rpn10Δ, sgf11Δ, swr1Δ, rpn4Δ, med1Δ, spt2Δ, mdm31Δ, env9Δ* and *ckb1* displayed a WT-like phenotype after 24 hours of nitrogen starvation conditions.

To quantify the effect on mitochondrial degradation of deleting *UBC4* and *SWC5*, we decided to measure the amount of mCherry protein signal inside the vacuole of each yeast population by imaging analysis using ImageJ Software (WT n=435 yeast cells; *ubc*4Δ n=411 yeast cells; *swc5*Δ n=328 yeast cells). As expected, the mCherry-signal distribution comparison between WT and *ubc4*Δ strains, and between WT and *swc5*Δ indicated a significant reduction of the mCherry signal inside the vacuole in both mutant yeast strains (Wilcoxon ranked-sum test, p-Value =1.3e-42; p-Value= 5.1e-64, respectively) (Figure 19).

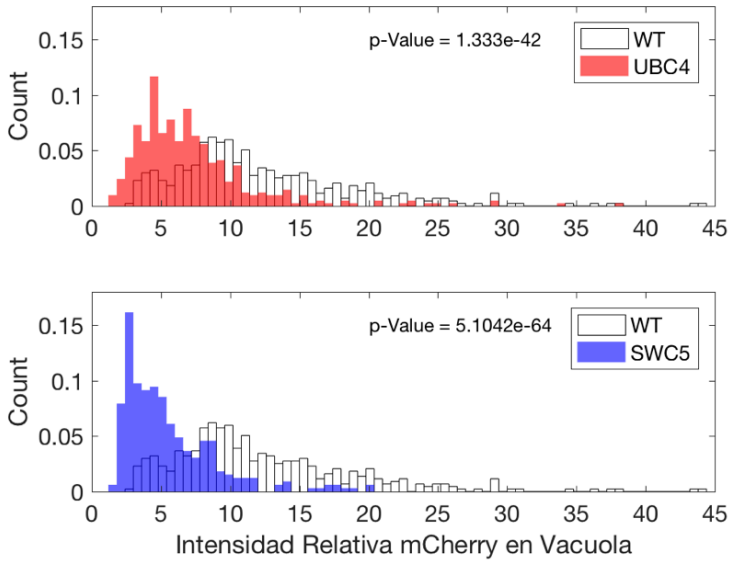


Figure 19 - Histogram showing the distributions of the red fluorescent protein signal inside the vacuole of a *ubc4*Δ knockout population (upper panel) and *swc5*Δ knockout population in comparison with the WT strain. Both mutant distributions display a reduced mean value of red fluorescent protein, indicating a reduction in mitochondrial autophagy.

With these validated results, we conclude that mitochondrial autophagy is severely compromised in the absence of the *UBC4* and *SWC5* longevity genetic factors, and confirmed a crosstalk between ubiquitin system and chromatin remodeling process with the mitochondrial autophagy pathway.

## Discussion

Mitochondrial dysfunction has been extensively described as one of the hallmarks that trigger aging (López-Otín et al., 2013). Therefore, cells need to implement reliable organelle quality control mechanisms that permits discrimination of damaged mitochondria from the healthy mitochondrial population, and their subsequent elimination. Selective mitochondrial degradation through autophagy (mitophagy) is a conserved catabolic system in eukaryotes and the major defective-organelle degradation mechanism that plays an essential role in life span determination. However, the molecular mechanisms underlying this degradation pathway remain uncharacterized (Kanki T et al., 2015).

In this study, we implemented a novel high-throughput flow-cytometry based approach to monitor the mitochondrial degradation capacity of yeast knockout strains affected in stationary-phase survival phenotype (chronological life span) to further understand the crosstalk between mitochondrial autophagy and life span determination.

It has to be remarked that previous attempts to measure mitophagy using flow cytometry analysis have been established in mammalian cells (Mauro-Lizcano et al., 2015). However, the main disadvantage of this method relies on the fluorescence-dependence of the mitochondrial dye to the mitochondrial potential and ROS levels presence inside cell (Xiao et al., 2016), limiting the implementation of this approach in yeast knockout strains with uncharacterized mitochondrial potential and ROS levels phenotypes. On the other hand, even though Rosella biosensor has previously been used for mitophagy analysis using a flow cytometry based method in mammalian cells (Sargsyan., et al 2015), our developed approach is the first large scale flow cytometry screening that attempts to quantify mitochondrial degradation by autophagy in a yeast knockout collection. However, it needs to be clear that our approach does not identify if the mitochondrial degradation is through selective or non-selective macroautophagy machinery.

Due to the close link between life span determination and mitochondrial autophagy, we hypothesized that some of the genetic factors that determine longevity were also

involved in modulating the mitochondrial degradation capacity. To test this hypothesis, we developed a high-throughput flow cytometry-based approach to monitoring the mitochondrial degradation in a life span-altered yeast collection using an autophagy fluorescent biosensor.

Our high-throughput approach revealed that nearly 10% of the genetic factors involved in life span determination affect mitochondrial degradation, implicating that the deregulation of mitochondrial degradation by autophagy during the aging process is independent of the majority of longevity factors. Nevertheless, we realized that mitochondrial degradation is affected by different biological process, some of them with no previous connection with this degradative pathway (Figure 17).

Several studies have been demonstrated a close link between mitochondrial autophagy and aging (Rubinsztein et al., 2011; Martinez-Lopez et al., 2015). Garay et al. (2014) reported that yeast mutants affected in the core autophagy machinery display a short-lived phenotype (Figure 16 - *atg10*Δ, *mon1*Δ, *atg5*Δ, *vps30, and rav2*Δ), while mutants affected in negative-regulation of autophagy increased life span phenotype (*lst4*Δ *and lst7*Δ). Moreover, Richard et al. (2012) demonstrated that yeast mutant specifically affected in mitochondrial autophagy pathway (*atg32*Δ) clearly display a short CLS, demonstrating the importance of this maintaining pathway in life span determination. This evidence suggests that life span determination depends heavily on the autophagy activity. However, our results revealed that there is not a linear correlation between both phenotypes (Figure 17).

We found that this link between mitochondrial autophagy and aging is not bidirectional. An alteration in the autophagy pathway will produce an alteration in life span phenotype. However, due to aging is a multifactorial and complex process, altered life span phenotype does not necessarily represent a deregulation in the autophagy pathway.

Using our proposed approach, we detected two new actors in this degradation pathway. Both Ubc4, an E2 Ubiquitin-conjugating protein, and Swc5, a component of Swr1 complex protein, which are required for efficient activation of the mitochondrial degradation pathway. Further validation using confocal microscopy, confirmed that *UBC4* and *SWC5* are new genetic factors implicated in the complete

activation of mitochondrial autophagy. Interestingly, there is no previous evidence of the action of the proteins encoded by these genes in mitochondrial autophagy pathway.

The ubiquitin-proteasome system is crucial to tag aggregates of damaged proteins for efficient degradation by the proteasome. Nevertheless, if this pathway is overwhelmed, cells can activate a selective autophagy of protein aggregates by ubiquitin-labeled recognition. Selective ubiquitin-cargo recognition for autophagy degradation has only been shown in higher eukaryotes (Schreiber and Peter, 2013). Recent evidence in yeast revealed that selective degradation of ribosomes and mitochondria by autophagy (ribophagy and mitophagy) depend on the ubiquitination/deubiquitination machinery (Kraft C et al., 2008; Müller et al., 2015).

Our findings suggest the existence of a conserved Ubiquitin-signaling pathway for selective degradation of defective mitochondria in eukaryotes. It has been proposed that in yeast, ubiquitination of mitochondria could be a signaling mark for degradation by autophagy. In support of this hypothesis, the elimination of Ubp3-Bre5 deubiquitination complex increased mitophagy rates (Müller et al., 2015). However, the presence of a molecular pathway of Ubiquitin-selective autophagy in yeast remains to be characterized.

Mitophagy induction in higher eukaryotes is highly dependent on the Pink1 (Serine/Threonine ubiquitin-kinase) and the Parkin (E3 ubiquitin ligase) ubiquitin signaling pathways. Damaged mitochondria recruit Pink1 to the outer mitochondrial membrane to promoting Parkin recruitment. This E3 ligase binds ubiquitin chains to outer mitochondrial membrane proteins, resulting in the recruitment of autophagy receptor and mitochondrial degradation by autophagy (Lazarou M et al., 2015). In agreement, knockdown of E2 enzymes in HeLa Cells delays ubiquitination and degradation of damaged mitochondria (Fiesel et al., 2014). Pink1 and Parkin homologous have not yet been identified in yeast (Schreiber A & Peter M, 2013).

In addition, two E2 ubiquitin-like proteins are involved in autophagosome formation: Atg3 and Atg10 have a fundamental role in the biogenesis of the autophagosome membrane by cleaving essential Atg proteins and lipids in an E2

ubiquitin-like reaction. Atg3 and Atg10 allow the correct expansion and closure of the sequestrating membrane, indispensable for cellular cargo isolation and delivery to the vacuole of the cell (Nakatogawa H, 2013).

Based on our results and previous reports, we propose that Ubc4 is a key signaling factor for selective degradation of mitochondria by autophagy via two possible scenarios: i) As part of ubiquitination-tagged process of damage mitochondria in a Pink1/Parkin-like manner; or ii) By taking part in autophagosome formation in an Atg3/Atg10 manner during early steps in the autophagosome biogenesis.

We also identified *SWC5* as a genetic factor that influences mitochondrial-degradation. Swc5 is a subunit of the chromatin remodeling SWR1 complex, involved in nucleosome remodeling, transcriptional regulation, gene silencing, chromosome segregation and DNA repair (Morillo-Huesca M et al., 2010). It has been proven that histone modifications can regulate the transcription of autophagy-related proteins (Fahrenkrog B, 2016). Deacetylation of histone H3 and histone H4 are two well-characterized histone modifications linked to autophagy regulation (Füllgrabe J et al., 2014). However, the mitochondrial autophagy de-regulation via histone modification generated by Swr1-complex has not been previously observed.

In yeast, the SWR1 complex replaces histone H2A with the variant H2A.Z. H2A.Z histone variant is present near activated-gene promoters and is required for rapid-response transcription activation (Tramantano et al., 2016). Based on this knowledge, we hypothesize that the interaction between *SWC5* and the autophagy could be a direct association. Indeed, the *swc5*Δ mutant shows a systematic transcriptional deregulation that could include some autophagy-related genes, causing the inactivation of the mitochondrial autophagy (Morillo-Huesca et al., 2010). Therefore, *swr1*Δ yeast strain should also display a *swc5*Δ-like mitochondrial autophagy-reduced phenotype. Unexpectedly, the *swr1*Δ yeast mutant strain showed a WT-like mitochondrial degradation phenotype after 24 hours of mitochondrial autophagy induction (Figure 18 - l).

Swr1 has been proposed to be the key protein responsible for dissociating the histone octamer, helping Swc5 to achieve the histone replacement (Morillo-Huesca et al., 2010). A global protein-protein interaction in *S. cerevisiae* (Krogan et al., 2006),

demonstrated that Swc5 could also interact with Cka1, an alpha subunit of the Casein kinase 2. This serine/threonine kinase is responsible for the phosphorylation of the mitochondrial membrane protein Atg32, an essential step for the signaling of mitochondria to be degraded (Kanki et al., 2013). We hypothesized that both genetic mutation, *swr1*Δ and *swc5*Δ, causes transcription deregulation and genetic instability (Morillo-Huesca et al., 2010), generating the activation of stress-response pathway such as autophagy. However, in *swc5*Δ yeast mutants, mitochondrial autophagy signaling is affected due to an inefficient phosphorylation step of Atg32 (Figure 18 – d; Figure 19).

Strikingly, both yeast mutant strains *ubc4*Δ and *swc5*Δ have a long-lived phenotype but show a decreased mitochondrial autophagy. The question of how these mutations result in an extended life span without an efficient mitochondrial degradation pathway remains. We propose two possible explanations for this unexpected result: The long-lived phenotype in diminished mitochondrial degradation mutants could be due to a feedback mechanism resulting in i) compensatory effects from other selective autophagy types; or ii) compensatory effects from another proteolytic system.

Our first proposal is based on previous reports that suggests a "regulatory switch" between different types of selective autophagy (Kraft et al., 2008; Müller et al., 2015). In an ubiquitination-deficient machinery strain (such as in *ubc4*Δ yeast mutant) cells cannot label damaged mitochondria with ubiquitin, avoiding the recognition for the autophagy machinery. However, ribophagy could be efficiently activated during these stress conditions. The selective degradation of the ribosome by autophagy may extend the CLS by ribosomal degradation because it promotes the downregulation of the translation machinery and reduces the chance of forming damaged protein aggregates via $H_2O_2$ produced by damaged mitochondria (Weids A et al., 2016).

In addition, under nuclear DNA-stress conditions, such as in *swc5*Δ depleted yeast mutant (Morillo-Huesca et al., 2010), selective degradation of nucleus-derived materials by autophagy, named nucleophagy, could be activated (Mijaljica and Devenish, 2013). Hence, nucleophagy could repress other types of autophagy to ensure optimal concentrations of nuclear-autophagy components, which could, in turn, extend the chronological life span by removing damaged nuclear components

(Vessoni et al., 2013).

Our second proposal that the cause of life span extension in mitochondrial autophagy-deficient mutants relies on the crosstalk between both degradation pathways, ubiquitin-proteasome, and autophagy. It has been extensively reported that if one degradative pathway is blocked, the other displays a stronger response (Park and Cuervo, 2013). In support of this hypothesis, we highlight one specific case that our high-throughput genetic approach identified. A *spg5*Δ yeast mutant strain is unable to correctly form the proteasome machinery. In response to this proteasome-deficient capacity, the cell displays a hyper-activation of mitochondrial autophagy leading to an extension in CLS. This evidence could also explain the unexpected *ubc4*Δ and *swc5*Δ results. Possibly the extension in the life span of these mutant strains is produced by a compensatory effect between degradative pathways, upregulating the activation of the other degradation system in reduced-mitochondrial autophagy.

Finally, our flow-cytometry data analysis suggested that the *rpn10*Δ, *sgf11*Δ, *swr1*Δ, *rpn4*Δ, *med1*Δ, *spt2*Δ, *mdm31*Δ, *env9*Δ and *ckb1*Δ knockout strains would be autophagy-null mutants. However, we found that these mutants showed a WT-like autophagy activity under confocal microscopy analysis, suggesting a poor correlation between the two approaches. We assumed that this discrepancy in our results is because mitochondrial autophagy was monitored at different time point depending on the analysis approach (flow cytometry - 8 hours after induction; confocal microscopy - 24 hours after induction). We hypothesized that those mutants that displayed an atg1Δ-like phenotype after 8 hours of autophagy induction, but a WT-like phenotype after 24 hours of induction, could be affected in the signaling pathway of the mitochondrial autophagy, delaying their completely activation.

## Concluding remarks

In conclusion, our methodology allowed us to identify previously reported mitophagy genetic factors (Kanki et al., 2009; Okamoto et al., 2009; Müller et al., 2015) demonstrating that our high-throughput approach is a reliable and straightforward tool for elucidating the main molecular mechanisms that rule this degradation pathway. Importantly, our genetic screen allowed us to identify that the genes that encode Ubc4, an E2 ubiquitin-conjugating enzyme, and Swc5, a

chromatin remodeling subunit, as possible new molecular players in mitochondrial autophagy. Our approach is not limited to monitoring mitochondrial degradation and can be implemented for further genetic screenings of the different types of selective autophagy (nucleophagy, ribophagy, bulk-autophagy).

## Perspectives

As further perspective of this study, we suggest validating the top candidates which did not show a clear *atg1*Δ-like deficient phenotype (*rpn10*Δ, *sgf11*Δ, *swr1*Δ, *rpn4*Δ, *med1*Δ, *spt2*Δ, *mdm31*Δ, *env9*Δ and *ckb1*Δ) by using Om45-GFP (Kanki et al., 2009) or mt-alkaline phosphatase delivery assays (Mendl et al., 2011). These western-blot based approaches allow the identification of partially and slightly defective mitophagy yeast mutants.

Even though we have not yet implemented an assay for validating top yeast candidates, confocal microscopy allowed us to identify two unreported longevity genetic factors involved in selective mitochondria degradation, *UBC4* and *SWC5*. It would be interesting to unreveal why *ubc4*Δ and *swc5*Δ have a deficiency in mitochondrial autophagy. For this purpose, we could monitor these mutant strains using the GFP-Atg8 marker. GFP-Atg8 marker would reveal if the general machinery of autophagy pathway is altered in these mutants, or if the observed behavior is a specific error in the mitochondrial degradation pathway, by incorrect tagging or damage signaling (Klionsky et al., 2016).

Finally, our study sets the basis for screening the entire yeast deletion collection and to perform a genome-wide screening analysis for identifying new modulators of specific autophagies in *S. cerevisiae*, focusing not only mitochondrial degradation but also on that of other organelles such as peroxisomes, nucleus, ribosomes, or the endoplasmic reticulum.

## References


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