**Universidade do Minho**

Escola de Ciências da Saúde

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# The emerging role of autophagy/mitophagy in α-synuclein-induced toxicity: studies on the yeast chronological aging model

# O papel emergente da autofagia/mitofagia na toxicidade induzida por α-sinucleína: estudos no modelo de levedura de envelhecimento cronológico

Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da

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**Setembro de 2012**

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**Título da dissertação:** The emerging role of autophagy/mitophagy in α-synuclein-induced toxicity: studies on the yeast chronological aging model

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Professora Doutora Paula Cristina da Costa Alves Monteiro Ludovico

**Ano de conclusão:** 2012

**Designação Ramo de Conhecimento do Doutoramento:**

Ciências da Saúde – Ciências da Saúde



Universidade do Minho, 25 de Setembro de 2012

Assinatura: _____


The work presented in this thesis was mainly done within the Microbiology and Infection Research Domain in the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho. The financial support was given by Fundação para a Ciência e Tecnologia by means of a project PTDC/BIA-MIC/114116/2009 and also by a grant, SRFH/BD/41674/2007.

## AGRADECIMENTOS

No fim do percurso tenho de recuar no tempo e agradecer a quem comigo percorreu estes 4 anos de trabalho. E, embora este seja o meu trabalho, muitas pessoas contribuíram para a sua concretização. Assim, gostaria de agradecer:

À Professora Doutora Paula Ludovico devo o meu maior agradecimento. Ao longo dos anos para além de minha orientadora, excelente nessa função, tornou-se também uma amiga. Quero agradecer a confiança que depositou em mim no dia em que aceitou ser minha orientadora. Obrigada pelos conselhos, pelos ensinamentos, criticas e sugestões. Quero também pedir desculpa pelas minhas asneiras e dizer que prometo que vou aprender com todos os "raspanetes". Obrigada chefe, por me ensinares a crescer como investigadora e como pessoa.

À Professora Patrícia Maciel gostaria de expressar o meu reconhecimento pelo auxílio prestado.

Ao Professor Fernando Rodrigues que me abriu as portas para a investigação. Obrigada por todos os ensinamentos, conselhos e discussões científicas...e pelas críticas, que apesar de no momento me parecerem absurdas, agora percebo que eram válidas e que me ensinaram a crescer cientificamente.

À Professora Cecília Leão pelo apoio científico e amigo que me ofereceu desde o início da minha caminhada no ICVS e que tem sempre uma palavra amiga para aconselhar e alegrar os dias menos bons.

Ao ICVS, na pessoa do Professoro Jorge Pedrosa, queria agradecer a oportunidade e o privilégio de trabalhar neste excelente instituto que permite produzir ciência de qualidade.

À Fundação para a Ciência e Tecnologia pelo financiamento que permitiu a realização deste trabalho.

Ao ID9, a todos os que fizeram parte desse laboratório, por todos os momentos partilhados, pela disponibilidade e amizade. No entanto tenho de realçar algumas pessoas: a Cris, a Xana, o Bruno e o Carvalho. Quero-vos agradecer toda a ajuda e amizade que facilitaram a minha vida durante este tempo. Vocês realmente fazem parte de toda a minha jornada diária, dos maus e principalmente dos bons momentos, dos dias em que me apetecia atirar a ciência ao lixo e dos dias "cor de rosa". Foi uma honra e um privilégio conhecer-vos, sinto a vossa falta todos os dias, mas sei que se precisar de alguma coisa posso contar com vocês. Muito obrigada AMIGOS.

Aos MIRD, antigos e recentes amigos e colegas, por toda a ajuda, partilha de informação e amizade. Por todo o tempo perdido a ouvir as minhas lamúrias. Obrigada a todos.

Aos antigos e recentes amigos e colegas dos NERD e Ciências Cirúrgicas, por toda a ajuda, partilha de informação (e reagentes) e amizade. Obrigada a todos.

Aos meus três amores, o Paulo e meus gémeos. A ti Paulo quero agradecer por todo o apoio incondicional e por toda a compreensão de que por vezes (muitas, eu sei!!!) o meu trabalho veio primeiro do que tudo resto. Obrigado por compreenderes que a minha vida no laboratório também é muito importante, e por isso peço-te também desculpas por todo o tempo roubado à nossa vida. Quero também agradecer todo o tempo perdido a contar as minhas plaquinhas, eu sei custa um pouco, mas repara o resultado até foi bom!!! OBRIGADA AMOR!

Por último, mas não menos importante, quero agradecer a toda a minha família, especialmente aos meus pais, por todo o apoio que me deram ao longo destes quatro anos, ou melhor, ao longo da minha vida. Peço-vos desculpa por todas as minhas ausências, e por todo o trabalho que sobrecarrego em vocês. Obrigada pelos conselhos e por todo o apoio e ensinamento, afinal parte do que sou e do que alcancei foi graças a vocês. MUITO OBRIGADA.

## ABSTRACT

α-Synuclein (α-syn) misfolding and aggregation is strongly associated with both idiopathic and familial forms of Parkinson disease (PD). Evidence suggests that α-syn has an impact on cell clearance routes and protein quality control systems, such as the ubiquitin-proteasome system (UPS) and autophagy. Recent advances in the key role of the autosomal recessive *PARK2/PARKIN* and *PINK1* genes in mitophagy, highlighted this process as a prominent new pathogenic mechanism. Nevertheless, the role of autophagy/mitophagy in the pathogenesis of sporadic and autosomal dominant familial forms of PD is still enigmatic. The yeast *Saccharomyces cerevisiae* is a powerful "empty room" model that has been exploited to clarify different molecular aspects associated with α-syn toxicity, which combines the advantage of being an established system for aging research. Here, we showed that the heterologous expression of α-syn WT or the A53T mutant form induced toxicity, additionally the toxic phenotype was more aggravated when the α-syn expression was induced at the stationary cells growth phase. At this growth phase, it was demonstrated that the induction of the toxic α-syn variants led to the stimulation of the unfolded protein response (UPR), by the activation of the Ire-Hac1 signaling pathway. The activation of this pathway is probably responsible by the induction of the *ATG6* and *ATG8* mRNA levels, linked with autophagy and *ATG32*, *ATG33* and *DNM1* mRNA levels, which are genes specifically associated with mitophagy, suggesting that autophagy and mitophagy are stimulated under α-syn-induced toxicity. In fact modulation of autophagy, by its inhibition with chloroquine or 3 methyladenine, reverted the α-syn toxic phenotype detected by an extension of in the chronological life span (CLS) of cells expressing the α-syn WT or A53T mutant forms. Additionally, with the specific method to quantify the autophagy or mitophagy activities, the alkaline phosphatase assay, it was confirmed that the toxicity due to the heterologous expression of the toxic α-syn variants was accompanied by exacerbated stimulation of both autophagy and mitophagy. Modulation of mitophagy, by its impairment, achieved by deletion of *ATG11* or *ATG32* resulted in a CLS extension, accompanied by physiologic autophagic levels, further implicating mitophagy in the α-syn toxicity.

It is established that reactive oxygen species, particularly superoxide anion ($O_2^{2-}$), are associated with autophagy induction. In the experimented conditions, the stimulation of autophagy/mitophagy under α-syn expression was accompanied with $O_2^{2-}$ accumulation. Nevertheless, cells with impaired mitophagy display lower levels of $O_2^{2-}$. Thus, the findings do not

rule out $O_2^{2-}$ as inducer of autophagy in these conditions. Other molecules that have been associated with the regulation of autophagy in higher eukaryotic cells are the sirtuins, particularly the SIRT1. Deletion of *SIR2*, the yeast homologue of SIRT1, essential for α-syn-induced toxicity, abolished autophagy and mitophagy, thereby rescuing cells from α-syn toxicity. These data show that Sir2 functions as a regulator of autophagy, like its mammalian homologue, SIRT1, but also of mitophagy, mediating the *ATG32* mRNA levels.

Caloric restriction (CR) is a physiologic intervention known to promote life span extension, by reducing the activity of various signal transduction pathways either directly or through the decrease in the activity of nutrient-sensing pathways. Wild type cells submitted to CR experienced a CLS extension accompanied by physiological levels of autophagy/mitophagy, independently of the expression of α-syn toxic variants. In fact, in α-syn-induced toxicity conditions, autophagy/mitophagy induction was significant lower in comparison with the same cells submitted to non-CR conditions, supporting the hypothesis that induction of autophagy/mitophagy are directly related with the α-syn-induced toxicity. CR, like mitophagy abrogation, has the capacity to reduce α-syn-induced toxicity. The observed high CLS of *atg11Δ* and *atg32Δ* cells expressing α-syn WT or A53T mutant form in comparison with the wild type cells submitted to the same conditions, suggested that CR controls autophagy/mitophagy independently, or at least partially independent, of the pathways regulating mitophagy . Similar results were observed in *sir2Δ* cells, in which CR is able to further increase the CLS of *sir2Δ* cells expressing α-syn toxic variants, suggesting that under CR conditions, the activated signaling pathways are independent of Sir2. Altogether our work highlights that α-syn expression is associated with autophagy, particularly mitophagy stimulation mediated by the regulation of *ATG32* by Sir2, an important phenomenon linked to α-syn-induced toxicity during aging. These findings open new insights to the study of the mechanism associated with PD pathogenesis in higher eukaryote organisms.

## RESUMO

A acumulação intracelular da proteína α-sinucleina (α-sin) com alterações conformacionais leva à formação de agregados que estão associados com as formas idiopática e familiar da doença de Parkinson (DP). Evidências sugerem que a α-sin interfere com as vias de degradação proteica e com o sistema de controlo de qualidade das proteínas, tais como o sistema ubiquitina-proteasoma e a autofagia. Um papel fundamental das proteínas *PARK2/PARKIN* e *PINK1*, cujos genes estão envolvidos em formas autossómicas recessivas da DP, na regulação da mitofagia, destacam este processo como um novo proeminente mecanismo patogénico da DP. No entanto, o papel da autofagia/mitofagia na patogénese das formas esporádica e familiar autossómica dominante da DP é ainda enigmático. A levedura *Saccharomyces cerevisiae* é um poderoso modelo, que tem sido explorado para esclarecer diferentes aspectos moleculares associados com a toxicidade da α-sin, combinando a vantagem de ser um sistema estabelecido para o estudo do envelhecimento. Este trabalho demonstra que a expressão heteróloga da α-sin selvagem ou da forma mutante A53T induz toxicidade, que é mais evidente quando a expressão da α-sin foi induzida em células envelhecidas (pós-mitóticas em fase estacionária de crescimento). A expressão das variantes tóxicas de α-sin levou à ativação de uma resposta específica da célula através da via de sinalização Ire-Hac1. A activação desta via é, provavelmente, responsável pelo aumento dos níveis de mRNA dos genes *ATG6* e *ATG8,* associados com o processo autofágico, e também dos genes *ATG32*, *ATG33* e *DNM1*, especificamente associados com a mitofagia, sugerindo que a autofagia e a mitofagia estão implicadas na toxicidade induzida pela expressão de α-sin. De fato, a modulação da autofagia, através da sua inibição com cloroquina ou 3 metiladenina reverteu o fenótipo tóxico da α-sin, detetado através do aumento da longevidade cronológica das células. Paralelamente, com o auxílio de um método específico para a quantificação da atividade autofágica ou mitofágica, confirmou-se que a toxicidade devida à expressão heteróloga das variantes tóxicas de α-sin foi acompanhada pela estimulação exacerbada do processo autofágico e mitofágico. A modelação da mitofagia, através da sua inibição, por deleção dos genes *ATG11* ou *ATG32*, resultou numa extensão da longevidade cronológica, acompanhada por níveis autofágicos fisiológicos, implicando a mitofagia na toxicidade induzida pela α-sin.

Está estabelecido que as espécies reactivas de oxigénio, em particular o anião superóxido ($O_2^{2-}$), regulam a indução de autofagia. Nas condições em estudo, a estimulação da autofagia/mitofagia em células a expressar α-sin foi acompanhada pela acumulação de $O_2^{2-}$. No

entanto a acumulação de $O_2^{2-}$ não é observada em células incapazes de realizar mitofagia. Assim, os resultados não descartam a possibilidade de $O_2^{2-}$ estar a contribuir para a indução da autofagia, em condições de toxicidade induzida pela α-sin. Outras moléculas que têm sido associadas com a regulação da autofagia, em células eucariotas superiores, são as sirtuinas, particularmente SIRT1. A deleção de Sir2 (proteína de levedura homólogo de SIRT1), cujo seu papel foi previamente demonstrado como sendo crucial para a toxicidade da α-sin, resultou na supressão da autofagia e da mitofagia, e consequentemente numa melhor performance das células. Estes dados mostram, pela primeira vez, que Sir2 para além de ser uma molécula reguladora da autofagia, tal como o seu homólogo de mamífero, SIRT1, regula também a mitofagia, controlando os níveis de mRNA do gene *ATG32*.

A restrição calórica (RC) é uma intervenção fisiológica que promove a extensão da longevidade, reduzindo diretamente a atividade de várias vias de transdução de sinal, ou diminuindo a actividade das vias de sinalização de nutrientes. As células da estirpe selvagem submetidas a RC demonstraram uma extensão na longevidade cronológica, acompanhada por níveis fisiológicos de autofagia/mitofagia, independentemente da expressão das variantes tóxicas de α-sin. No entanto, esta indução foi significativamente menor quando comparada com as mesmas células submetidas a condições de não-RC, corroborando a hipótese de que a RC tem a capacidade de reduzir a toxicidade induzida por expressão da α-sin. Adicionalmente, células deletadas no gene *ATG11* ou *ATG32*, a expressar α-sin selvagem ou a forma mutante A53T, quando sujeitas a RC apresentaram uma maior longevidade cronológica em comparação com as células da estirpe selvagem submetidos às mesmas condições, o que sugere que a RC tem a capacidade de controlar a toxicidade, devido a expressão de α-sin, por mecanismos independentes, ou pelo menos parcialmente independentes, dos mecanismos que estão na base da regulação da mitofagia. Os mesmos resultados foram observados para as células deletadas no gene *SiR2*, sugerindo que a RC regula a autofagia/mitofagia por vias de sinalização independentes de Sir2. Em suma, o nosso trabalho sugere que a expressão de α-sin promove a estimulação da autofagia, particularmente da mitofagia, e que esta indução é regulada pelos níveis de mRNA do gene *ATG32*, através da acção da proteína Sir2, um evento relevante ligado à indução da toxicidade de α-sin, durante o envelhecimento. Estes resultados abrem novas perspectivas para o estudo de mecanismos associados com a patogénese da DP, em organismos eucariotas superiores.

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ABBREVIATIONS

| | |
|---|---|
| **3-MA** | 3-Methyladenine |
| **α-Syn** | α-Synuclein |
| **Act1** | Actin 1 |
| **AD** | Alzheimer disease |
| **ALD** | Amyotrophic lateral sclerosis |
| **ALP** | Alkaline phosphatase |
| **Ams1** | α-Mannosidase |
| **Ape1** | Aminopeptidase 1 |
| **ATG** | Autophagy related |
| **BSA** | Bovine serum albumin |
| **CCCP** | Carbonyl cyanide m-chlorophenylhydrazone |
| **cDNA** | Complementary deoxyribonucleic acid |
| **CLS** | Chronological life span |
| **CMA** | Chaperone-mediated autophagy |
| **CPY** | Carboxypeptidase Y |
| **CQ** | Chloroquine |
| **CR** | Caloric restriction |
| **Cvt** | Cytoplasm-to-vacuole targeting |
| **DHE** | Dihydroethidium |
| **DHR** | Dihydrorhodamine 123 |
| **DNA** | Deoxyribonucleic acid |
| **DTT** | Dithiothreitol |
| **DUBs** | De-ubiquitylating enzymes |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EM** | Electron microscopy |
| **ER** | Endoplasmic reticulum |
| **ERAD** | ER-associated degradation |
| **ERCs** | Extrachromossomal ribosomal DNA circles |
| **FACS** | Fluorescence Activated Cell Sorting |
| **GFP** | Green fluorescence protein |

| | |
|---|---|
| **HD** | Huntington disease |
| **HSP** | Heat shock protein |
| **HSF** | Heat-shock factor |
| **IRC** | Index of respiratory competence |
| **IRE1** | Inositol-requiring kinase 1 |
| **LAMP-2A** | Lysosomal-associated membrane protein 2A |
| **LB** | Lewy bodies |
| **LR** | Lewy neuritis |
| **Min** | Minutes |
| **mtDNA** | Mitochondrial deoxyribonucleic acid |
| **mtDsRed** | Mitochondrially-targeted DsRed |
| **mTOR** | Mammalian target of rapamycin |
| **NVJs** | Nucleus-vacuole junctions |
| **PAGE** | Polyacrilamide gel electrophoresis |
| **PAS** | Phagophore assembly site |
| **PBS** | Phosphate-buffered saline |
| **PCR** | Polymerase chain reaction |
| **PD** | Parkinson disease |
| **PDA1** | Alpha subunit of pyruvate dehydrogenase |
| **PE** | Phosphatidylethanolamine |
| **PI** | Propidium iodide |
| **PINK1** | PTEN-induced kinase-1 |
| **PMN** | Piecemeal microautophagy of the nucleus |
| **PN** | Proteostasis network |
| **PtdIns3K** | Phosphatidylinositol 3-kinase |
| **PtdIns(3)P** | Phosphatidylinositol (3)-phosphate |
| **qPCR** | Quantitative Polymerase chain reaction |
| **RLS** | Replicative life span |
| **Rpm** | Rotations per minute |
| **RNA** | Ribonucleic acid |
| **ROS** | Reactive oxygen species |

| | |
|---|---|
| **RT-PCR** | Real-time Polymerase chain reaction |
| **SC** | Synthetic complete |
| **SEM** | Standard error of the mean |
| **SIR2** | Sirtuin 2 |
| **SOD1** | Superoxide dismutase 1 |
| **SOD1** | Superoxide dismutase 2 |
| **TBS** | Tris-buffered saline |
| **TBST** | Tris-buffered saline with 0.1% Tween 20 |
| **TDH2** | Isoform 2 of glyceraldehyde-3-phosphate dehydrogenase |
| ***TPI1*** | Triose phosphate isomerase |
| **TORC1/2** | Target of rapamycin complex 1 or 2 |
| **ULK1/2** | Uncoordinated 51-like kinase 1 or 2 |
| **UPR** | Unfolded protein response |
| **UPRE** | Unfolded protein response element |
| **UPS** | Unfolded protein system |
| **UVRAG** | Ultraviolet irradiation resistant-associated gene |
| **WT** | Wild type |
| **XBP1** | Box binding protein 1 |
| **YPG** | Yeast peptone Glycerol |
| **YEPD** | Yeast Extract Peptone Dextrose |

# OBJECTIVES AND OUTLINE OF THE THESIS

In the last decade, several piece of evidence established a connection between the cell clearance routes and protein quality control systems, such as the ubiquitin-proteasome system (UPS) and autophagy, and the human physiology. Particularly, deregulation of autophagy is emerging as the common basis of numerous pathologies such as neurodegenerative diseases as the Parkinson disease (PD), the most common neurodegenerative movement disorder. PD is mainly a sporadic disease, nevertheless, monogenic familial forms of the disease, characterized by an autosomal dominant or recessive pattern of inheritance have also been identified. One of the genes associated with familial PD is the gene that codifies to α-synuclein (α-syn). α-Syn is a ubiquitously expressed protein in the brain with an intrinsic ability to bind to lipids and membranes. The function of α-syn is still unclear, but it regulates synaptic plasticity, dopamine neurotransmission and endoplasmic reticulum-Golgi trafficking, and acts as a molecular chaperone. In normal conditions, the soluble forms of α-syn are degraded by chaperone-mediated autophagy and by UPS, whereas insoluble and aggregated forms, due to overexpression or mutations in gene codifying α-syn, inhibit these protein control quality systems leading to the pathogenesis of PD associated with an upregulation of autophagy. In addition, oxidative stress and mitochondria dysfunction have also been implicated in the pathogenesis of PD. It was recently proposed that this could be a consequence of the loss of mitochondria due to overactivation of autophagy. Recent advances in the key role of the autosomal recessive PARK2/PARKIN and PINK1 genes in mitophagy highlighted this process as a prominent new pathogenic mechanism. Nevertheless, the role of autophagy/mitophagy in the pathogenesis of sporadic and autosomal dominant familial forms of PD is still enigmatic. The yeast *Saccharomyces cerevisiae* is a powerful "empty room" model that has been exploited to clarify different molecular aspects associated with α-syn toxicity, which combines the advantage of being an established system for aging research.

The work reported in this thesis aimed to get new insights on the contribution of autophagy/mitophagy for the toxicity induced by the heterologous expression of the human wild-type α-syn gene and the clinical A53T mutant during yeast chronological life span (CLS).To drive the reader through the main achievements, this thesis was organized in four chapters:

In **chapter 1**, a general introduction, mainly centered on the knowledge of the cellular quality control systems that mediate cellular proteostasis, with particular emphasis on autophagy, will be given. In addition, a revision on the actual acknowledge about the contribution of autophagy in particularly for PD pathogenesis will be made. Finally, the impact of aging as a main risk factor for neurodegenerative diseases will be discussed.

In **chapter 2**, all the materials and methods used in this work are referred.

In **chapter 3**, results are presented and discussed in two sections. The results presented in the section 3.1 show evidence indicating that α-syn toxicity is associated with autophagy and mitophagy in yeast aged cells. The findings on the caloric restriction effects in the toxicity induced by the presence of α-syn in aged cells will be also described in section 3.2.

Finally, **chapter 4** comprises an integrative discussion focused on the main contributions of the present work combined with future perspectives.

# CHAPTER I

# GENERAL INTRODUCTION

## 1.1 The protein quality control system

Cells encompass millions of proteins, which are packed into different subcellular localizations [1]. Proteins are the most abundant molecules in cells and regulate/participate in almost all physiological processes on which cells live depends [2]. Inside the cells, a large fraction of newly synthesized proteins needs to be efficiently folded in order to be functionally active. The protein folding beginnings in the moment that proteins are synthetized in the ribosomes, continues through structural intermediates and finished when the native state is reached [3-5]. This statement implies that the folding environment in the cells is a critical step to define the cell health, and consequently, cells have a hard task of maintaining proper protein homeostasis, also referred to as proteostasis [1]. To maintain proteostasis, cells have an adaptive network commonly called protein quality control. Protein quality control describes a collection of pathways, which include the molecular chaperones, the unfolded protein response (UPR), the ubiquitin-proteasome system (UPS) and the lysosomal proteolysis system [6]. Protein quality control system is responsible to maximize the protein folding capacity, to minimize the intrinsic and extrinsic protein deregulations and to degrade misfolded proteins [7]. Indeed, protein quality control system is viewed as an elaborate network of molecular chaperones and protein degradation factors that continually monitor and maintain the integrity of the proteome [8], and also as an efficient process due to the functional redundancy and capacity of molecular chaperones and clearance machines (the UPS and the lysosomal proteolysis system) [9]. Nevertheless, beyond the protein quality control system, proteostasis integrates RNA metabolism and processing, protein synthesis, folding, translocation, assembly/disassembly, and clearance [10, 11]. In this sense, proteostasis constitute a modular, elaborated and integrated, network (Fig. 1) that "governs the life of proteins" from the genesis to their decease, being dynamically regulated by several signaling pathways and in response to developmental signals, genetic changes, epigenetic marks, environmental stress and aging [10, 11].

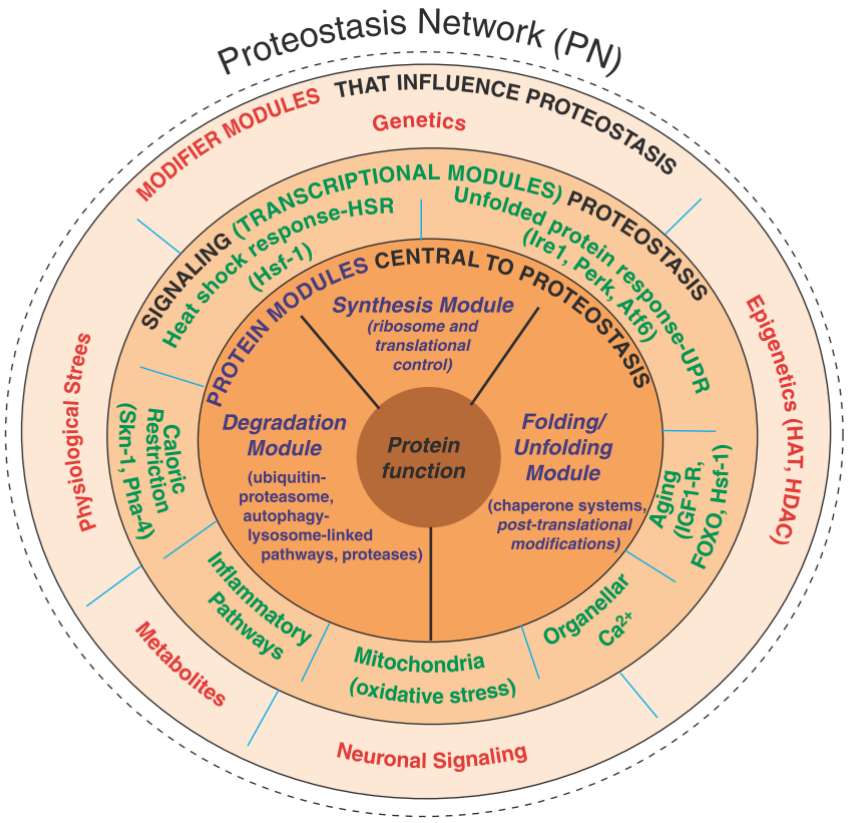


**Figure 1.** The proteostasis network (PN). In the center (blue font) are described the processes that are responsible for the control of the protein homeostasis. The next layer (green font) includes the signaling processes that influence the level and activity of the central players. In the outer layer is described the modifiers that influence the players shown in the other two layers. From [11].

### 1.1.1 Molecular chaperones

Molecular chaperones are proteins that have the function of interact, stabilize and help other proteins to acquire their functional active conformation [12]. In fact, cells possess a complex chaperone network that has the capacity to assure that proteins acquire a stable folded conformational state. Molecular chaperones also function as a surveillance mechanism that targets the proteins for degradation, when the folding process fail (Fig. 2) [13]. Essentially, chaperones are responsible to fold newly synthesized polypeptides and translocate them across membranes, and to refold the denatured substrates. Additionally, they also play an important role in targeting misfolded proteins for degradation as well as preventing aggregation (Fig. 2) [8]. Eukaryotic cells have two distinctly regulated chaperone networks to play these distinct functions: the chaperones that are

linked to protein synthesis (CLIPS), which are functionally and physically linked to the translation machinery and assist folding of newly translated proteins [14] and the heat shock proteins (HSPs), which were first identified in *Drosophila melanogaster* due to the ability of the fly cells to respond to non-permissive heat treatments [15].

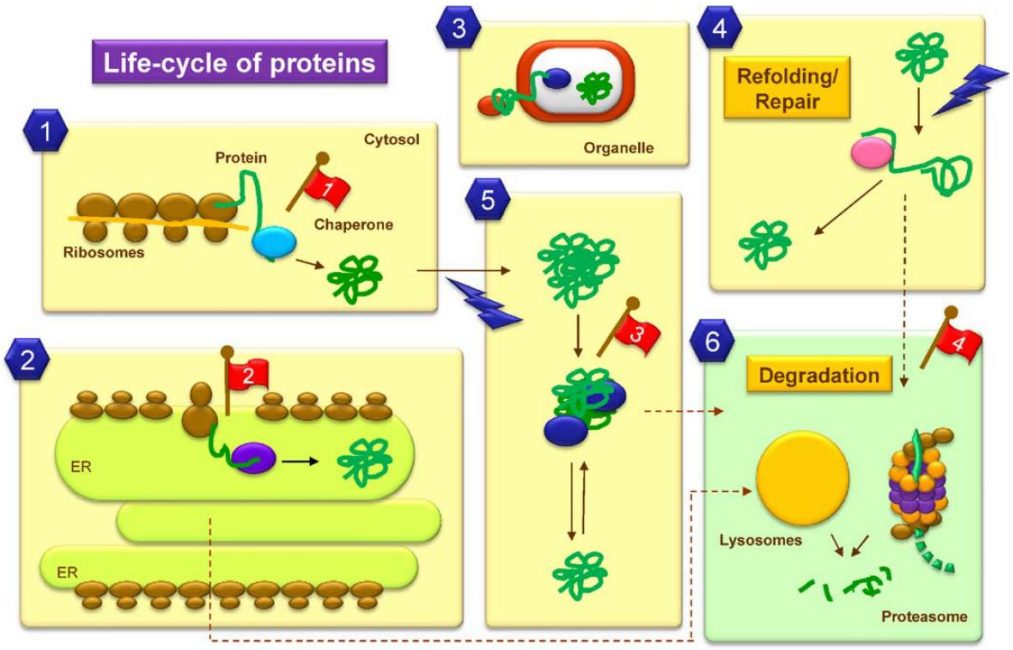


**Figure 2.** Life-cycle of proteins. 1) and 2) After synthesis, proteins usually fold naturally, but those that fail a proper folded conformation are aided by chaperones that are also able to promote the folding of proteins synthesized in the endoplasmic reticulum (ER) and cytosol. 3) Unfolding of previously folded proteins is required for their trafficking through membranes. The proteins can also suffer damage (4 and 5) that consequently results in their unfolding and/or aggregation. In these events, it is necessary the chaperone assistance to refold (4) or disaggregate (5) the impaired proteins. When the proteins damage is irreversible, molecular chaperones mediate the protein degradation of the altered proteins by the different proteolytic systems (6). From [13].

Chaperones are evolutionary conserved, from bacteria to mammalian, and can be classified, according to their molecular weight, in different classes: HSP100, HSP90, HSP70, HSP60, HSP40 and the small heat shock proteins (sHSP) [7, 12, 13, 16-18]. They are considered stress factors that are rapidly induced in response to high temperatures and other stress stimuli, which can operate in the cytosol or in different cellular sub-compartments (Fig. 2), contributing to the protection/control of different subsets of proteins [7, 12, 13, 16]. Essentially, the vast majority of chaperones recognizes

and binds to nascent polypeptide chains and partially folded proteins. The folding process is promoted through cycles of substrate binding and release that are regulated by their ATPase activity and by cofactor proteins [8]. The heat-shock response is controlled in eukaryotes by specific heat-shock transcription factors (HSFs) that are expressed and maintained in a non-DNA-binding state under non-stressed conditions, resulting in basal transcription levels of their downstream targets. Under stress conditions, like the presence of misfolded proteins, the HSFs are activated to a trimeric DNA-binding state. Many HSF genes (HSF1, HSF2, and HSF4) are expressed in mammals while *D. melanogaster*, *Caenorhabditis elegans* and yeast only express HSF1 [19, 20].

The cells must accomplish and maintain a correct balance between protecting folding intermediates, to guarantee that proteins reach the native state, and enforcing the rapid and efficient clearance of misfolded species. Alteration in this equilibrium can lead to an imbalance of cellular homeostasis.

### 1.1.2 Unfolded protein response (UPR)

Proteins that are synthetize in the endoplasmic must be correctly folded and assembled before transit to intracellular organelles and to the cell surface. When the molecular chaperones are unable to maintain their vital function, cells can undergo several stress conditions such as perturbation in calcium homeostasis or redox status, elevated secretory protein synthesis, accumulation of misfolded proteins or carbon source deprivation that lead to the accumulation of unfolded or misfolded proteins in the ER lumen, resulting in the activation of the ER stress [21]. Under ER stress, two distinct and interconnected mechanisms can be activated in an attempt to respond to the presence of misfolded proteins. The first is an ER stress response called the unfolded protein response (UPR) that acts by increasing its folding capacity [22]. The second mechanism is termed ER-associated degradation (ERAD), which has the capacity of recognize misfolded proteins and retrotranslocate those proteins through the ER membrane into the cytosol, to be degraded by the ubiquitin-proteasome machinery [23].

In the last years, it becomes clear that the UPR pathways are remarkably conserved from yeast to mammalian. In yeast, the folding capacity of the ER is only monitored by the conserved pathway that is transduced by the yeast Ire1p (inositol-requiring kinase 1) [24] and the mammalian

homologues IRE1α [25] and IRE1β [26]. Ire1p is a highly conserved transmembrane kinase that contains an unfolded protein sensor domain in the ER lumen connected to an effector domain in the cytosol and to a ribonuclease domain [27]. Upon accumulation of misfolded proteins in the ER lumen, the Ire1p is activated and consequently promotes the non-conventional splicing of the mRNA of *HAC1* (a leucine zipper family transcription factor), via its ribonuclease domain [28]. Then the spliced Hac1p travels to the nucleus, where it mediates the transcription of UPR targets, such as ER chaperones, ERAD machinery and several secretory proteins [29]. Likewise, in mammalian cells the Hac1p orthologue, the X-box binding protein 1 (XBP-1), which share few sequence homology with Hac1, undergoes also the non-conventional splicing of its transcripts mediate by IRE1 [30]. Similarly, in the nucleus, XBP-1 can activate the UPR targets [31].

Although, it is suggested a conservation of UPR signaling mechanisms, the pathway is far more complex in higher eukaryotic cells. Thus, additionally to the IRE1, higher eukaryotes present two other UPR sensors, the ER transmembrane kinase PERK and the ER transmembrane transcription factor ATF6. PERK contains a lumenal sensor that is strong similar to that of IRE1, but presents a dissimilar catalytic domain, consisting of an eIF2α (α subunit of eukaryotic initiation factor 2) kinase [29, 32]. Since eIF2α phosphorylation is able to inhibit the general cap-dependent translation [32], it is suggested that PERK might associate ER stress with translational control. In fact, activation of the PERK by the presence of misfolded proteins results in a generalized inhibition of translation [33]. Therefore, in this sense UPR can reduce stress by restricting new protein synthesis, giving time to ER to reestablish homeostasis without lose capacity with newly synthetized proteins, which must be folded, modified and sorted [32].

Accumulation of misfolded proteins also activates ATF6, which like Hac1p is a leucine zipper DNA binding protein, whose activity is regulated post-transcriptionally. But unlike Hac1p, ATF6 is constitutively expresses as a 90 kDa protein with a large lumenal domain and a cytosolic DNA-binding domain. Under ER stress, ATF6 is processed down to a 50 kDa cytosolic fragment, which allows it to enter to the nucleus and mediate UPR targets activation [34-36].

Hence, UPR acts as a sensitive and flexible pathway that regulates ER activity and adapts cells to different physiological conditions. Furthermore, it has the ability to decide the cells fate, since when homeostasis fails, the UPR as the capacity of drive cells to death.

### 1.1.3 Proteolytic systems

In all eukaryotic cells, the production and degradation of proteins, commonly known as protein turnover, is a constant, ongoing process that is crucial for cellular renewal. In fact, protein degradation is important for cell homeostasis. Different rates of proteins turnover are necessary to allow their levels to change rapidly in response to external stimuli. Furthermore, beyond the complex network of the chaperone system, several proteins are unable to reach their native stable state and need to be degraded. In this line, cells have to ensure the proteostasis quality and for that cells possess mechanisms that promote the elimination of the non-functional and potential toxic proteins. Thus, protein degradation assumes a major role in the regulation of cellular homeostasis in all eukaryotic cells, and processes like DNA transcription, repair and cell cycle are coordinately controlled with protein degradation [37].

Cellular protein degradation can occur in different compartments such as cytosol, lysosome/vacuole and endoplasmic reticulum. Eukaryotic cells encompass two major pathways - the ubiquitin-proteasome pathway (UPS) and the lysosomal proteolysis system - mediated protein degradation. UPS is highly selective and recognizes only ubiquitinated substrates, like short-lived proteins, while organelles, aggregated proteins and long-lived proteins are usually degraded by the lysosomal proteolysis system [38, 39].

#### 1.1.3.1 Ubiquitin-proteasome system (UPS)

The UPS is one of the main proteolytic systems that participate in the protein quality control system. In fact, UPS is the principal system responsible for the ATP-dependent degradation of short-lived, misfolded and truncated proteins found in the nucleus, endoplasmic reticulum and cytosol of all eukaryotic cells [13, 40]. Degradation of proteins by the UPS is a highly complex process that requires the coordinated action of a large number of proteins. These proteins participate in the multiple steps of the UPS pathway, being progressively involved in the recognition and the conjugation of multiple ubiquitin molecules to the subtract, to produce the poly-ubiquitin chains, and in the destruction of these poly-ubiquitylated substrates by the proteolytic machinery, namely by the 26S proteasome [41, 42].

Most of the proteasome subtracts are target for degradation through the linkage of ubiquitin, called ubiquitination, which consists on the formation of an isopeptide bond between specific lysines of the target protein and ubiquitin. Targets of the ubiquitin system are ubiquitinated by a cascade of three enzymes, generally known as E ligases, the E1, ubiquitin-activating enzyme, the E2, ubiquitin-carrier enzyme and finally the E3 ubiquitin-protein ligase (Fig. 3), which act sequentially to activate ubiquitin [13, 43]. Repeated cycles of ubiquitinization produce the poly-ubiquitin chains that are recognized to degradation. In fact, the degradation of ubiquitinated proteins requires poly-ubiquitin chains of more than 4 ubiquitins [44]. Nevertheless, the ubiquitination of a specific protein could not be sufficient to determine its degradation, since mono-ubiquitinated or even poly-ubiquitinated proteins can experiment post translational modifications that are related to other various cellular functions, including DNA repair or membrane trafficking [45].

Multi-ubiquitinated proteins are degraded by the 26S proteasome (Fig. 3), which consists of a multicatalytic complex formed by 4 rings containing two types of catalytic subunits ($\alpha$ and $\beta$), known as 20S, capped on both ends by a multisubunit regulatory complex, the 19S, that is composed by chaperones, ATPases and enzymes that allows the substrate recognition and the ubiquitin tags removal [13]. Three major types of proteolytic activity have been described in the catalytic subunit that were identified as trypsin-like, which performs the cleavage after hydrophobic bonds, chymotrypsin-like, which cleaves at basic residues and postglutamylpeptide hydrolase-like or caspase-like activities, which cleaves after acidic amino acid [46, 47].

After poly-ubiquitinated with at least 4 ubiquitins, the target protein has the possibility to bind either directly to the ubiquitin receptors in the 19S regulatory complex of the 26S proteasome or to adaptor proteins that contain both polyubiquitin-binding and proteasome-binding domains (Fig. 3) [48]. Next the target protein is unfolded by unfoldases in the regulatory complex, followed by the removal of the polyubiquitin chain by proteasome-associated de-ubiquitylating enzymes (DUBs), and then is translocated into the central proteolytic chamber, where it is cleaved into short peptides (Fig. 3) [13, 49].

The UPS is an intracellular protein degradation system that is responsible for the protein turnover within the cell, playing an important role in a large variety of biological processes including regulation of cell cycle, protein misfolding, transcription, replication, mitochondrial biogenesis and cell death [41]. Therefore, it is conceivable that deregulation of the UPS function could lead to

harmful cellular consequences. In fact, imbalances of the UPS can underlie several diseases such as the neurodegenerative disease that will be discussed hereafter.

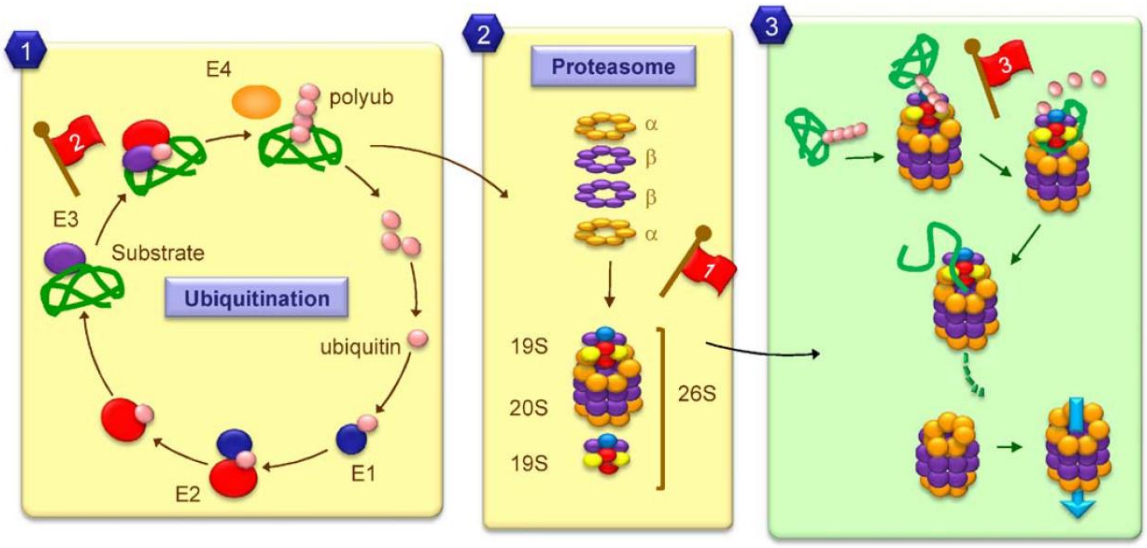


**Figure 3.** The ubiquitin-proteasome system (UPS). 1) The ubiquitination process, which consists in the covalent attachment of 4 or more ubiquitins through the coordinated action of the catalytic enzymes, E1, E2 and E3, which act sequentially to activate the ubiquitin and ligate it to the substrate presented by the E3. 2) The proteasome, which is composed by a catalytic core (the 20S) formed by 4 rings containing two types of catalytic subunits (α and β) and a regulatory complex, the 19S. 3) The polyubiquitinated chains are recognized by components of the regulatory subunit. Next the deubiquitinases present allow the release of the ubiquitin for recycling, followed by the substrate unfold by unfoldases in the regulatory complex. The ATPases in this complex provide the energy required for the injection of the substrate protein into the catalytic barrel. From [13].

### 1.1.3.2 Lysosomal proteolysis system

As above described, the turnover of proteins and of misfolded proteins that can generate insoluble forms and aggregates, essentially due to an impairment of the chaperone system and/or the overload or deregulation of the UPS, are processed by the lysosomal proteolysis, particularly via the autophagic pathway [7, 50, 51]. In higher eukaryotes, lysosomal proteolysis system could be divided in three main sub-systems: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. In CMA the target proteins are translocated across the membrane of the lysosome complexed with chaperones that allow the recognition by the lysosomal membrane receptor

lysosomal-associated membrane protein 2A (LAMP-2A), resulting in the target proteins unfolding and degradation (Fig. 4) [52]. In microautophagy, the vacuole membrane invaginates and directly engulfs small components of the cytoplasm (Fig. 4) [53, 54]. Macroautophagy, hereafter referred to as autophagy, is the most well characterized process of the three. It is a process in which the cellular components are sequestered within vesicular structures (autophagosomes) and delivered to the vacuole/lysosome for degradation (Fig. 4) [55]. The autophagy molecular mechamism and regulation processes will be discussed in more detail hereafter.

In the yeast *Saccharomyces cerevisiae*, the lysosomal, which in yeast is vacuolar, proteolysis system occurs only by two main sub-systems: macroautophagy (commonly referred as autophagy) and microautophagy [53, 54]. Additionally, a similar pathway in many respects to CMA has been described in yeast, in which the vacuolar protein import is dependent on cytosolic hsp70 family and on protease-sensitive components on the outer surface of vacuoles. Protein degradation by this pathway depends on a functional vacuolar ATPase [56]. Nevertheless, this pathway is still poorly explored.

Autophagy (macroautophagy) is a physiological process that is conserved among eukaryotic cells, which is crucial to maintain the balance between anabolism and catabolism in order to confer the normal growth and development [57]. For instances, autophagy is active at basal levels to promote the turnover of long-lived proteins and for the removal of unnecessary or damage organelles. On the other hand, the process is induced in response to several stress conditions like starvation, heat and oxidative stress, being also associated with cellular development and differentiation [58]. Like the UPS, autophagy deregulation causes detrimental cellular consequences, being also associated with several diseases. These aspects will be focused hereafter.

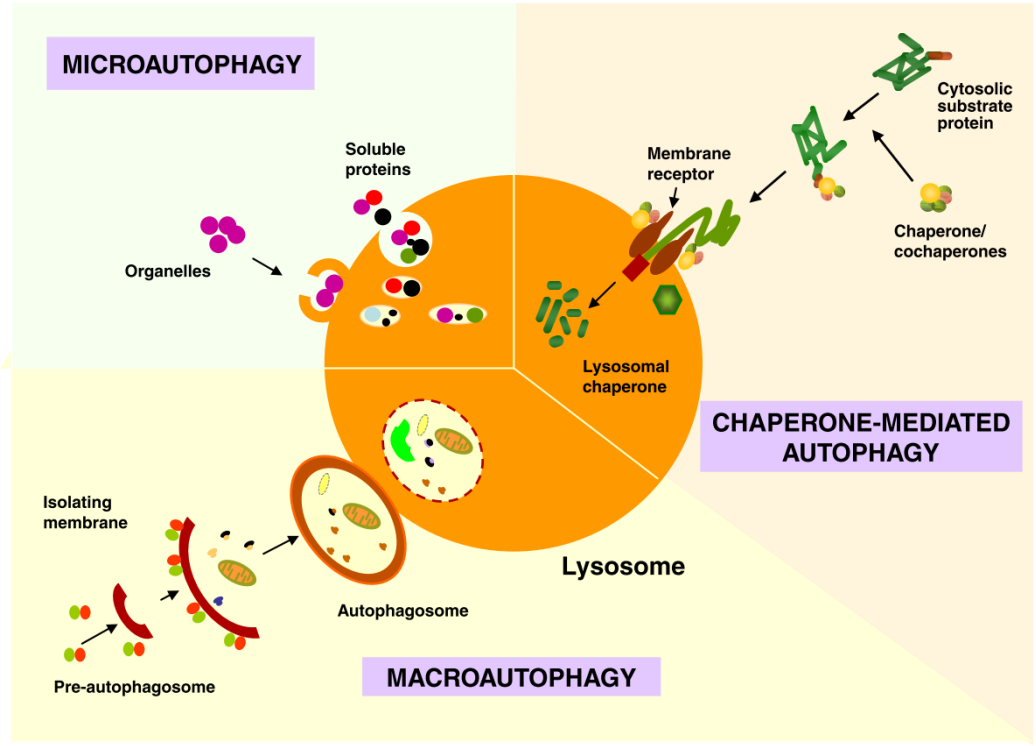


**Figure 4.** Lysosomal proteolysis system. three fundamentally different types of lysosomal proteolysis systems: macroautophagy (commonly referred as autophagy), microautophagy and chaperone-mediated autophagy, have been described. From [59].

## 1.2 Autophagy (macroautophagy)

Autophagy is an essential, homeostatic process by which cells degrade their own components. The most primordial function of autophagy is adaptation to nutrient starvation. Nevertheless, it is now established that it also orchestrates diverse pieces of the cellular response pathways to other dangerous stimuli [60].

### 1.2.1 Autophagic machinery

The lysosome in mammalian cells or the vacuole in yeast is the organelle responsible for the turnover of long-lived proteins, degradation of the misfolded proteins and dysfunctional organelles. In mammalian cells this process has been phenomenological observed for a long time, nevertheless the molecular autophagic components have been identified in the yeast *S. cerevisiae*, just in the last

decade [61-63]. Over 35 different AuTophaGy-related (ATG) genes have been identified in yeast, and many of them have homologues in higher eukaryotic cells [39, 64]. The Atg proteins are divided in different groups according to their physiological functions in the autophagic process [65]. Conceptually, the autophagy process can be divided into discrete steps: induction, cargo recognition (for specific types of autophagy) and packaging, vesicle nucleation, vesicle expansion and completion, vesicle fusion with the vacuole, degradation and export of the metabolic building blocks (Fig. 5) [66]. The Atg proteins can be included into different functional groups due to their participation in the various steps of the pathway: i) the Atg1-Atg13-Atg17 kinase complex, which participates in the autophagy induction; ii) the class III phosphatidylinositol 3-kinase (PtdIns3K) complex I, integrating Vps34, Vps15, Atg6 and Atg14 that are implicated in the autophagosome formation; iii) two ubiquitin-like protein conjugation systems, Atg12 and Atg8, which participate in the expansion of autophagosome membranes; iv) Atg9 and its recycling system that potentially contributes to the delivery of membranes to the forming autophagosome; and v) the proteins that contribute to the last steps of the process (Fig.5) [67]. Furthermore, in yeast, autophagy machinery is concentrated at a perivacuolar site, called phagophore assembly site (PAS), and the action of the autophagy machinery at the PAS results in the phagophore expansion and autophagosome formation (Fig. 5) [68].

In yeast, autophagy induction is initiated by the activation of the target of rapamycin (TOR)-Atg1-Atg13-Atg17 kinase complex, being TOR a negative regulator of the complex [55]. TOR is a serine/threonine kinase that in response to amino acids and growth factors coordinates different aspects of cell growth, playing a crucial role in the autophagy regulation [69]. Yeast harbors two TOR complexes with distinct functions, TOR complex 1 and 2 (TORC1 and TORC2), nevertheless only TORC1 has a central role in autophagy regulation [69]. TORC1 regulation is dependent on the nutritional conditions. Thus under nutrient rich conditions it is active, promoting autophagy inhibition, while under nutrient starvation it is inactive allowing the activation of the autophagy pathway [69]. TORC1 directly or indirectly regulates the Atg1-Atg13-Atg17 kinase complex through the Atg13 phosphorylation state (Fig. 6A) [70]. Under favorable nutrient conditions, with active TORC1 complex, Atg13 is hyperphosphorylate and has low affinity for Atg1 and Atg13. Since Atg17 is unable to interact with Atg1 without the presence of Atg13 (Fig. 6A) [71-73], the complex is blocked and consequently autophagy is inhibited. Upon nutrient deprivation, the TORC1 complex is inactive and Atg13 is rapidly dephosphorylated, resulting in the interaction between Atg1 and Atg17,

the consequent complex formation and the activation of Atg1 kinase activity. The Atg1 is upregulated during autophagy induction, suggesting an important role for Atg1 in the autophagy process [72].

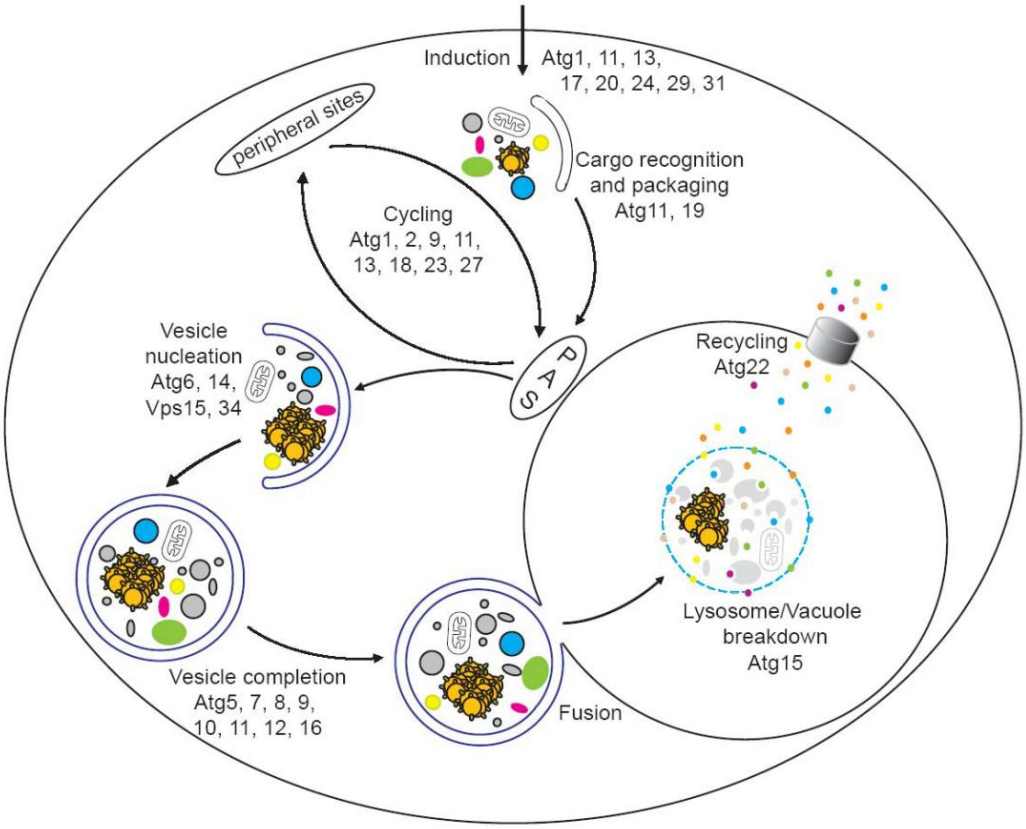


**Figure 5.** Schematic representation of the autophagic pathway in yeast cells. Autophagy can be divided in several steps including induction, cargo recognition (for specific types of autophagy) and packaging, vesicle nucleation, vesicle expansion and completion, vesicle fusion with the vacuole/lysosome, degradation and export of the metabolic building blocks. The Atg proteins can be included into different functional groups due to their participation in the various steps of the pathway (see in the text). From [65].

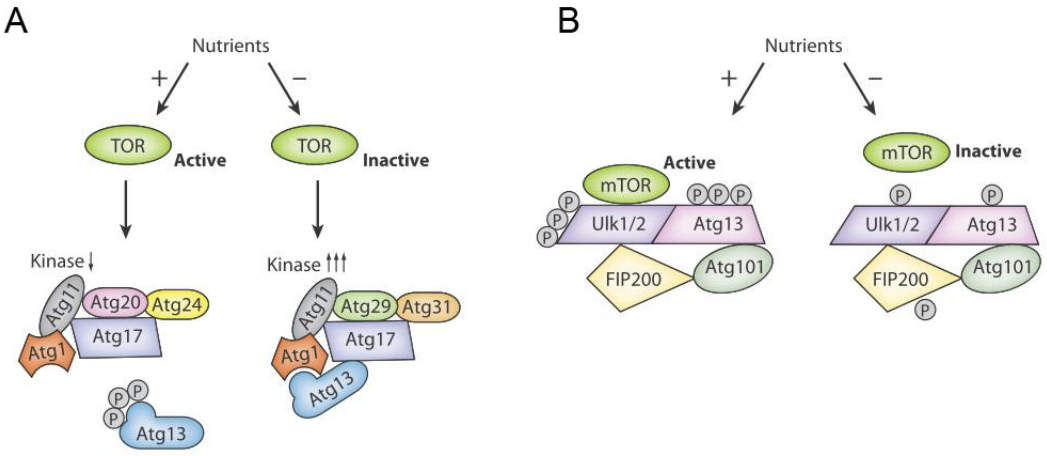


**Figure 6.** The Atg1 complex as a regulator of autophagy in (A) yeast and (B) mammalian cells. Adapted from [74].

Homologues of Atg1 were identified in various organisms, such as *D. melanogaster* [75], *C. elegans* [76] and mammalian cells [77, 78], being also involved in the regulation of autophagy. In mammalian, the uncoordinated 51-like kinase 1 and 2 (ULK1 and ULK2) appear to be the functional similar to yeast Atg1 homologues, since some data already showed that the knockdown of ULK1 and ULK2 inhibits autophagy induction [79, 80]. Like in yeast, ULK1 and ULK2 are part of a complex in which the mammalian homologue of Atg13, mAtg13, and the mammalian orthologue of Atg17, the protein FIP200, are also integrated (Fig. 6B) [80-82]. In conditions that lead to autophagy induction, a decrease in mTOR activity results in the dephosphorylation of ULK1, ULK2, and mAtg13. In this situation, ULK1 and ULK2 are activated promoting the phosphorylation of mAtg13 and FIP200 (Fig. 6B) [80, 82].

The nucleation and assembly of the initial phagophore membrane requires the PtdIns3K complex. In yeast, there are two PtdIns3K complexes that share the only PtdIns3K protein present. Each complex contains three common components, Vps34 (the only PtdIns3K protein in yeast), Vps15 and Vps30/Atg6 (Fig. 7) [83]. The function of Vps34 is dependent on Vps15, which is required for Vps34 membrane association and activity [84]. Additionally, each complex contains another specific component, which makes the distinction between the two complexes. Thus, the complex I also integrates Atg14 and in the complex II, Vps38 is present (Fig.7). The complex I, Vps15-Vps34-Vps30-Atg14, localizes to the PAS and is involved in autophagy and in the cytoplasm-to-vacuole (cvt) pathway, which is a yeast selective type of autophagy (that will be discussed

hereafter) (Fig. 7) [85], while the complex II functions at the endosome and participates in the sorting of carboxypeptidase Y (CPY), which is normally transported from the late Golgi to the vacuole through the CPY pathway (Fig. 7).

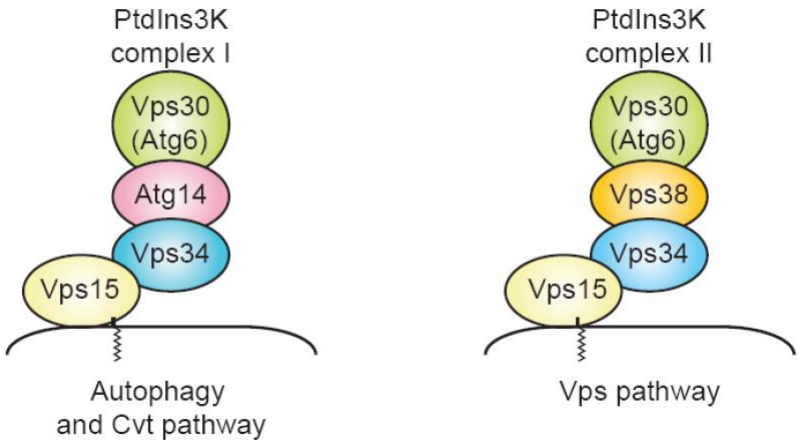


**Figure 7.** The two phosphatidylinositol 3-kinase (PtdIns3K) complexes in yeast. Each complex contains three common players, Vps15, Vps34 (the PtdIns3K) and Vps30/Atg6. Additionally, another specific component, Atg14 or Vps38 participates in complex I or complex II, respectively. From [54].

Apparently, the role of PtdIns3K is to produce phosphatidylinositol (3)-phosphate (PtdIns(3)P) at the PAS to recruit PtdIns(3)P-binding proteins, which in turn recruit additional downstream effectors to the PAS [86, 87]. It is described that PtdIns(3)P recruits Atg18, Atg20, Atg21, Atg24 and Atg27 to the PAS [88-90]. On the other hand, Atg24 and possibly Atg20 interact with Atg17 [88], and Atg20 interacts with Atg11 [91]. These data suggests that Atg20-Atg24 complex might interact with the Atg1 kinase complex.

In contrast to yeast, mammalian cells present two types of PtdIns3K proteins: class I and III. Class III PtdIns3K, hVps34, which is similar to the yeast Vps34, generates PtdIns(3)P and plays a stimulatory role in autophagy. It also forms a complex with p150, the homologue of Vps15, and with its accessory protein Beclin 1 (the homologue of Vps30/Atg6) [92, 93]. Furthermore, the orthologues of Atg14 and Vps38 are already identified and designed as Atg14-like protein (Atg14L or Barkor) and ultraviolet irradiation resistant-associated gene (UVRAG), respectively [94-96].

The role of the enzymatic product of hVps34 is not well understood, however it likely participates in diverse phases of the autophagic process contributing to the autophagosomal membrane formation [97], to the targeting of PI3P–binding proteins, such as Atg18, to the autophagic membrane [98], to the localization of LC3 (Atg8) lipidation [99] and to autophagosomal maturation into autolysosomes [100]. Additionally, Beclin 1, a key regulator of autophagy, interacts with functionally distinct hVps34-containing protein complexes. Therefore, when complexed with Atg14L, the complex plays a role in initiation [94, 101], and when associated with UVRAG/VPS38, the complex participates in the maturation of the autophagosome [94, 100]. On other hand, the participation of Beclin1 in autophagy is predominantly done by B-cell lymphoma/leukemia-2 (Bcl-2). Bcl-2 is an anti-apoptotic protein that inhibits autophagy by binding and sequestering Beclin1 [55].

In yeast, the phagophore expansion and nucleation that leads to the autophagosome formation also requires the participation of the PtdIns3K complex I (Vpas34-Vps15-Atg6/Vpas30-Atg14), which together with the Atg1 kinase complex is a platform to recruit two interrelated ubiquitin-like conjugation systems, Atg12-Atg5-Atg16 and Atg8-PE (phosphatidylethanolamine) to the phagophore (Fig. 8) [102, 103].

In yeast, Atg12 is activated by the Atg7 (E1 ubiquitin-activating enzyme) and then by Atg10 (E2 ubiquitin-conjugating enzyme) staying covalently attached to Atg5 [104]. This conjugation further interacts with Atg16 which links the Atg12-Atg5-Atg16 complex into a multimeric structure and attaches it to the phagophore [105, 106]. Apparently, the Atg12-Atg5-Atg16 complex has the capacity to induce curvature into the growing phagophore through asymmetric recruitment of Atg8-PE (Fig. 8) [107].

In the other conjugation system, Atg8 is first processed by the cysteine protease Atg4. In the same way as in the Atg12-Atg5 conjugation system, E1 enzyme Atg7 activates Atg8 and transfers it to the E2 enzyme Atg3. Finally, Atg8 is conjugated to the target lipid PE through an amide bond [99, 108]. In favorable nutritional conditions, Atg8 is cytosolic, although upon autophagy induction, Atg8 is conjugated with PE being tightly associated with membranes. In fact, Atg8-PE is localized in both sides of the phagophore during the autophagic process [109, 110].

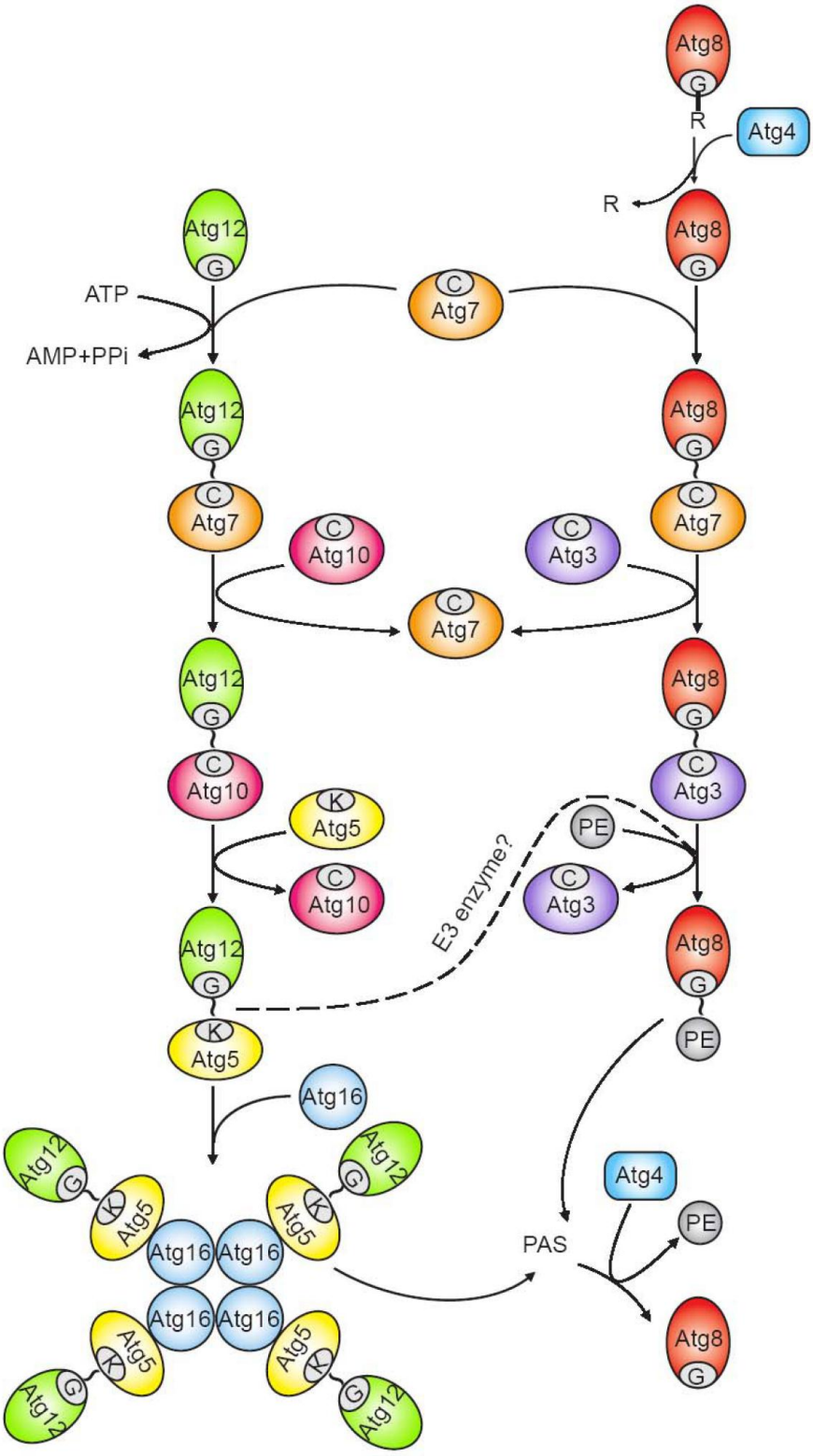


**Figure 8.** The two ubiquitin-like protein conjugation systems in yeast required for the phagophore expansion and nucleation. From [54].

Contrarily to Atg12-Atg5 conjugation, Atg8-PE association is a reversible process, being possible to make the recycling of the Atg8. Once again the Atg4 cysteine protease has the capacity of release Atg8 from its target lipid, which is recycled and used in another conjugation reaction, allowing the efficient progression of the autophagic process (Fig. 8) [110].

The Atg12 and Atg8 conjugation systems are evolutionarily conserved and mammalian homologues for each component of the yeast Atg12-Atg5 and Atg8-PE conjugation systems, with similar function, have been described [111-113]. In mammalian cells, it is observed the existence of at least four Atg8 yeast homologues, MAP1LC3, GATE16, GABARAP and Atg8L. All the refereed proteins share a conserved glycine residue near their C terminus, and are conjugated to PE, an event similar to the one observed in yeast, catalyzed by Atg4, Atg7 and Atg3 [109, 114-117]. MAP1LC3, commonly known as LC3, is the most abundant in the autophagosomal membrane, being established as a central marker to assess the autophagosome and autophagic activity [118]. Like the yeast Atg8-PE, the mammalian LC3-PE (also named LC3-II) localizes in the membrane outer face, and is cleared from the membrane due to a cleavage promoted by Atg4, while the inner population remains associated to the vesicle and is delivered into the lysosome where it is degraded [109, 110, 119].

One of the main puzzling questions regarding autophagy process is the origin of the autophagosome membrane. During autophagy, the formation of double-membrane vesicles is required, and apparently these vesicles do not bud from a pre-existing organelle, rather the vesicles are supposed to be form by the expansion of a membrane core, of yet unknown origin, called phagophore (Fig. 9) [120-122]. Some evidence indicates that the ER membrane, plasma membrane, mitochondrial outer membrane and Golgi complex contribute to the vesicle formation [123]. Apparently, in yeast, the phagophore membrane formation occurs at or is organized around the PAS [124]. In fact, although the role of the PAS is not entirely understood, it is proposed that it serves to facilitate the nucleation and/or expansion of the phagophore, by the recruitment of the necessary Atg proteins [103, 120]. Thus, Atg11, which is essential for PAS organization under vegetative conditions, and Atg17, which role is critical during starvation, are the initial factors responsible for subsequent recruitment of the remaining Atg proteins. Atg17, in addition to the Atg1-Atg13-Atg17 complex, interacts also with Atg29 and Atg31 and this association is essential for recruitment of Atg proteins to the PAS to generate autophagosomes (Fig. 9). Furthermore, Atg1-Atg13 is also required for PAS organization (Fig. 10) [125].

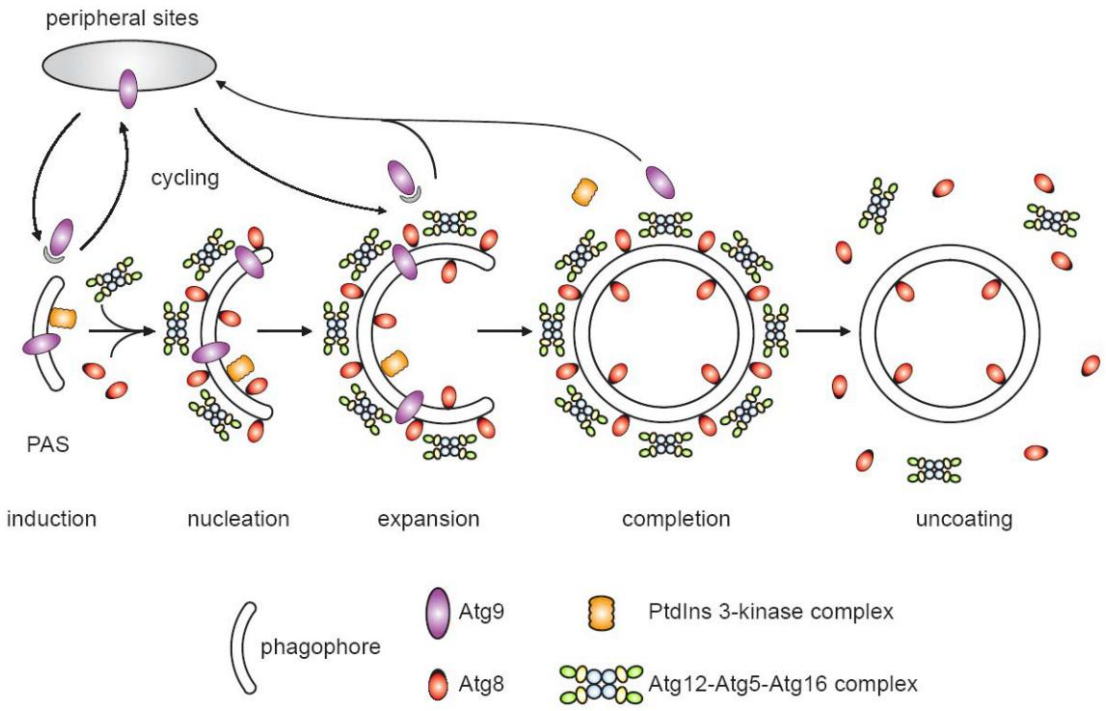


**Figure 9.** Schematic representation of the double membrane formation in yeast. From [54].

Another protein that is recruited relatively early to the PAS is Atg9. In fact it is considered a "membrane carrier" during the assembly process [121, 126]. During autophagy, Atg9 is present in multiple punctate structures, like PAS and subjacent or at the mitochondria surface, and also a portion is dispersed to the cytosol being apparently associated with membranes (Fig. 10). The cycling of the Atg9 between PAS and the other structures is crucial for the autophagosome formation, since it is suggested that the membrane could be transported to the PAS by this shuttling process [127, 128]. This travelling, called anterograde movement, involve several Atg proteins (Fig. 10). Atg11 is essential, particularly for the specific organelles degradation, since its absence blocks the transport of Atg9 to PAS [126, 129]. Additionally the efficient movement also involves the peripheral membrane protein Atg23 and the type I transmembrane protein Atg27. They form an essential interdependent complex with Atg9. Atg9, Atg23 and Atg27 are also found in the PAS and in other punctate structures (Fig. 10).

Another collaborator on the Atg9 anterograde movement is the actin cytoskeleton [126]. Particularly, an actin-related protein, Arp2, which is one subunit of the Arp2/3 complex, the nucleation factor of branched actin filaments. Hence, the data suggests that Arp2/3 complex brings

the power to push the cargo, composed by Atg9 and its associated membranes, from the membrane donor to the forming autophagosome (Fig. 10) [126, 130, 131].

The above referred recruitment of the two ubiquitin-like conjugation systems, Atg12-Atg5-Atg16 and Atg8-PE to the phagophore by the PtdIns3K complex I is also assisted by the Atg9. The absence of this interaction does not hamper the conjugates formation, nevertheless they completely diffuse in the cytosol, losing the punctate localization [132].

The retrieval of Atg9 from the PAS to the peripheral sites also requires the participation of several Atg proteins including the Atg1-atg13 kinase complex, Atg12, Atg18, and the PtdIns3K complex I. The absence of any of these players culminates in the accumulation of Atg9 in the PAS [128]. In the same way, the retrieval of Atg23 and Atg27 is also coupled to the participation of the Atg1-atg13 kinase complex (Fig. 10) [133, 134].

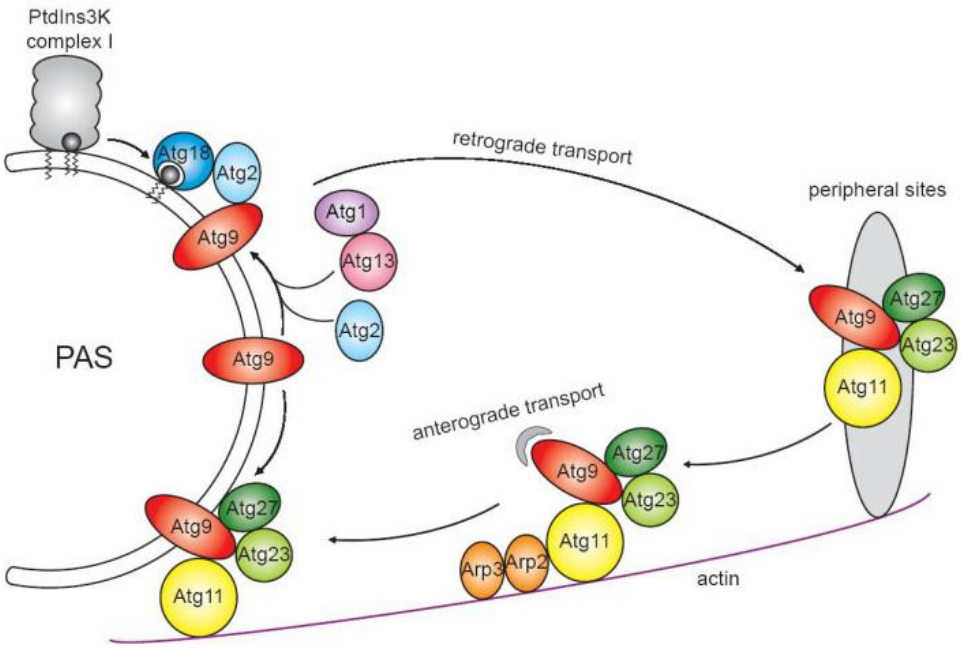


**Figure 10.** Cycling of Atg9. During autophagy, Atg9 cycles between the PAS and non-PAS punctate structures, such as mitochondria surface, and also a portion is dispersed to the cytosol being apparently associated with membranes. From [54].

In mammalian cells there is no evidence for the existence of the PAS. The phagophore membranes seem to initiate from the ER [135, 136], together with other cytosolic membrane structures, like Golgi and late endosomes [60, 97, 137]. However, colocalization of the Atg proteins was also observed, nevertheless in multiple sites, instead of only one place as in yeast cells. The data suggests that probably the mammalian cells present multiple PAS [138, 139]. In fact, mammalian Atg9 homologue, mAtg9 resides in the *trans*-Golgi network and late endosomes. Deprivation nutrient conditions induce the redistribution of the mAtg9 to peripheral sites, overlapping with LC3 positive autophagosomes. Furthermore, like in yeast, the mAtg9 cycling is also dependent of the ULK1, since its absence restrings mAtg9 to the *trans*-Golgi network [139].

In yeast, the machinery involved in the autophagosome-vacuole fusion includes the Rab family GTPase Ypt7, the NSF homologue Sec18, the SNARE proteins Vam3, Vam7, Vti1, Ykt6, the class C Vps/HOPS complex proteins, and two other proteins, Ccz1 and Mon1 [140-142]. The autophagosome inner membrane vesicle, called autophagic body, is released to the vacuole lumen, after fusion. Upon release into the vacuole, the autophagic body is degraded dependently on appropriate vacuole function and on the activity of the resident vacuolar acid hydrolases, like Pep4 and Prb1, and the lipase Atg15 [143-145]. The resulting molecules from degradation must be released back to the cytosol for maintenance of the cellular functions. Apparently, the efflux of the molecules across the vacuolar membrane is played by Atg22, a putative amino acid effluxer on the vacuolar membrane [146], together with the vacuolar permeases Avt3 and Avt4 [146, 147].

In mammalian cells, the autophagosome-lysosome fusion is mediated by the same machinery that is involved in the vesicular trafficking pathways, and the fusion event involves the lysosomal membrane protein LAMP-2A and the small GTPase Rab7 [148, 149].

### 1.2.2 Selective types of autophagy

Autophagy is commonly recognized as a non-selective process, in which cytosolic components are randomly surrounded by the phagophore membrane and included in the autophagosome. However, in certain scenarios autophagy plays a biosynthetic role by mediating the specific degradation of unnecessary or damage organelles or the trafficking of some molecular enzymes [150]. Some types of selective degradation have been detected and explored, like the

selective removal of aggregated proteins [151] and of damage organelles, for example mitochondria [152, 153], peroxisomes [154] and ER [155].

#### 1.2.2.1 The Cytoplasm-to-vacuole (Cvt) pathway

The best described type of selective autophagy is the Cvt pathway that is responsible for the specific delivery of at least two hydrolases, α-mannosidase (Ams1) and aminopeptidase I (Ape1), to the vacuole, the site where they will be enzymatically processed into their mature form. This pathway operates constitutively under growing conditions in *S. cerevisiae* [61]. In this sense, and contrarily to autophagy, the Cvt pathway is a biosynthetic process, however the machinery used during the process is almost identical to the one used by bulk autophagy but with the participation of some additional factors. The transport of ApeI and Ams1 to the vacuole is dependent of participation of Atg19 and Atg11. In fact, Atg11 sequester the complex enzyme-Atg19 and drive it to the PAS, suggesting that Atg11 is associated with selective types of autophagy [91, 156].

#### 1.2.2.2 Mitophagy

Mitochondria are organelles that have essential functions for the cell, including ATP production via oxidative phosphorylation and calcium homeostasis, however their continued presence within the cell may sometimes result in cell death. In fact, mitochondria deterioration is accomplished with the production of excessive reactive oxygen species (ROS) leading to the release of pro-apoptotic proteins, like cytochrome *c* and apoptosis-inducing factor (AIF), triggering the damage of the neighboring mitochondria and the entire cell [157]. Furthermore, if the cellular energy intake is low, an excessive number of mitochondria could result in the generation of high ROS levels and consequently cellular damage [158]. In this sense, as a quality control system, the cells have to proceed with the controlled removal of excessive, unstable or damage mitochondria by specific autophagy, mitophagy, supporting the crucial role of mitophagy in cell survival [50, 159, 160].

In the yeast *S. cerevisiae*, mitochondria are sequestered, and delivered into the vacuole. This process is performed by the core of Atg proteins essential for autophagosome formation, nevertheless can occur without nutrient starvation and does not involve the Atg proteins that are specific for bulk autophagy (macroautophagy) [161, 162].

The first protein identified as being involved in mitophagy was the Uth1p [163], an outer mitochondrial membrane protein that in coordination with the core of Atg proteins mediate the selective removal of mitochondria. Next a second mitophagy-related protein was found, the Aup1 [164], a putative protein phosphatase present in the mitochondrial intermembrane space. In fact, it has recently been proposed that Aup1 regulates mitophagy by regulating the retrograde signaling pathway [165]. Nevertheless, later studies did not confirm the need of Uth1p and Aup1p to the process of mitochondria elimination [161, 166], however, this could be partially explained by the different yeast backgrounds used in the studies or even in the detection methods [160]. In this sense, the participation of Uth1p and Aup1p in mitophagy is still matter of controversy.

More recently, two other proteins were identified as being associated with mitophagy, Atg32 and Atg33 [161, 166]. Atg32 is a mitochondrial anchored protein that is specific and crucial to mitophagy (Fig. 11A), but not for autophagy or other types of selective autophagy, since *ATG32* mutant cells are only competent in the autophagy and Cvt pathways. Atg32 is induced and anchored on the outer membrane of damage mitochondria, functioning as a protein receptor that interacts with the adaptor protein Atg11, resulting in the mitochondria sequester to the PAS (Fig. 11A). Additionally, Atg32 owns an evolutionary conserved motif (WXXI/L) that is crucial for direct binding to Atg8, an interaction that is required for mitochondrial recruitment by the phagophore (Fig. 11A) [161, 166].

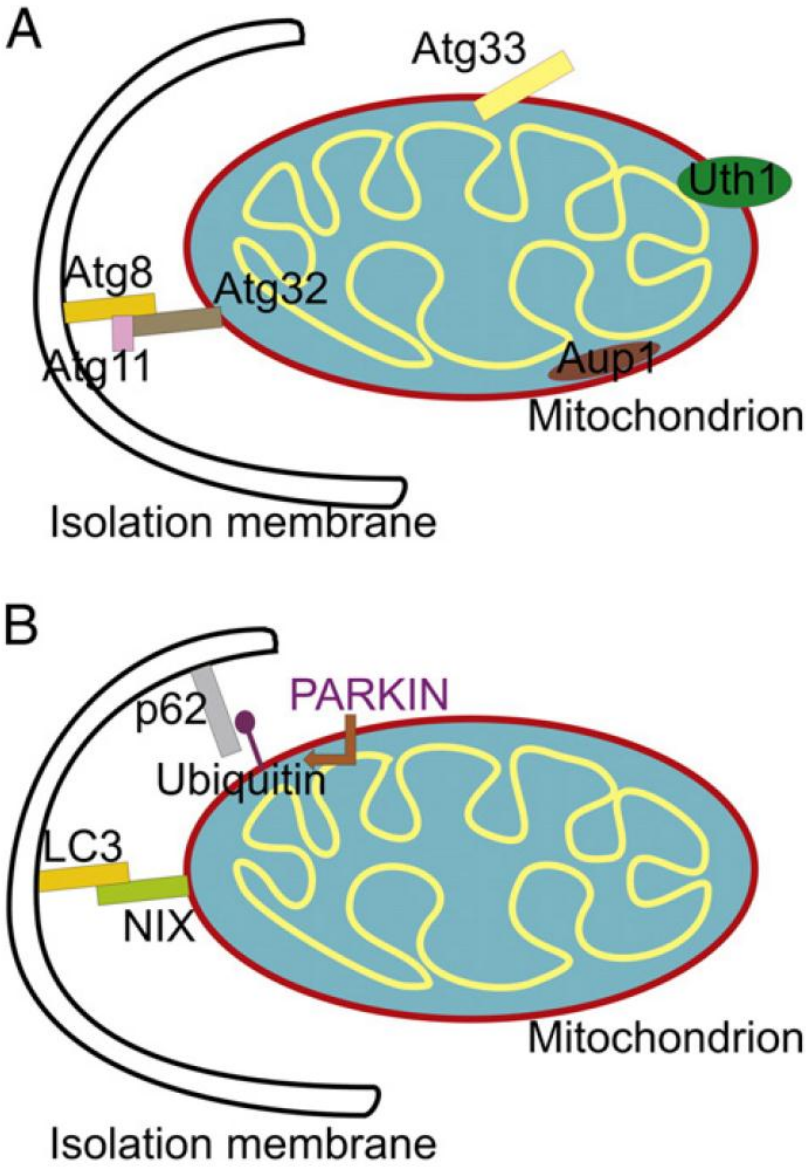


**Figure 11.** Schematic representation of mitophagy in (A) yeast and (B) mammalian cells. From [160].

However, important questions remains open, particularly the consequences on the mitophagy process under the absence of Atg32. Mounting evidence revealed that under determined conditions, the lacking of Atg32 did not alter the mitochondrial mass neither the DNA levels, suggesting a critical role for Atg32 in the removal of excessive mitochondria [167]. In contrast, in the presence of damage mitochondria, Atg32 seems to be critical for mitophagy induction [166].

Atg33 is an outer mitochondrial membrane protein, which when absent is responsible for the blockage of mitophagy during nitrogen starvation and almost completely during the stationary

phase, suggesting that it is probably required for the recruitment of aged mitochondria by the PAS (Fig 11A) [160]. Nevertheless, further studies are necessary to prove this hypothesis.

Another pre-requisite that apparently is essential to mitophagy is mitochondrial fission. This event is required to remove the damage mitochondria and also to prepare them to fit into the autophagosomes. Nevertheless, the fission process must be associated with a concomitant mitochondrial dysfunction or even with unclear signals [160]. In yeast, it is still controversial if mitophagy is strictly dependent on mitochondria fission and thus alternative pathways for mitochondrial quality control by mitophagy must be considered [168].

In mammalian cells it is well established that loss of mitochondrial membrane potential causes mitophagy. Additionally, mitophagy is also triggered during differentiation of specific tissues [169, 170]. The process occurs with the participation of ULK1 and ATG7 [171, 172]. Additionally, NIX, a BPNIP3-like protein is crucial for the complete mitochondrial removal [169, 170]. In fact, NIX has been proposed to be a receptor protein, having the same function as Atg32 in yeast cells. NIX also possesses the LC3-interacting region (Fig. 11B) [173, 174]. Nevertheless, in determined conditions, mitophagy is also induced in the absence of NIX, suggesting the existence of other redundant pathways. Indeed, mounting evidence indicate that PARKIN, an E3 ubiquitin ligase that when mutated is linked to autosomal recessive forms of Parkinson disease, is translocate from the cytosol to mitochondria in cells submitted to carbonyl cyanide m-chlorophenylhydrazone (CCCP) or paraquat treatments. PINK1, a mitochondrial kinase also linked to autosomal recessive forms of Parkinson disease, seems to assist the translocation of PARKIN to the damage mitochondria both in mammalian cells [175-177] and in *D. melanogaster* [178]. Furthermore, PINK1 is rapidly degraded in healthy mitochondria, whereas it accumulates on the surface of damaged mitochondria (Fig. 11B) [179]. Next PARKIN catalyzes poly-ubiquitination of diverse substrates, recruiting the autophagy receptor p62/SQSTM1, which that simultaneously binds ubiquitin and LC3/GABARAP proteins (Fig. 11B) [177].

Some evidence propose that NIX is able to interact with PARKIN, inducing its translocation for damage mitochondria [180], however further studies are needed to validate this hypothesis

### 1.2.2.3 Other selective types of autophagy.

Both peroxisomes and ribosomes are selectively eliminated via autophagy in yeast cells [181]. Peroxisomes are organelles involved in lipid metabolism and in the elimination of peroxides. In cells the balance between biogenesis and degradation of peroxisomes is highly regulated, and when the peroxisomes population is in excess or damaged, they are committed to degradation by an autophagy-related process called pexophagy. Both yeast and mammalian cells use essentially macropexophagy (Fig. 12), in which the peroxisomes are delivered into the lysosome or vacuole by the engulfment of the autophagosome, but direct engulfment by the vacuole, micropexophagy (Fig. 12), has also been demonstrated [182]. During macropexophagy, Atg30 is an adaptor that interacts with Pex3 and Pex14 (peroxisome proteins), with the selective autophagy adaptor Atg11 and also with Atg17 (Fig. 12) [107, 183].

Ribosomes are also selectively degraded during starvation in a process called ribophagy. This process depends on the core of Atg proteins from autophagy [184], and is also dependent on the catalytic activity of the ubiquitin protease Ubp3p and its cofactor Bre5p [185], suggesting that ubiquitin is involved in this process.

*S. cerevisiae* cells are also able to degrade non-essential portions of the nucleus into the vacuole in response to starvation conditions, a process called piecemeal microautophagy of the nucleus (PMN). In this process patches between the nucleus and the vacuole, the called nucleus-vacuole junctions (NVJs), are formed by the interaction between Vac8 (vacuolar membrane protein) and a nuclear membrane protein Nvj1 [186]. During PMN, part of the NVJ buds resulting in a bleb that is released into the vacuole. Besides the already referred specific proteins, PMN requires proteins from the autophagy machinery (Fig. 13) [187]. Usually PMN is induced upon nutrient starvation, although it does not contribute to cell survival during starvation conditions, it might be important concerning the adjustment of determined processes in the nucleus, as for instance transcription or ribosome biogenesis (Fig. 13) [188].

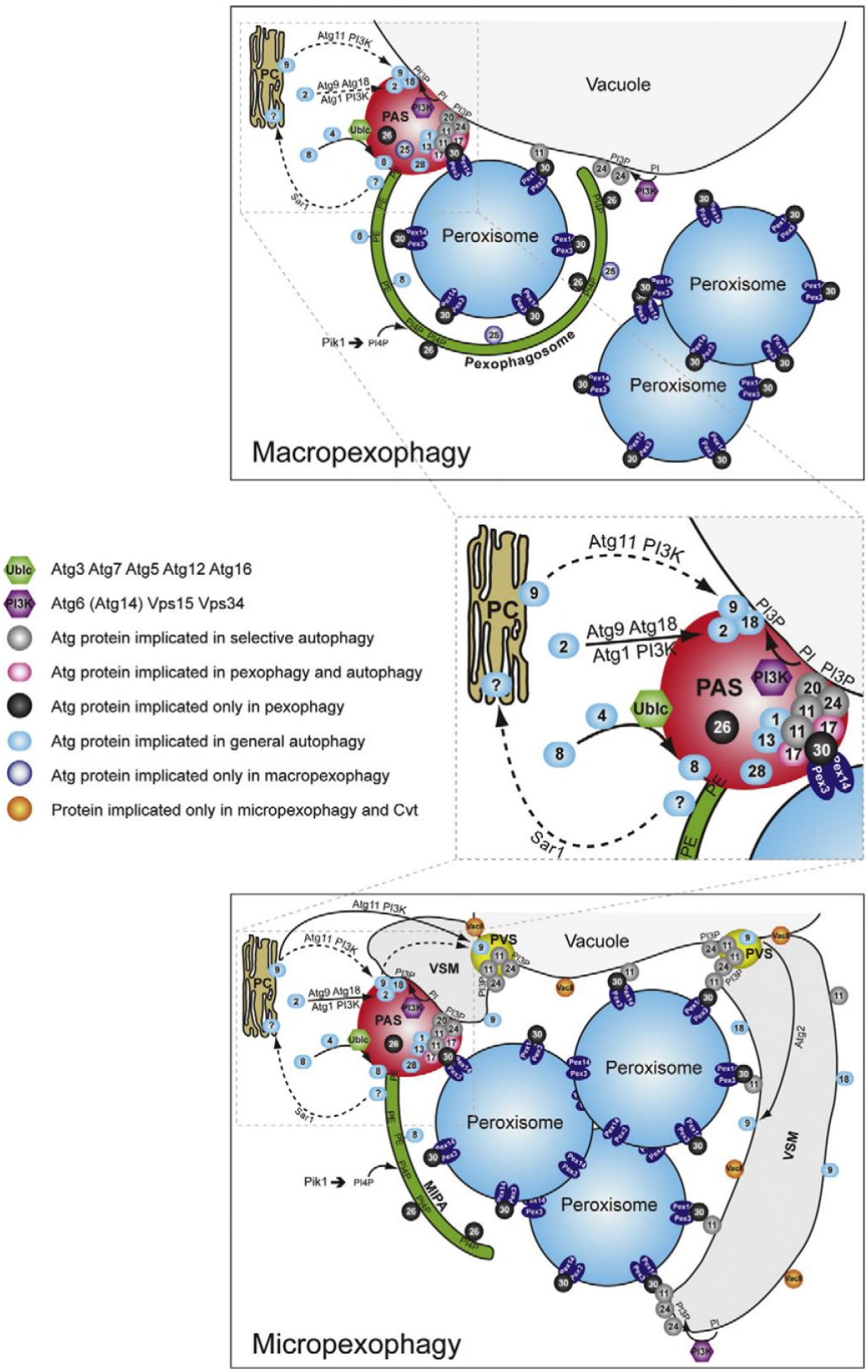


**Figure 12.** Schematic representation of pexophagy (see in the text). From [50].

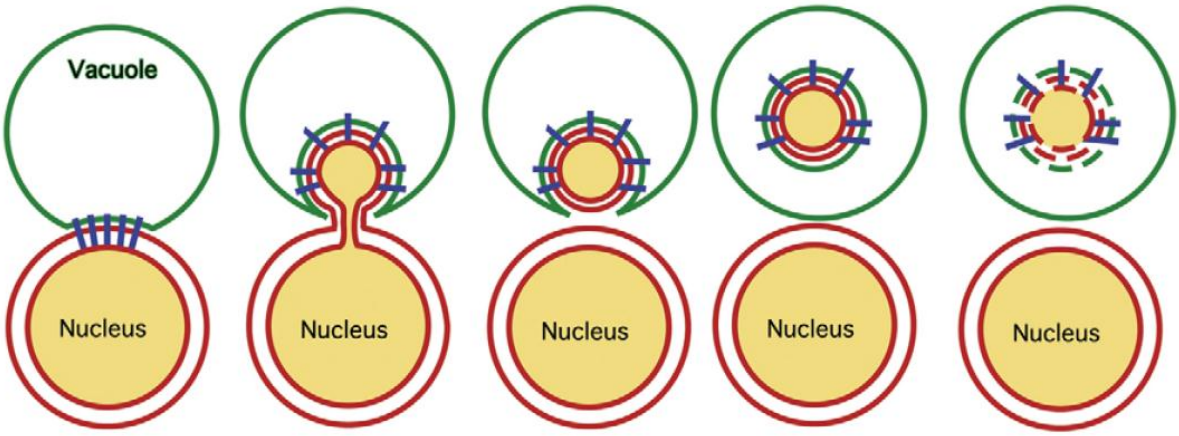


**Figure 13.** Schematic representation of piecemeal microautophagy of the nucleus, which allows the degradation of a portion of the nucleus (see in the text). From [50].

### 1.2.3 Transcriptional/translational and post-translational regulation of autophagy

Autophagy genes are regulated at a transcriptional level in response to stress. In fact, autophagy induction under starvation conditions greatly stimulates the expression levels of some *ATG* genes. Under starvation, *ATG8* levels are extremely high, nevertheless, the transcription factor(s) that regulate the expression level of the *ATG8* gene are still poorly understood [110], being Gln3, a nitrogen catabolite repression-sensitive GATA-type transcription factor, the only regulator factor described [119, 189]. On other hand, the *ATG14* mRNA levels are increased more than 20-fold in nitrogen starvation conditions and are also regulated by Gln3, but the relationship between the *ATG14* mRNA levels and autophagy induction is not established (reviewed in [190]). In addition to *ATG8* and *ATG14*, a microarray analysis demonstrated that *ATG1*, *ATG3*, *ATG4*, *ATG5*, *ATG7*, *ATG12* and *ATG13* are upregulated under nutrient deprivation conditions [191]. Alongside, under amino acid starvation, yeast triggers a general amino acid control in an attempt to induce the transcription of several genes. In this response, Gcn4 plays a central role, since it is a transcriptional activator of gene expression [192]. Its synthesis is mainly regulated at the translational level, particularly by the protein kinase Gcn2 that phosphorylates the only known substrate eIF2α, which transcriptionally stimulates autophagy, since the transcription of *ATG12*, *ATG1* and *ATG13* was found upregulated in these conditions (reviewed in [55]). Recently, it was demonstrated that the life span extension induced by the acetylase inhibitor spermidine requires the participation of essential

autophagy genes. Indeed, the increased longevity is linked to alterations in the acetylation of nuclear histones and to a transcriptional increase of different *ATG* genes, including *ATG7*, *ATG11* and *ATG15* [193]. Furthermore, as hereafter described, ER stress is also associated with autophagy stimulation by upregulating the expression of specific *ATG* genes [194, 195]. Although, autophagy could be transcriptionally regulated, this type of alteration/regulation is not the main mechanism of autophagy regulation.

Stimulation of autophagy in conditions, in which protein synthesis is blocked, for example with cycloheximide, results in the formation of abnormally small autophagosomes, suggesting that protein synthesis is not directly correlated with induction of autophagy, but it is crucial for the generation of normal sized autophagosomes [196]. Furthermore, it is proposed that higher levels of Atg8 are necessary to mediate the increased number of membrane fusion events occurring during the formation of the large autophagosomes. Indeed, this hypothesis was sustained by the finding that deletion of *ATG8* results in cells with abnormally small autophagosomes [196, 197]. Furthermore, it is also proposed that translational initiation factors, like eIF4GI, that are associated with the translation of proteins controlling cell growth and proliferation, block the stimulation of autophagy downstream of TOR [198], while eEF-2 (eukaryotic elongation factor-2) kinase and the eIF2α kinase signaling pathway that induce translational arrest, positively regulate autophagy [199, 200].

Several distinct stimuli and toxic agents can induce a rapid homeostatic autophagy, and there is increasing evidence that initially autophagy induction does not require transcriptional reprogramming and changes in the expression of the autophagic genes, suggesting that the cells have enough Atg proteins levels to rapidly response to cellular injuries. In fact, instead of transcriptional reprogramming, autophagy depends on post-translational modifications of proteins, including phosphorylation, acetylation and ubiquitination. The autophagic process comprises ubiquitin-like modification of Atg12 and Atg8/LC3, and TOR/PKA-dependent phosphorylation of the Atg1/ULK kinase complex, as above described. Additionally, in mammalian cells, it was demonstrated that several Atg protein, including Atg5, Atg7, Atg8, and Atg12, are acetylated in nutrient-rich conditions and in contrast they are deacetylated during starvation. In this sense, deacetylation modification of some autophagy players is important for autophagy stimulation [201]. Additionally, the deacetylation process is dependent on the NAD-dependent deacetylase SIRT1 (a sirtuin family member) [201], which expression is induced by caloric restriction (CR) and direct

related with promotion of autophagy and longevity [202]. Furthermore, assays with the acetylase inhibitor, spermidine and the deacetylase activator, resveratrol, both inducers of autophagy, showed that they induce changes in the acetylation of several proteins linked to the autophagy network. Importantly, in the cytoplasm, both agents induce essentially convergent protein deacetylation, whereas in the nucleus, acetylation is dominantly triggered by spermidine and resveratrol. Thus it is suggested that multiple post-translational modifications of the acetylproteome can probably regulate autophagy [203].

Collectively, the findings suggest that autophagy at basal conditions is regulated by nuclear-independent responses, however, during time, to maintain an increased autophagic activity, transcriptional reprogramming is required.

### 1.2.4 Signaling pathways that regulate autophagy

Autophagy occurs at basal levels in the majority or even all cells, but it can be induced under several conditions. Indeed, extracellular stimuli, such as starvation or therapeutic treatment, and also intracellular stimuli, including the accumulation of misfolded proteins, have the capacity to modulate the autophagic response. Additionally, autophagy is sensitive to nutrient availability and is negatively regulated by signaling pathways. In fact, the link between autophagy and nutrient-sensing pathways that regulate life span has been demonstrated in many studies. These aspects will be explored hereafter.

#### 1.2.4.1 Nutrient Signaling

Nutrient starvation is able to rapidly induce the formation of autophagosomes. In yeast and mammalian cells, the two main signaling cascades that sense nutrient status and negatively regulate autophagy are the TOR and Ras-cAMP-PKA pathways.

**TOR complex 1 (TORC1)**. A key player in the nutrient sensing and in the regulation of cell growth and the autophagic process is the TOR kinase. As aforementioned, inhibition of autophagy in

yeast cells can be done by TORC1, dependent on the nutritional conditions (Fig. 14). Particularly, TORC1 regulates the Atg1 kinase complex by the Atg13 phosphorylation state [71-73].

Beyond the direct regulation of autophagy by the interaction with the Atg proteins (as above described), TOR can also act through its downstream effectors to control autophagy. Under nutrient rich conditions, TOR has the capacity of phosphorylate the protein Tap42, which promotes its association with PP2A. The association between Tap42-PP2A results in the activation of the catalytic subunits of PP2A, a negative regulator of autophagy [204]. PP2A is a phosphatase that acts on several TOR substrates, including glutaminase (Gln3). Dephosphorylation of Gln3 by PP2A culminates in the activation of the transcription of several genes [205, 206], including *ATG8* and *ATG14* [119, 189], as above referred. On the other hand, under starvation or rapamycin treatment TOR becomes inhibited. This inhibition causes dephosphorylation and dissociation of Tap42 from PP2A, allowing the PP2A to acquire the capacity of dephosphorylate its targets, leading eventually to a variety of anti-proliferative responses and also to the induction of autophagy [204].

**Ras/cAMP-dependent protein kinase A (PKA).** Besides TORC1, the Ras/PKA also regulates autophagy [207]. This pathway plays a key role in cell proliferation, stress response, and longevity [208]. In yeast, PKA contains a heterotetramer that is composed by a regulatory subunit Bcy1 and three apparently redundant catalytic subunits, Tpk1, Tpk2, and Tpk3. Thus, in response to nutrient rich conditions, two redundant Ras GTPases, Ras1 and Ras2, are activated and subsequently stimulate adenylate cyclase to produce cAMP. Then cAMP is able to bind to the regulatory PKA subunit, Bcy1, allowing its dissociation from the PKA catalytic subunits [209], and consequently the activation of PKA. When this activation occurs, the autophagy induced by TOR inhibition is suppressed, indicating that PKA is a potent negative regulator of autophagy (Fig. 14) [210, 211]. Additionally, it was demonstrated that Atg1, Atg13, Atg18 and Atg21 are PKA substrates. Apparently, in the presence of nutrients, PKA phosphorylation promotes the presence of Atg1 and dissociated from the PAS, whereas in contrast, during nutrients depletion, Atg1 is dephosphorylated and it is localized to the PAS. However, it is still unknown if the phosphorylation of these proteins by the PKA is linked to autophagy regulation [211].

Additionally, PKA may function in parallel with Sch9 in the control of several physiological events, such as growth, cell cycle progression, and glycolysis [208, 212]. Sch9, a serine/threonine protein kinase, which is the yeast homologue of Akt and S6K, is also involved in the nutrient sensing.

[213]. It is described that Sch9 is a negative regulator for autophagy (Fig. 14), since, hyperactivation of Sch9 moderately suppresses autophagy when TOR becomes inactivated by rapamycin. Furthermore, Sch9 also cooperates with PKA to regulate autophagy, thus inactivation of both PKA and Sch9, independently of TORC1 regulation, triggers autophagy. Additionally, as the inactivation of Sch9, PKA and TORC1 has an additive effect, probably the three, at least in part, cooperate in parallel in the autophagy regulation (Fig. 14) [210]. The common downstream targets of PKA and Sch9 pathways, the stress resistance transcription factors Msn2/Msn4 and the protein kinase Rim15, are required for autophagy induced by inactivation of these pathways, but not for autophagy induced by inactivation of TORC1 [210].

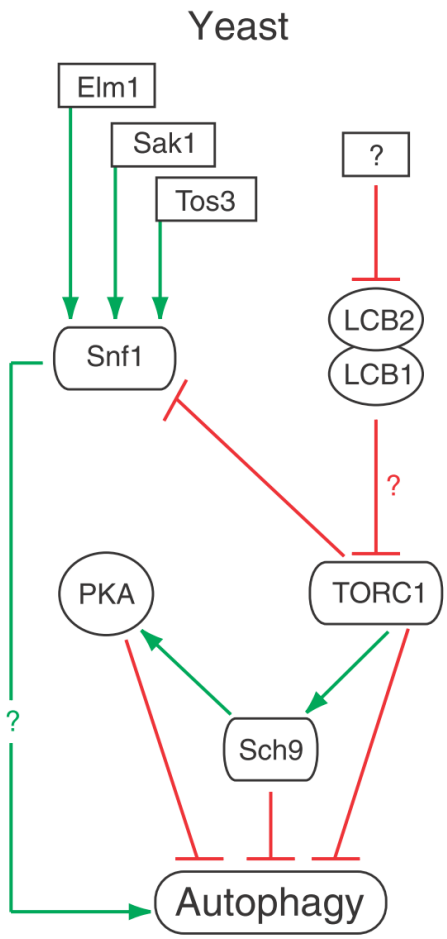


**Figure 14.** The signaling pathways that regulates autophagy in yeast cells. Adapted from [214].

In yeast, the abrogation of the conserved TOR, Ras/cAMP-dependent protein kinase A (PKA) or Sch9 proteins, that integrate the network of nutrient sensing pathways, is known to promote longevity (Fig. 14) [214-217]. These signaling pathways are negative regulators of autophagy, reinforcing that autophagy and aging are coordinately regulated by a complex network of different signaling pathways, with partial overlapping branches and yet undisclosed hierarchic connections.

#### 1.2.4.2 Energy sensing

**SNF1.** Snf1 is the yeast homologue of the mammalian AMP-activated protein kinase (AMPK), and functions as an energy sensor that is able to reprogram the cellular metabolism in order to restore the normal energy levels essential to sustain cell metabolism and to support a stress response. Snf1 is activated in response to glucose depletion in attempt to allow transcription of glucose-repressed genes, however its activity increases in aged cells even when glucose is abundant (reviewed in [218]).

It is established a crosstalk between this energy sensing protein and the nutrient-sensing players. In fact, Sch9 was shown to be a common downstream target of the intrinsic aging defense pathway controlled by Snf1 kinase and the extrinsic nutrient-sensing pathway regulated by TORC1, additionally, Snf1 is also regulated by PKA [219]. In contrast to AMPK, Snf1 is negatively regulated by TORC1, but it can positively regulate autophagy (reviewed in [218]). The molecular mechanism underlying Snf1 regulation of autophagy is still elusive, but appears to be partially dependent on the inactivation of TOR kinases [220] and on the direct Atg1 phosphorylation [214]. Apparently, Snf1 is associated with the DNA replication stress, and several evidence suggests a strong interconnection between DNA replication stress, autophagy and nutrient signaling pathways. Nevertheless, the underlying crosstalk between DNA replication stress, autophagy and nutrient sensing pathways and the role of snf1 in this crosstalk remains to be elucidated.

### 1.2.4.2 Stress response

**ER stress.** As above referred, the ER is the major compartment in the cell that facilitates folding of newly synthesized proteins. Several evidence suggests that autophagy is induced by the ER stress (Fig. 15), and the signaling mechanisms associated with that regulation are dependent on the specific stress conditions and the organism in study. ER stress response is normally activated in an attempt to protect the cells from changes that alter the organelle homeostasis. In this sense, autophagy can help cells to handle with ER stress, contributing to the elimination of unfolded or aggregated proteins or even participating in the mechanisms of ER stress-induced cell death (reviewed in [221]). In yeast, chemical induction of ER stress triggers autophagy by the Atg1 kinase [139]. In fact, activation of the Ire1-Hac1 arm, particularly Hac1 is capable of inducing the expression of several *ATG* genes, like *ATG5, ATG7, ATG8*, *ATG14* and *ATG19* (Fig. 15) [194, 195]. Additionally, ER proliferation leads to the formation of autophagosome-like structures that are densely and selectively packed with membrane stacks derived from the UPR-expanded ER [155].

In mammalian, PERK and IRE1 have been implicated as mediators of ER stress-induced autophagy. It is demonstrated that the ER stress response to the presence of aggregates in the cytosol, particularly PERK or its downstream target eIF2$\alpha$, upregulates Atg12 expression and induces autophagy confirmed by the conversion of LC3-I to LC3-II [194] (Fig. 15).

Under certain scenarios, when the intensity or duration of the ER damage cannot be restored, ER stress can also lead to cell death via both apoptosis and autophagy [222]. Therefore, depending on whether pharmacological or genetic inhibition of autophagy improves or prevents cell death, stimulation of autophagy induced by ER stress can generate a protective or a cytotoxic response.

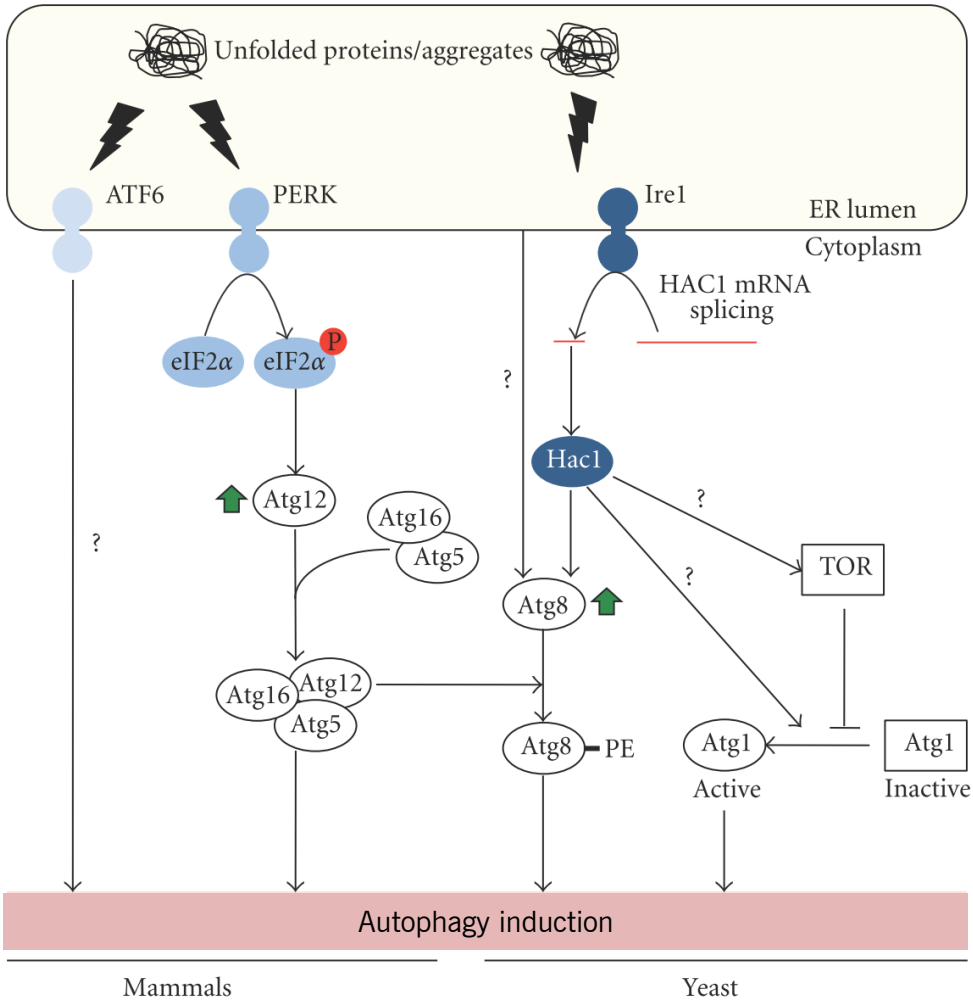


**Figure 15.** Signaling cascades inducing autophagy upon ER stress. The transmembrane protein Ire1 (in yeast and mammalian cells), ATF6 and PERK (mammalian cells) sense the accumulation of unfolded proteins and/or aggregates, triggering a general transcriptional response that disturbs the levels of proteins involved in autophagy. From [223].

**Oxidative stress.** A common intracellular stress that apparently leads to induction of autophagy is the formation of ROS, which are essentially generated in mitochondria, as above mentioned. The link established between ROS and autophagy induction is via the cysteine protease Atg4, responsible for the cleavage of Atg8/LC3 from the autophagosome (as above described). In fact, ROS inhibit Atg4 protease activity and promote the lipidation of Atg8/LC3 [224]. Additionally, stimulation of autophagy induced by starvation, inhibitors of mitochondrial electron transfer chain or even addition of exogenous hydrogen peroxide ($H_2O_2$) is correlated with increased superoxide anion ($O_2^{2-}$) production along with $H_2O_2$ decrease suggesting that $O_2^{2-}$ is the major ROS involved in

autophagy modulation. Furthermore, the scavenging of $O_2^{2-}$ accumulation by overexpression of Sod2 is able to inhibit autophagy and increases starvation-induced cell death (reviewed in [225]).

Even though, ROS are involved in autophagy induction, autophagy can also have an impact on ROS production. Therefore, it is possible that autophagy induction results in the removal of specific proteins, like the detoxifying enzymes, generating a self-sustaining loop, which enhanced ROS production by mitochondria that promotes an over-activation of autophagy (reviewed in [225]). Therefore, ROS acts as positive regulators of autophagy, while autophagy induction can either reduce or increase oxidative stress.

### 1.2.5 Autophagy in cell survival and cell death

Autophagy is a crucial process to maintain cell homeostasis, particularly important in post-mitotic cells, since they do not have the capacity to get rid of their waste through cell division [226, 227]. Autophagy is a process that is present at basal levels, in most cells, contributing to the turnover of cytoplasmic components. Usually, it is induced during starvation in an attempt to maintain the energy homeostasis. Nevertheless, its induction is not only restricted to starvation, the autophagy network is likewise involved in the clearance of damage organelles, the degradation of intracellular pathogens and also protein aggregates that cannot be removed by the UPS (reviewed in [228]).

An apparent counter intuitive aspect is that besides the autophagy cytoprotective role, it is also participating in cell death, probably due to uncontrolled over stimulation of autophagy (reviewed in [229]). Autophagic activity above a certain threshold engulfs largely portions of the cytosol and organelles, which could result in an irreversible type of cellular atrophy and cause a total collapse of cellular functions. In fact, it was shown that during intense autophagic activity, the total area of autophagic vacuoles may equal that of the cytosol and organelles outside the vacuole (reviewed in [228]). Paradoxically, autophagy has also been implicated in a type of programmed cell death (type II PCD), designed by autophagic cell death that is different from apoptosis and necrosis, which was first described as a type of cell death with the presence of autophagosomes [230], but they may also accumulate as a result of inhibition of the processing of autophagosomes rather than an upregulation of autophagy. In this sense, recently new criteria were defined, and then autophagic

cell death is characterized by: (i) cell death that occurs without the involvement of the apoptosis machinery; (ii) increase of autophagic flux, and not only an increase of the autophagic markers, in the dying cells; and (iii) suppression of autophagy via pharmacological inhibition and genetic approaches can be able to rescue or prevent cell death [231].

It is now accepted that autophagy can participate in cell survival under determined circumstances and also promotes cell death in other, being context-dependent, depending on features such as the nature of the stimuli, the cell type and the presence or activation of other autophagy-related factors. Nevertheless, the primordial function of autophagy is associated with a rapid cellular rearrangement in an attempt to promote the cell survival.

### 1.2.6 Autophagy and aging

Aging is a complex, continuous and dynamic process that begins immediately after birth and ends with death [232]. It is associated with a general and progressive decline of cellular processes that ultimately culminate in cell death. Aging, at the molecular level, is characterized by a progressive accumulation of damage in DNA, RNA, lipids and proteins. In fact, the most related aging symptom at the molecular level is the accumulation of altered proteins, both polypeptide chain length and amino acid composition [233, 234]. In this sense, the age-dependent decrease in the activity of the quality protein control system such as autophagy is believed to be the main cause for the accumulation of dysfunctional organelles and damaged molecules, particularly critical in post-mitotic cells [235, 236]. The genetic and pharmacological inhibition or enhancement of autophagy is respectively correlated to decreased or increased longevity. Indeed, the vast majority of interventions found to increase longevity are autophagy-dependent, suggesting a crucial cytoprotective role of autophagy and the tight relation between autophagy and aging.

In yeast, the abrogation of the conserved TOR, Ras/cAMP-dependent protein kinase A (PKA) or Sch9 proteins, which integrate the network of nutrient sensing pathways, is known to promote longevity [214-217]. These signaling pathways are negative regulators of autophagy, supporting that autophagy and aging are coordinately regulated. Another important aspect to consider when analyzing the regulation of autophagy and aging by nutrient sensing pathways is the dietary intervention, caloric restriction (CR). CR is the only non-genetic intervention that, until date, is the

best approach used to delay the onset of aging. CR consists in the reduction of calories intake while maintaining appropriate levels of essential nutrients such as minerals and vitamins. CR regime changes from glucose or amino acids limitation and incubation in water for yeast, axenic medium or bacterial deprivation for worms, reduced availability of yeast for flies and calories or amino acid restriction or alternated day feeding for rodents. It is responsible for 20% to more 100% life span expansion in these organisms [237-246]. Nevertheless, although in several models the CR beneficial in the prevention of aging, a direct effect of CR on the life span extension in human and primates is still under discussion [247]. Importantly, CR exerts its effects by reducing the activity of various signal transduction pathways either directly or through the decrease in the activity of nutrient-sensing pathways, such as the TOR, PKA and Sch9. The transduction pathways are able to activate a stress response and particularly to induce the antioxidant defenses [248], by inducing catalase activity and the superoxide dismutases (SODs) [249, 250]. Thus, CR restriction regulates several signaling pathways that are linked to autophagy regulation, suggesting that CR is also regulating autophagy. In this sense, it is proposed that autophagy is one of the mechanisms associated with the extension of life span by caloric restriction [251], since in cells where the expression of different *ATG* genes was abolished, the anti-aging effects of CR were reverted [252, 253].

Although TORC1 is the most studied regulator of autophagy, molecules such as the acetylase inhibitor, spermidine, and the deacetylase activator (Sirtuin1), resveratrol, prolong life span of multiple species in a TOR-independent autophagy-dependent fashion [193]. Interestingly, in yeast the longevity effects of CR are also associated to the sirtuins activity (reviewed in [202, 254-256]), particularly the yeast replicative life span, pointing to a complex and elaborated network regulating autophagy and aging. Sirtuins are a family of deacetylases that function either as deacetylases or as ADP ribosylase enzymes, which are regulated by the cofactor $NAD^+$ and thus may serve as sensors of the metabolic state of the cell [257]. In yeast, while high Sir2 activity has been reported to extend replicative life span by inhibiting the progressive enlargement and fragmentation of the nucleoli in old cells resulting from the accumulation of toxic extrachromossomal ribosomal DNA circles (ERCs) [239], in chronological life span, Sir2 appears to have a different role. Under non-CR conditions, deletion or overexpression of Sir2 has no effects on the CLS, nevertheless it is described that the CLS extension experienced by cells under extreme CR is also dependent on the role of Sir2. In this sense, the findings suggest that in *S. cerevisiae*, Sir2 deacetylases play both pro- and anti-aging roles in different contexts (reviewed in [257]).

Mammalian cells possess seven sirtuins, in which SIRT1 is the closest to the yeast Sir2, nevertheless the aging phenotype associated with SIRT1 overexpression and abrogation has not yet been determined. However, several studies have reported that also in mammals sirtuins may affect aging [258].

Together, the accumulating findings, above described, point to the existence of an elaborated network signaling autophagy and aging, and pharmacological manipulation of autophagy appears as an attractive strategy to delay aging and has been motivating the development and the search for drugs that directly or indirectly, regulate autophagy activity. Nevertheless, the pleiotropic nature of nutrient signaling pathways regulating autophagy and the dual and opposite functions, cytoprotective or detrimental according to the context, of autophagy generate serious limitations.

### 1.2.7 Monitoring autophagy

Given the impact of autophagy in several physiological and pathological processes, it was necessary to establish methods to correctly identify, quantify and manipulate the autophagic process. Thus, in the last years, the identification of the autophagic machinery players and the possibility to experimentally manipulate the autophagic process allowed the development of innumerous techniques to monitor and modulate autophagy in order to prove its role in determined processes [259].

To evaluate autophagy, it is necessary to have in attention that an increased number of autophagosomes is not directly related with an induction of autophagy, since autophagosome is an intermediated structure and the number, at determined time point, is a function of the balance between the rate of their generation and the rate of their conversion into autolysosomes. Therefore, autophagosome accumulation may denote either autophagy induction or, alternatively, inhibition if a blockage of the process occurs before the autophagosome formation [259].

Microscopy, in particular electron microscopy (EM), is an old methodology, that it is still indispensable for the monitorization of autophagy. The EM has been used to quantify the autophagosomes number. Nevertheless, although the autophagosome is a double membrane structure that contains undigested cytoplasmic material including organelles, whereas autolysosome is a single membrane structure containing cytoplasmic components at various stages of

degradation, it is not always possible to distinguish the autophagosomes from autolysosomes, and there is no specific biochemical marker that distinguishes the both organelles [260]. In this sense, new adaptations to the EM methodology were developed, using the combination of positive and negative markers, such as immunogold labeling studies [118]. Since assessment of the autophagosome by EM techniques requires specialized expertise and is very expensive, alternative methodologies were developed. In the last years, it became widely used the quantification of autophagosomes by counting the GFP-Atg8/LC3 punctate, using fluorescence microscopy, both in yeast and in mammalian cells. As above referred, Atg8/LC3 is a unique autophagic marker, since their lipidated form remains attached to the autophagosome until the fusion with the lysosome/vacuole. Nevertheless, the quantification of the autophagosomes by this technique has several constrains. Since Atg8–PE/LC3-II is associated with all types of autophagic organelles, including the pre-autophagosomal phagophores, and its formation/lipidation/delipidation kinetics is not well established, it is almost impossible, at least with the currently available antibodies, to distinguish between Atg8/LC3-I and Atg8-PE/LC3-II [109]. Thus, the autophagosomes quantification may not be entirely correct. Surprisingly, in yeast, it is possible to assess, by light microscopy, the accumulation of undigested autophagic bodies in the vacuole. In this assay the cells must be treated with protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF), to inhibit the degradation of autophagic bodies, or it can also be used vacuolar protease mutant strains [261]. This method cannot be applied to mammalian cells because the lysosome is too small.

More recently, the measurement of autophagy flux by the LC3 turnover was introduced, essentially in mammalian cells. In this assay cells must be treated with lysosomal inhibitors that will impair the LC3-II degradation resulting in LC3-II accumulation. Therefore, the differences in the LC3-II amount between samples in the presence and absence of lysosomal inhibitors represent the amount of LC3 that is delivered to lysosomes for degradation [262]. Another developed method is based on the disappearance of total LC3/Atg8 that will be directly correlated with autophagy flux. In this method, which can be applied to mammalian and yeast cells, the reduction of total LC3/Atg8 can be quantitatively and sensitively monitored by flow cytometry [263] or by immunoblot analysis and is inversely correlated with autophagic flux. To perform this technique, the LC3/Atg8 must be fused with tag proteins, like GFP. Additionally, other autophagy substrates like p62 can be used to monitor autophagy flux [264].

Due to the different issues associated with the microscopic methodologies, biochemical assays to assess the autophagosome number were also developed. In mammalian cells, LC3 is also used in biochemical assays to assess autophagosome numbers. The conversion from endogenous LC3-I to LC3-II and from GFP-LC3-I to GFP-LC3-II can be detected by immunoblotting with antibodies against LC3 or GFP, respectively. In this assay it is established that the amount of LC3-II is correlated with the number of autophagosomes [109]. Nevertheless, not all LC3-II is present on autophagic membranes and some population of LC3-II seems to be ectopically generated in an autophagy-independent manner [259].

Besides the methodologies described to assess the number of autophagosomes, the autophagic flux assays were also designed to reveal if the autophagosome number is correlated with autophagy induction or inhibition. One of the most traditional methods to evaluate autophagy flux, that can be a performed in yeast and mammalian cells, is the measurement of the degradation of long-lived proteins [265]. It is possible to measure the bulk degradation of long-lived proteins, by incubating cells with radioactive amino acids to label all cellular proteins. The accumulated radioactivity is directly correlated to the degradation of long-lived proteins. It should be noted that the distinction between long-lived and short-lived proteins is not always possible and that a considerable fraction of the long-lived proteins degradation will, therefore, reflect non-autophagic proteolysis [266]. In this sense, some long-lived proteins and even other macromolecules that appear to be exclusively degraded by autophagy, can be used to specifically measure autophagy including lactate dehydrogenase (LDH) and lactose [267].

All the above described methods are based on the disappearance of a signal/protein, nevertheless there are inherent difficulties because the reduction of the protein levels is generally so small and occurs so slowly that the detection of the changes is hampered by the high background of the remaining protein. In this sense, to overcome this difficulty, in yeast was developed a method that allows the quantification of the autophagic flux based on the incremental increase of a signal, particularly it is based in the measurement of an enzyme activity. Yeast cells own a vacuolar alkaline phosphatase, Pho8, which contains a N-terminal transmembrane domain that acts as an internal uncleaved signal sequence allowing translocation into the ER, but as the majority of the resident vacuolar hydrolases, Pho8 is delivered into the vacuole through a portion of the secretory pathway [268]. When Pho8$\Delta$60, which lacks the membrane spanning region and cytosolic tail, is expressed in cells where the endogenous Pho8 was abrogated, the modified inactive protein will be detected in

the cytosol. When autophagy is induced the Pho8Δ60 is transported to the vacuole via autophagy, where it is activated, and this conversion can be enzymatically measured, for instances through the alkaline phosphatase (ALP) assay [269].

To accomplish a more specific measurement of autophagy, it is important to execute the above described methodologies in the presence of autophagy modulators [259]. Actually, in autophagy research, one of the most severe problems is the lack of highly specific autophagy modulators, both inhibitors and activators. Nonetheless, some modulators are available, and it is also possible to perform genetic manipulation techniques, as schematized in figure 16 [270]. In yeast and mammalian cells, the commonly used pharmacological approaches to inhibit autophagy involve the use of PI3-kinase inhibitors such as wortmannin or 3-methyladenine (3-MA). These drugs, inhibitors of class III phosphatidylinositol 3-kinases, block the autophagosome formation, nevertheless, they will also interfere with a variety of cellular processes [271, 272], including DNA synthesis and endocytosis, being necessary a careful analysis of the results. The other class of pharmacological inhibitors blocks autophagy at later stages of and include bafilomycin A1, chloroquine (CQ), and lysosomal protease inhibitors such as E64d and pepstatin A, which raises the lysosomal/vacuole pH leading to the inhibition of lysosome/vacuole-autophagosome fusion and to lysosomal/vacuole protein degradation. One major limitation of these inhibitors is also the fact that they affect other cellular processes besides autophagy, such as mitosis and endocytosis [259]. A more specific inhibition of the autophagy pathway is achieved by the knockout or knockdown of different *ATG* genes, which as observed in figure 16, can be applied in the several autophagy steps, allowing the study of a specific autophagy phase. In yeast, this approach can be easily performed. In mammalian cells, it is possible to implement the RNAi mediated approaches to induce the knockdown of certain Atg proteins, nevertheless, since the protein levels are not completely suppressed, the results must be carefully analyzed. Furthermore, the autophagy inhibition can results in mild phenotype, probably due to the presence of isoforms that can compensate for the deficiency [259].

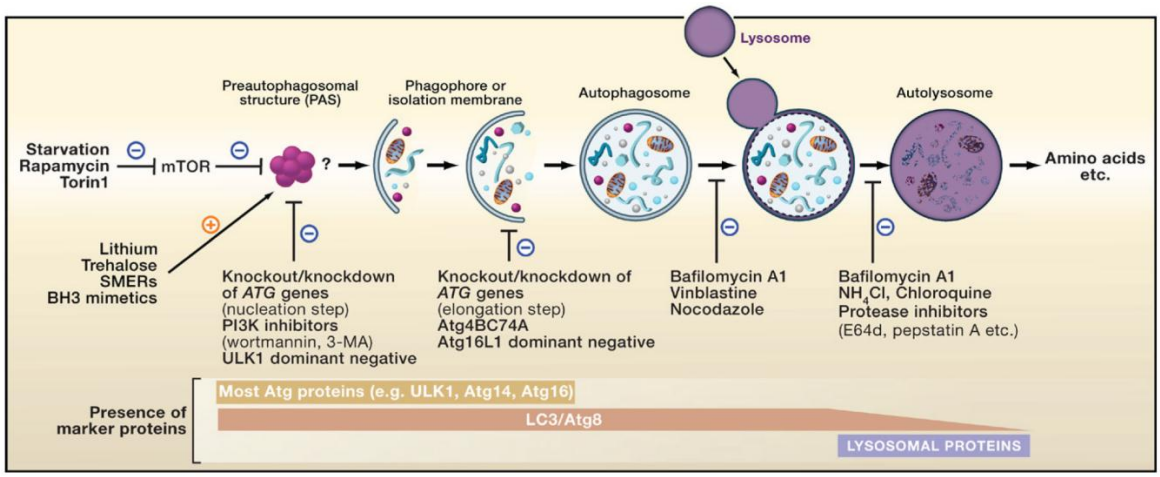


**Figure 16.** Schematic representation of the most common treatments and reagents used to modulate the autophagy process. From [259].

Autophagy can also be stimulated. Like the autophagy inhibitors, the autophagy inducers lack specificity to the autophagic pathway. The most efficient physiological inducer of autophagy is starvation. Another way to activate autophagy is through the modulation of the nutrient-sensing signaling pathways, in which the most used target is the TOR kinase. It can be used rapamycin and its analogs, such as CCI-779, that will inhibit TOR and activating autophagy. However, TOR inhibition is not a specific inducer of autophagy, since other cellular responses, particularly protein synthesis and cellular metabolism, are also affected [259].

Currently, to measure autophagy, it is available a large number of methods, however none of them is a perfect method to evaluate the autophagosome number and autophagy flux. Therefore, to perform an accurate and rigorous evaluation of the autophagic activity it is necessary to apply multiple approaches.

## 1.4 The protein misfolding related synucleinopathies

The protein misfolding related synucleinopathies, in which Parkinson disease (PD) is included, is the most common neurodegenerative movement disorder, affecting millions of elderly persons worldwide [273-275]. PD is clinically characterized by major motor difficulties including uncontrollable resting tremor, muscular rigidity, impaired postural reflexes, and bradykinesia, which

differ between patients [273, 276]. Although, PD is a prevalent degenerative illness that affects the central nervous system and also the peripheral and enteric systems [276], the main neuropathology that underlie PD motor deficits is the loss of dopaminergic neurons in the *substantia nigra* pars *compacta*, which results in a severe depletion of striatal dopamine (DA) [273], as well as the presence of cytoplasmic, Lewy bodies, (LB) and neuritic, Lewy neuritis (LN) inclusions in the surviving dopaminergic neurons [277].

The PD etiology remains poorly understood, however the pathogenesis of this disease appears to be converging on common contexts: aging, oxidative stress, mitochondrial dysfunction and protein aggregation [51, 273]. PD is mainly a sporadic neurodegenerative disease. However, rare monogenic familial forms of the disease, characterized by early-onset and an autosomal dominant or recessive pattern of inheritance have also been identified [278]. Until now, 6 genes have been linked to familial PD. α-Syn and LRRK2/dardarin mutations are responsible for autosomal dominant forms of PD, whereas mutations in Parkin, PINK1, DJ-1 and ATP13A2 are associated with the autosomal recessive forms of PD. Additionally, the genes that codify for the ubiquitin carboxy-terminal esterase L1 (UCHL1), the mitochondrial serine protease (Omi/HtrA2) or the synaptic protein Synphilin-1, are also associated with PD, nevertheless these associations remain poorly understood [279-282]. α-Syn is a small protein that contains an imperfect sequence (KTKEGV) repeated about 6 times throughout the N-terminal half of the protein [283], a hydrophobic central region and an acidic C-terminal region (Fig. 17) [284]. This protein is natively unfolded, however it can be found in several aberrant conformational states such as oligomer, protofibril, and amyloid fibril [285]. The focus on α-syn emerged at the time that α-syn was identified as the major constituent of the Lewy bodies (LB). In fact, three autosomal dominant mutations, A53T, A30P and E46K, have been identified and linked to the familial forms of PD. Additionally, the duplication and triplication of the α-syn locus leads to earlier disease onset and faster progression [286]. Furthermore, excessive posttranslational modifications including phosphorylation, ubiquitination, nitration, oxidation, and the increase propensity to interact with other proteins modulate the predisposition of α-syn to fibrillize [285]. For instance, α-syn is essentially in a non-phosphorylated state in normal conditions, although in disease conditions, it is extensively phosphorylated. Indeed, in the post-mortem brain tissue from PD patients the majority of the α-syn presents in the LB is phosphorylated at Ser129. Thus, it is hypothesized that the phosphorylation at Ser129 serves as the priming event that leads to the LB formation [287].

The α-syn function is poorly understood, however mounting evidence indicates that α-syn is able to interact with some pathways, like the vesicular trafficking, the lipid metabolism, the protein quality control system and with the mitochondrial homeostasis. α-Syn regulates the membrane trafficking via vesicle budding or turnover. Thus, overexpressed or mutated α-syn allows the efficient vesicles budding from the ER, however hampers the vesicles dock and fusion with Golgi membranes [288], resulting in vesicles accumulation. In addition, endocytosis is also impaired by α-syn, triggering the obstruction of post-vesicle internalization steps and defects in vacuolar fusion. It is also described that α-syn inhibits the phospholipase D (LPD), which is involved in the lipids metabolism, causing the accumulation of lipids droplets. Besides lipids, α-syn appears to interact with several proteins, modifying their activity, what suggests that it acts as a chaperone protein. Moreover, expression of a-syn was shown to induce ubiquitin accumulation, impairment of the proteasome, mitochondria fragmentation and accumulation of ROS (reviewed in [289-291]).

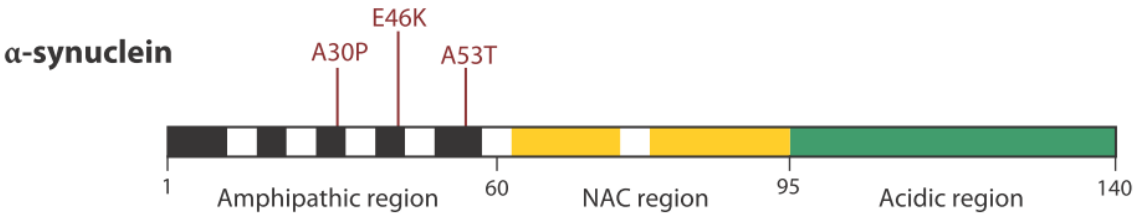


**Figure 17.** α-Synuclein protein domains and pathogenic mutations. Adapted from [292].

### 1.4.1 Synucleinopathies and the proteolytic systems

The proteolytic systems, namely UPS and autophagy, have been associated with the pathogenesis of PD. Depending on the α-syn conformation state, it can be degraded by the UPS and/or by autophagy. The α-syn soluble form is degraded by by the chaperone mediated autophagy (CMA) and can also been degraded by the UPS (Fig. 18a). However, the misfolded α-syn aggregates leads to proteosomal dysfunction, probably because α-syn aggregates sequester ubiquitinated proteins, Hsp and components of the proteasome [293, 294]. This is also supported by data showing that overexpression of α-syn in cultured cells is able to inhibit the proteosomal activity, with

more strong effects observed with the α-syn clinical mutants [295, 296], and that aggregated α-syn interacts with the 19S cap [297]. The mutant forms of α-syn, particularly α-syn A53T, impair the CMA. They are able to bind to the lysosomal membrane with high affinity, but they aren't translocated into the lysosome. Additionally, the binding to the CMA receptor leads to the block of the uptake and degradation of other CMA substrates and consequently to a general CMA blockage [298]. In PD, the blockage of CMA by α-syn prevents also the activation of protective proteins such as myocyte enhancer factor 2D (MEF2D), which is a transcription factor that plays a role in the neuronal survival. Indeed, the blockage of the CMA induces the upregulation of UPS and some compensatory autophagy (macroautophagy) (Fig. 18d), which appears to contribute to neuronal cell death [299]. Several reports showed that autophagosomes structures accumulate in the brain of PD patients [300] and animal models of PD [301], leading to the question of whether autophagy is a cause or a protective factor of neurodegeneration. On the other hand, it is also showed that aberrant α-syn is also able to inhibit autophagy through RAB1A, a GTPase involved in the early secretory pathway, ER-to Golgi transport. Additionally, the RAB1A inhibition leads to the mislocalization of Atg9 and to the inhibition of autophagosome formation [302].

Altogether these findings support the hypothesis that autophagy dysfunction is an important mechanism of PD pathogenesis. Importantly, aging is a major risk factor for neurodegenerative disorders, such as PD. During aging, an inefficient function of several processes becomes evident. Thus, the CMA loses efficiency with downregulation of LAMP-2A receptors, and consequently the UPS becomes the route of α-syn elimination (Fig. 18b). Additionally, upregulation of α-syn results in the failure of the CMA and in the UPS, and consequently in the induction of autophagy (macroautophagy) [303] (Fig. 18c).

Recently, it was observed that in cells overexpressing α-syn or expressing α-syn A53T mutant form occurs a colocalization between autophagosomes and normal, polarized mitochondria, thus these cells presented a decrease number and length of mitochondria [304], suggesting that mitophagy may play a role in the PD pathogenesis. Moreover, this process of clearance apparently is dependent of Parkin, other gene associated with PD [304].

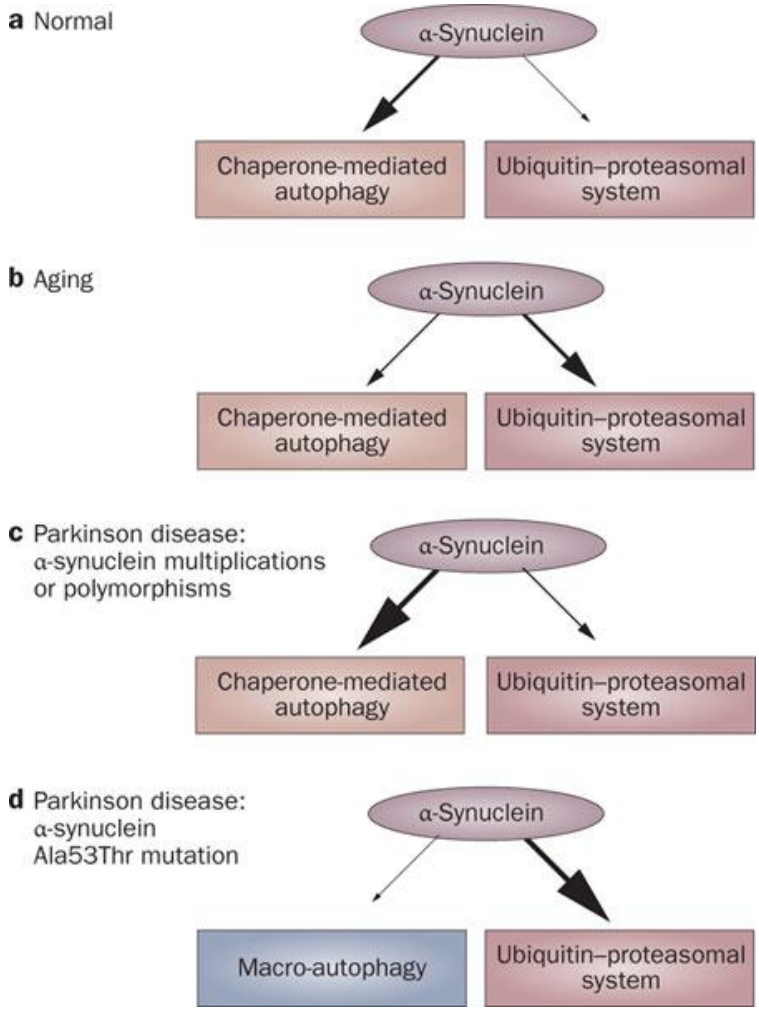


**Figure 18.** The proteolytic systems involved in α-synuclein degradation. a) Degradation of α-synuclein normally occurs though chaperone-mediated autophagy (CMA) with a smaller contribution from the ubiquitin-proteasome system (UPS). b) During aging the CMA becomes inefficient and the UPS is induced. c) Upregulation of α-synuclein induces the CMA activity, but over the time leads to the failure of the CMA and UPS, and autophagy (macroautophagy) adopts a more important role to compensate CMA and UPS failure. d) α-synuclein A53T mutant form blocks CMA, inducing the UPS and autophagy (macroautophagy). From [303].

Furthermore, Parkin plays a role in the specific recruitment and degradation of damage mitochondria by autophagy. The data demonstrated that the treatment of cells with the carbonyl cyanide m-chlorophenylhydrazone (CCCP) results in the relocation of PARKIN to mitochondria followed by mitochondria loss. Further observations also indicated that the PARKIN-marked mitochondrial fragments are LC3 positive, demonstrating that the clearance of damaged mitochondria occurs through autophagy [305, 306]. In this process mediated by Parkin other PD relate gene also interacts, PINK1. Under normal cellular conditions, PINK1 is imported to the inner

mitochondria, although upon mitochondrial depolarization, the import of PINK1 is blocked occurring its accumulation on the outer membrane of damaged mitochondria. Thus, in this scenario Parkin is recruited to the mitochondria and apparently PINK1/Parkin modulates mitochondrial trafficking [307]. Nevertheless, how the recruitment of PARKIN to damaged mitochondria can promote their degradation is still illusive, but it is well established that some of the disease-associated mutations linked to Parkin and PINK1 result in impaired mitophagy [175, 308, 309]. Furthermore, besides α-syn, PINK1 and Parkin, the other monogenic PD-related genes, LRRK2 [310] and DJ-1 [311] apparently are also associated with autophagy or mitochondrial dynamics, nevertheless further studies are necessary to elucidate that role in mitophagy.

Together, these findings imply that beyond autophagy, the specific degradation of mitochondria (mitophagy) might play a significant role or even be the convergence points for different monogenic PD-related mutations that give rise to similar symptoms. Nevertheless, the significance of the mitophagy role and if it a cause or a consequence of the PD pathogenesis is yet controversial.

## 1.5 Yeast models for synucleinopathies

Several model systems have been developed to study PD, particularly synucleinopathies, from high eukaryotic models, like mice, to simple organisms, like yeast. Modeling mice are indispensable for studying the molecular basis of neurological disorders, nevertheless this model entails certain limitations that include slow pace, high costs and complex intrinsic pathways. Thus, it is not surprising that in recent years numerous neurological diseases have been modeled in genetically tractable organisms, including *D. melanogaster*, *C. elegans* and yeast *S. cerevisiae* [312]. Indeed, yeast models presents advantage with respect to genome-wide experimental approaches. The short generation time and the cheap manutention, together with the availability of a collection of precise deletion mutants of every gene in the genome, and the rapidly evolving databases of yeast protein–protein interactions and gene expression patterns. Additionally the key cellular processes associated with the neurodegenerative diseases are conserved between yeast and the higher eukaryotic cells. In this sense, these large and easily accessible bodies of information, coupled with the ease with which yeast can be manipulated genetically, have led to dissection of novel molecular

mechanisms underlying the pathogenesis of synucleopathies, including PD. Furthermore, the yeast models are preferred systems for the screening of chemical libraries, identification and validation of novel drugs, mainly because the compounds/drugs assays are performed in a physiologically relevant environment and their use is cost-effective and user friendly. Nevertheless, the yeast model also possesses limitations. The more pronounced begins with the presence of a cell wall and the most obvious when thinking in neurodegenerative diseases is the lack of cell-cell interactions, like immune and inflammatory responses and synaptic transmission among others. Furthermore, several pathways are far more complex in mammalian cells than in yeast.

For modeling human disease in yeast, it is possible to follow two different approaches. When the human disease-related gene has a yeast homologue, the analysis can be made by the gene deletion or overexpression. On the other hand, if the human disease-related gene does not have a yeast homologue, the human gene is heterologous expressed in yeast and screens are designed against any relevant phenotypes that result from this expression [313]. In fact these strategies already prove to be useful. For example the yeast models of synucleinopathy, which are achieved by the heterologous expression of different forms of human α-syn in yeast [284, 291, 314-317], already allowed the study of the changes in α-syn distribution in living cells. Additionally, some central features of mammalian cellular pathology can be recapitulated in the yeast models, such as defective vesicle trafficking, proteasomal degradation, ROS accumulation, mitochondrial pathology and lipid-droplet accumulation [284, 291, 316, 318], permitting a more detailed study of the complex process in a simple organism.

The yeast *S. cerevisiae* is also an established model for aging study. In yeast is possible to evaluate aging by two different methods, by the replicative life span (RLS) or by the chronological life span (CLS). RLS is related with the number of daughter cells produced by a mother cell prior to senescence, and is thought to provide a model of aging in mitotically active cells, whereas the CLS corresponds to the length of time that a mother cell can survive in a non-dividing state, and is proposed to serve as a model for aging of post-mitotic cells in multicellular eukaryotes [257]. In a context of age-related diseases, this model as the advantage of being an easily manipulable model with a short life cycle. In fact, the only studied in which was explored the effects of the expression of α-syn in aged cells was performed in yeast [319].

The humanized yeast *S. cerevisiae* models provides a platform to explore fundamental processes that induce neurodegeneration, being an important tool to disclose protein function or

cellular pathways that are involved in misfolding, aggregation and subsequent toxicity of related-protein diseases, in both proliferative and aged cells.

This chapter attempts to summarize the relevant findings on the regulation of protein quality control system and its connection in a proteotoxicity context associated with α-syn toxicity. However, how different pathways of protein quality control influence each other, how they are regulated in a coordinated manner, and their contribution to aging under proteotoxicity remains to be answered. On the next chapters, results will be presented in an attempt to get new insights on the mechanisms associated with the toxicity induced by α-syn, during aging through the study of the effects of autophagy/mitophagy modulation during yeast CLS, hoping to contribute for the continuous knowledge on the regulatory mechanisms associated with synucleinopathies.

# CHAPTER 2

## MATHERIAL AND METHODS

## 2.1 Strains and plasmids

The yeast *Saccharomyces cerevisiae* strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

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

| Yeast Strain | Genotype |
|---|---|
| W303-1A | *MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* |
| BY4741 | *MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 *rho* 0 | *MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| *atg11Δpho8Δ* | *MAT a his3Δ1 leu2Δ0 met15Δ0 ura3v0 atg11::kanMX4 pho8::HPH* |
| *atg32Δpho8Δ* | *MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atg32::kanMX4 pho8::HPH* |
| *sir2Δpho8Δ* | *MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sir2::kanMX4 pho8::HPH* |

### 2.1.1 Generation of *rho* 0 strains

The BY4741 *rho* 0 strain used for lifespan analysis was generated by treatment with ethidium bromide. In a standard experiment cells are washed with sterile 10 mM $KPO_4$, pH 7 and inoculated into 10 ml of 10 mM $KPO_4$ phosphate, pH 7 buffer containing 10 µg/ml ethidium bromide. After incubation for 1 hour with shaking (150 rpm) at 26°C cells are spread on YEPD plates for single colonies growth. Small colonies should correspond to respiratory deficient mutants. Each experiment was determined for more than one *rho* 0 isolate in order to verify the observed phenotype. Absence of mitochondrial DNA was verified by fluorescence microscopy of log phase cells stained with DAPI and by the absence of growth on the non-fermentable carbon source, glycerol.

**Table 2.** Plasmids used in this study

| Plasmids | Type of Plasmid | Source |
|---|---|---|
| pRS304GAL-SynWT-GFP | Integrative | This study |
| pRS304 GAL-SynA53T-GFP | Integrative | This study |
| pRS304 GAL-SSynA30P-GFP | Integrative | This study |
| pRS306 GAL-SynWT-GFP | Integrative | This study |
| pRS306 GAL-SynA53T-GFP | Integrative | This study |
| pRS306 GAL-SynA30P-GFP | Integrative | This study |
| pCM252-SynWT-GFP | CEN | This study |
| pCM252-SynA53T-GFP | CEN | This study |
| pYX242-SynWT | 2µ | Winderickx, J [320] |
| pYX242-SynA53T | 2µ | Winderickx, J [320] |
| pYX242-SynA30P | 2µ | Winderickx, J [320] |
| pYX222-SynWT | 2µ | This study |
| pYX222-SynA53T | 2µ | This study |
| pYX222-SynA30P | 2µ | This study |
| pYX242-cytPho8 | 2µ | Reichert, AS [168] |
| pYX242-mtPho8 | 2µ | Reichert, AS [168] |
| pYX222-mtDsRed | 2µ | Gourlay, CW |
| pRS416-GFPAtg8 | 2µ | Reggiori, F |

### 2.1.2 Construction of the double mutants strains: *atg11Δpho8Δ*, *atg32Δpho8Δ* and *sir2Δpho8Δ*

The *ΔPH08* yeast cells from the Euroscarf, disrupted with a geneticin cassette were used as template. Therefore it was used the plasmid pAG34, which contains the hygromycin cassette and homologous regions relative to the locus of interest, to perform the homologous recombination. Linerized pAG34 with the XhoI restriction enzyme, were introduced in *ΔPH08* yeast cells (Euroscarf) by transformation with the LiAc method. Cells were then plated in selective YEPD medium containing hygromycin, to select the positive clones. The correct integration of the marker at the target locus in these transformants was then verified by PCR, using the internal

primers (3 and 4) paired with the external primers (5 and 6) (Table 3), which allowed the confirmation that the hygromycin cassette is located in the correct place.

Next, it was proceeded with the disruption of the *PHO8* gene in *atg11Δ*, *atg32Δ* or *sir2Δ* mutant cells For that, first it was amplified the hygromycin selection cassette using the internal primers (3 and 4) paired with the external primers (1 and 2) (Table 3). Primers 1 and 2 have about 50 oligonucleotides from the flanking region on either side of the *PHO8* gene, to allow the homologous recombination. The cassette was amplified in two separate PCR reactions to produce overlapping fragments, to minimized false positive appearance. The *PHO8* gene disruption was achieved by co-transformation, as described before. The resulting cells were then plated in selective YEPD medium containing hygromycin and geneticin. Once again, the resistant transformants were checked for correct integration of the marker at the *PHO8* locus, as above described.

**Table 3.** Primers used to construct the double mutants *atg11Δpho8Δ*, *atg32Δpho8Δ* and *sir2Δpho8Δ*

| Number | Primer name | Sequence |
|---|---|---|
| 1 | Pho8-AMP-FW | 5'-GCCAGCAAGTGGCTACATAAA-3' |
| 2 | Pho8-AMP-RV | 5'-CAGTACGTGTCATGCGGTTAG-3' |
| 3 | HYG-FW | 5'-CGCAAGGAATCGGTCAATAC-3' |
| 4 | HYG-RV | 5'-AAAGCATCAGCTCATCGAGA-3' |
| 5 | Pho8-CONF-FW | 5'-CGACATGAATAGCAGCATTGA-3' |
| 6 | Pho8-CONF-RV | 5'-TCACGCTATAGAATGCACCT-3' |

## 2.2 Media and culture conditions

Cells were maintained in YEPD agar medium consisting of 0.5% yeast extract, 1% peptone, 2% glucose and 2% agar. All experiments were performed in synthetic complete (SC) medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI) supplemented with the appropriate amino acids and bases for which the strains were 300 mg/L leucine, 50 mg/L histidine, 100 mg/L adenine, 100 mg/L tryptophan, 100 mg/L methionine or 100 mg/L uracil. Calorie restriction (CR) was accomplished by reducing the

glucose concentration from 2% to 0.5 in the initial culture medium. In a standard experiment, overnight cultures were grown in either media and inoculated into flasks with a ratio volume/medium of 3:1 at 26°C with shaking at 150 rpm. Growth was monitored by measuring the turbidity of the culture at 640 nm (OD640) on a spectrophotometer (Genesys 20/Thermo Spectronic) and viability was determined by counting colony-forming units (CFUs) after 2 d of incubation at 26 °C on YEPD agar plates.

### 2.3 Chronological life span

Yeast chronological life span (CLS) was measured as previously described [321]. In a standard experiment, overnight cultures were grown in SC medium containing different concentrations of glucose and then inoculated into flasks containing medium with the same concentration of glucose at a volume ratio of 1:3. These cultures were then incubated at 26 °C with shaking at 150 rpm.

Depending on the expression model used, different manipulations were performed. For the galactose-inducible (high toxicity) models, cells were grown until exponential, diauxic or stationary phases, harvested by centrifugation, washed twice with sterile water and then transferred to a SC medium containing 2% (w/v) galactose to induce α-syn expression. For the Tet-On models (low toxicity models), cells were grown at 26°C and 150 rpm, until exponential or stationary phases, and then doxycycline was added to the medium at a final concentration of 2 µg/ml in order to induce the expression of α-syn. Finally, the cells expressing α-syn under the control of the constitutive *TPI1* promoter (moderate toxicity model) were grown until stationary phase.

The survival of the cells from the inducible expression systems, Tet-On and *GAL1*, was assessed by counting colony-forming units (CFUs) after incubation of culture aliquots for 2 days at 30°C on YEPD agar plates. Regarding the moderate expressing system (constitutive *TPI1* promoter), cultures reached stationary phase two days later and this was considered day 0 of chronological life span (CLS). Survival was assessed by CFUs beginning at day 0 of CLS (when viability was considered to be 100%), and then again every 2-3 days until less than 0.01% of the cells in the culture were viable.

The CLS was also determined by flow cytometry, using the fluorescent probes propidium iodide (PI) and FUN1 beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100 %). Briefly, for PI staining $10^6$ cells were collected at indicated time points, washed twice, ressuspended in PBS and stained with 5 μg/ml PI for 30 min at 30°C in the dark. For FUN1 staining, aliquots of $10^6$ cells were collected at indicated time points, washed twice and ressuspended in sterile water supplemented with 2% glucose and stained with 0.5 μM FUN1 for 30 minutes at 37°C. The PI and the FUN1 signals were measured using FACSCaliber2 flow cytometer (BD-Biosciences) with a 488 nm excitation laser. Signals from 30,000 cells/sample were captured at a flow rate of 1,000 cells/s. Data collected with the FACSCaliber2 flow cytometer was processed with Flowjo software (Tree Star) and quantified with WinList software (Verity Software House).

### 2.3.1 Determination of maximum and minimum chronological life span

Mean, which represents 50% of cell survival, and maximum, which represents 10% of cell survival, chronological life spans were determined from curve fitting of the survival/CLS data, from pair matched, pooled experiments with the statistical software Prism (GraphPad Software).

## 2.4 Pharmacologic induction or inhibition of autophagy

Pharmacological induction of autophagy was accomplished by treating the cells at day 0 of CLS with 2 μg/ml of rapamycin. To perform the pharmacological autophagy inhibition assay, the cells were treated at day 0 of cells with 10 mM 3-methyladenine (3-MA), which inhibits the phosphatidylinositol 3-kinases (PI3K), blocking the autophagosome formation [322], or with 0.5 mM chloroquine (CQ), which raises the lysosomal/vacuole pH leading to the inhibition of lysosome/vacuole-autophagosome fusion and lysosomal/vacuole protein degradation [229, 323-326]. Next CLS was determined as described before, in section 2.2.

## 2.5 Index of respiratory competence (IRC)

The IRC reflects the respiratory status of mitochondria and thus mitochondrial functionality. It can be defined as the ability of cells to grow on non-fermentable substrates such as glycerol. The IRC was calculated by the ratio between the number of CFUs on non-fermentable carbon source (yeast extract/peptone and 2% glycerol, YPG) and the colonies on a fermentable carbon source (YEPD) agar plates.[327]

## 2.6 Superoxide dismutase (SOD) assays

For determination of superoxide dismutase activities, yeast extracts were prepared in 25 mM Tris buffer (pH 7.4) containing a cocktail of protease inhibitors. Protein content of cellular extracts was estimated by the method of Bradford (Bio-Rad) using bovine serum albumin (BSA) as a standard. Briefly, for determination of catalase activity 15 µg of proteins were separated by native PAGE. Then the gel was incubated in 2.5 mM nitro blue tetrazolium, during 20 min in dark, followed by an incubation with developed solution (36 mM phosphate potassium buffer, pH 7.8; 28 mM Temed and 86 µM riboflavin), during 15 min in dark. Next gel were transferred to deionized water and exposed to light until the appearance of bands. Superoxide dismutase activities were measured based on their ability to inhibit reduction of nitro blue tetrazolium to formazan in non-denaturing polyacrylamide gels [328]. Sod2 activity was distinguished from Sod1 activity based on the ability of 2 mM cyanide to inhibit Sod1, but not Sod2. Quantification of band intensities was performed by densitometry using Quantity One Basic Software from Bio-Rad.

## 2.7 RNA extraction

RNA was extracted with phenol, using the following procedure: 0.8 ml of cold TRIzol LS reagent (Invitrogen) were added to the yeast pellet and the resulting suspension was transferred to a microcentrifuge tube containing 0.5 g of sterile glass beads, 0.5 mm diameter, which had been soaked in concentrated nitric acid, washed thoroughly with water, and dried overnight in a

baking oven at 200°C. The suspension was shaken for 4 min in a vortex mixer; after 5 min at room temperature, 0.2 ml chloroform were added and the tubes shaken vigorously by hand. After 5 min incubation in ice, the suspension was centrifuged at 7000g for 15 min in the cold. The upper, aqueous phase was transferred to a new tube, 0.5 ml isopropanol were added, the mixture was incubated 10 min in ice and centrifuged at 11,000 g for 10 min in the cold. The supernatant was removed and the pellet washed with 1 ml cold 70% ethanol. After vacuum-drying for 5 min, the RNA pellet was dissolved in 50 µL of RNAse free water.

The purity of the sample was checked by the ratio A260/A280 [using the ND-100 UV-visible light spectrophotometer (NanoDrop Technologies)]. The RNA integrity was assessed by visual inspection, after electrophoresis on 1% agarose gel, in the presence of ethidium bromide.

### 2.8 Quantitative mRNA expression

The quantitative mRNA expression analysis was performed according to the MIQE guidelines (Minimum information for publication of quantitative real-time PCR Experiments [329]). First, the primers listed in Table 4 were constructed, by in silico analysis, in Beacon Designer 7.90 software (Premier Biosoft International). The primers were constructed in an attempt that all annealing temperatures were in a range between 53°C and 58°C, and preferentially located near the 3' terminal portion. This software includes a BLAST analysis against *S. cerevisae* genome sequence for specificity assurance, and uses Mfold server, to avoid positioning on risky secondary structures. To determine the optimal annealing temperature of the primers it was performed a quantitative real-time PCR (qPCR) at a range of around the calculated Tm of the primers, using a thermal gradient block. To check the specificity of the reaction, all melting curves were analyzed and checked the presence of one single shark peak. Also, the presence of a single fragment, with the expected sized of the reaction product (one sample per gene) was confirmed by 1% agarose gel electrophoresis. It was established the efficiency of the primers by a calibration curve using a sample of cDNA as template. The PCR efficiency of each primer pair (Eff in Table 4) was evaluated by the dilution series method using a mix of sample cDNAs as the template and was determined from calibration curves using the formula 10(-1/slope). After, a regular qPCR protocol was taken to each dilution, in duplicate to all primers

used in this study, the corresponding quantification cycle ($C_q$) values were determined. Then, the measured $C_q$ values were plotted against the log of the copy number of the template, creating the Cc which allowed the calculation of the efficiency from the slope and $R^2$ value.

**Table 4.** List of primers for quantification of mRNA expression and their efficiency (Eff)

| Gene | Primer sequence | Eff (%) |
|---|---|---|
| *ACT1* | F: GATCATTGCTCCTCCAGAA | 98.2 |
| | R: ACTTGTGGTGAACGATAGAT | |
| *PDA1* | F: TGACGAACAAGTTGAATTAGC | 94.8 |
| | R: TCTTAGGGTTGGAGTTTCTG | |
| *TDH2* | F: CCGCTGAAGGTAAGTTGA | 95.1 |
| | R: CGAAGATGGAAGAGTTAGAGT | |
| *ATG6* | F: TGTCTACTATGATGAGAATTTCA | 93.4 |
| | R: TGGCTTGTTGTGAGTGTT | |
| *ATG8* | F: AATATCTAGTTCCTGCTGACC | 98.2 |
| | R: CCGTCCTTATCCTTGTGTT | |
| *ATG32* | F: TTATTGTTTCCTCCAGTT | 91.8 |
| | R: GCTTCGTCATCATTATCTTCC | |
| *ATG33* | F: GTCTGTTTGTTTAGCCATCAC | 95.1 |
| | R: AAAGTGGTTAAAGTGGGAGTA | |
| *DNM1* | F: TCAACAGGAGAATGGACAA | 98.4 |
| | R: CATCATCATCGTAGTCACTG | |

To normalize the data of qPCR of our target genes we tested four putative reference genes: *ACT1* (actin), *PDA1* (alpha subunit of pyruvate dehydrogenase), *TDH2* (isoform 2 of glyceraldehyde-3-phosphate dehydrogenase) and *TUB2* (β-tubulin) in same experimental conditions. We used as reference, genes with $C_q$ differences ($\Delta C_q$) between all the time points less than 0.5. Results are listed in Table 5.

**Table 5.** Quantification cycles differences ($\Delta C_q$) of the tested reference genes.

| Gene | $\Delta C_q$ |
|---|---|
| *ACT1* | 0.31 |
| *PDA1* | 0.36 |
| *TDH2* | 0.33 |
| *TUB2* | 0.63 |

The qPCR was used to measure the mRNA transcripts of the *ATG8*, *ATG6*, *ATG32*, *ATG33* and *DNM1* genes. Three reference genes [ACT1 (actin), PDA1 (alpha subunit of pyruvate dehydrogenase) and TDH2 (isoform 2 of glyceraldehyde-3-phosphate dehydrogenase)] were selected due to their stable expression and were tested in the same experimental conditions allowing expression normalization.

Total RNA (300 ng) was reverse-transcribed into cDNA in a 20 µL reaction mixture using the iScript™ cDNA synthesis kit (Bio-Rad). Then, 22.5 ng of cDNA of each sample were tested in duplicate in a 96-well plate (Bio-Rad), in a 20 µL reaction mixture using the SsoFast Evagreen Supermix™ kit (Bio-Rad) and processed according to the manufacturer's instructions in a CFX96™ Real Time System (Bio-Rad). A blank (no template control) was also incorporated in each assay. The thermocycling program consisted of one hold at 95°C for 1 min, followed by 39 cycles of 15 min at 95°C, 20 s at 57°C and 20 s at 72°C. After completion of these cycles, melting-curve data were collected to verify PCR specificity, contamination and the absence of primer dimers. Relative expression levels were determined with efficiency correction, which considers differences in the efficiencies between target and reference genes, using the gene expression module of the CFX manager Software (Bio-Rad).

## 2.9 Determination of HAC1 mRNA splicing

To determine the *HAC1* mRNA splicing, real time reactions were carried out in the same manner as those used for quantification of mRNA expression (above described), using the primer pair: F: ATGACTGATTTTGAACTAACTAG and R: CAATTCAAATGAATTCAAACCTG (NCBI Gene ID:

850513). Afterward, the respective fragments were fractionated by electrophoresis on a 1% agarose gel.

### 2.10 Determination of UPR activity

Yeast strains transformed with the plasmids harboring the genes that codify to α-syn WT, A53T or A30P or the vector control were transformed with a second plasmid carrying UPRE-*LacZ* reporter gene. Then cells were cultured in the same conditions and collected at the specific time points. After cells were disrupted using glass beads in the Z-buffer [60 mM Na2HPO4.7H20, 40 mM NaH2PO4.H20, 10 mM KCl, 1 mM MgSO4.7H2O and 0.27% (v/v) β-mercaptoethanol] and assayed for β-galactosidase activity using the colorimetric substrate ONPG as previously described [330].

### 2.11 Autophagy and mitophagy activity

The monitoring of autophagy and mitophagy was performed according to the protocol described by Mendl and co-authors [168] and Noda and Klionsky [269] Briefly, strains deleted for *PH08* gene were transformed with cytPho8- or mtPho8-expressing plasmids. At specific time points, $5x10^8$ cells were collected, harvested, washed in 2 ml of ice-cold water containing 0.85% NaCl, 1 mM PMSF, and resuspended in 300 µL lysis buffer (20 mM PIPES, 0.5% Triton X-100, 50 mM KCl, 100 mM potassium acetate, 10 mM MgSO4, 10 µM ZnSO4, and 1 mM PMSF). An equal volume of acid-washed glass beads was added and the cells were lysed by vortexing for 7 min. To start the assay, 100 µl of extract was added to 400 µL reaction buffer [250 mM Tris-HCl, pH 8.5, 0.4% Triton X-100, 10 mM MgSO4, 4 mM nitrophenyl phosphate (Sigma)], and samples were incubated 15 min at 37°C before terminating the reaction by adding 500 µL of stop buffer (2 M glycine, pH 11). Generation of nitrophenol was monitored by measuring absorbance at 405 nm using a microplate reader Model680 (Bio-Rad), and each sample was corrected with the time 0 blank. Protein concentration in the extracts was measured with the Bradford (Bio-Rad) according to the manufacturer's instructions.

## 2.12 Confocal microscopy

For mitochondrial morphology and Atg8 analysis, strains expressing the α-syn or carrying the vector control were transformed with pYX222-mtDsRed and pRS416-GFPAtg8. At specific time points, cells were collected and imaged using a confocal Olympus FLUOVIEW microscope. To visualize the α-syn foci, the cells were collected and fixed in 4% paraformaldehyde during 15 min. After washing, first with 100 mM cacodylate (pH 7.4) and then with TDES (100 mM Tris, pH 7.5, 25 mM DTT, 5 mM EDTA and 1.2 M sorbitol), cells were incubated 10 min with TDES, at room temperature, to soften the cell wall, followed by a wash step with 100 mM phoscitrate buffer (100 mM $K_2HPO_4$, 100 mM citric acid): 1 M sorbitol. Cells were then incubated 30 min with 100 mM phoscitrate buffer: 1 M sorbitol containing 50 µl of β-glucuronidase and 25 µl of 10 mg/ml zymolyase was added to produce spheroplasts. This was followed by a wash step with 100 mM cacodylate: 5 mM $CaCl_2$: 1 M sorbitol. Next, cells were permeabilized with 0.1% Triton-X100 contained in 1X with phosphate-buffered saline (PBS), during 10 min. After 5 min rinsing with 0.1% BSA in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$), cells were incubated 2 h with the antibody anti-α-syn (Cell signaling) (1:400), followed by rinsing with 0.1% BSA in PBS for 15 min. Next cells were incubated with the Pacific Blue conjugated goat anti-rabbit secondary antibody (1:100) for 4 h. Finally, cells were rinsed again with 0.1% BSA in PBS for 15 min, resuspended in PBS and visualized, at room temperature, by confocal microscopy. Images were acquired in a confocal Olympus FLUOVIEW microscope with an Olympus PLAPON 60X/oil objective, with a numerical aperture of 1.35. GFP and DsRed were excited with and argon laser and a helium-neon laser (GFP: 488 nm excitation; DsRed: 559 nm excitation). Pacific blue was excited with a UV laser (Pacific blue: 405 nm excitation). Background reduction was performed with appropriate saturation levels using software FV1000 (Olympus) and Adobe Photoshop CS. Image stacks for analysis were acquired with sequential steps of 0.25 to 0.5 µm per plane in the z-direction and a total thickness of 4-6 µm. The acquired stacks were rendered with FV1000 software.

## 2.13 Assessment of ROS accumulation

Free intracellular reactive oxygen species (ROS), specifically superoxide anions were measured using dihydroethidium (DHE) or dihydrorhodamine 123 (DHR). Aliquots of cells were collected at indicated time points and DHE was added to a final concentration of 5 µM from a 5 mM stock in DMSO and cell were incubated for 10 min at 30°C. For DHR staining, aliquots were taken at selected time points and DHR was added to a final concentration of 15 µg/mL and cells were incubated for 90 min at 26 °C. After incubation, cells were washed once with PBS. The DHE signals were measured using FACSCaliber2 flow cytometer (BD-Biosciences) with a 488 nm excitation laser. The DHR signal was collected through a 488-nm blocking filter, a 550-nm long-pass dichroic with a 525 nm band pass. Signals from 30,000 cells/sample were captured at a flow rate of 1,000 cells/s. Data collected with the FACSCaliber2 flow cytometer were processed with Flowjo software (Tree Star) and quantified with WinList software (Verity Software House).

## 2.14 Preparation of protein extracts and western blot analysis

For detection of protein levels by western blot, the total cellular extracts were collected at specific time points and extracted as previously described.[331] Briefly, cells were pre-treated with 2 M lithium acetate for 5 min at room temperature. After lithium acetate removal, 0.4 M NaOH was added for 5 min on ice. Next, the cell were resuspended in SDS-PAGE sample buffer and boiled for 5 min. Of total protein, 20 µg were resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane during 7 min at 25V. The membranes were blocked with Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% skim milk, followed by incubation with anti-α-syn (1:1000) primary antibody in TBST containing 1% skim milk and anti-actin (1:5000) (kindly provide by Dr. Gourlay, C) primary antibody. After washing with TBS, the membranes were incubated with the respective secondary antibody, HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG at a dilution of 1:5000 and detected by enhanced chemiluminescence (Thermo Scientific).

## 2.15 Statistical analysis

The results shown are mean values and standard error of the mean of at least three independent assays. Statistical analyses were determined using two-way ANOVA. A p-value of less than 0.05 was considered as a significant difference.

# CHAPTER 3

# RESULTS AND DISCUSSION

## Section 3.1

# α-Synuclein toxicity is associated with autophagy and mitophagy induction in yeast aged cells

***Part of the results described in this chapter were published as follow:***

Sampaio-Marques, B., Felgueiras, C., Silva, A., Rodrigues, F. and Ludovico, P. Yeast chronological lifespan and proteotoxic stress: is autophagy good or bad? Biochemical Society transactions 2011, 39:1466-1470.

Sampaio-Marques, B., Felgueiras, C., Silva, A., Rodrigues, M., Tenreiro, S., Franssens, V., Reichert, A.S., Outeiro, T.F., Winderickx, J. and Ludovico, P. SNCA (α-synuclein)-induced toxicity in yeast cells is dependent of sirtuin2 (Sir2)-mediated mitophagy. Autophagy 2012, 8:10, 1-16.

***The results described in this chapter were presented in the following national or international congresses:***

National congresses:

- Microbitec'11. Braga, Portugal. 2011. Mitophagy is contributing to alpha-synuclein, wild type and A53T mutant form, toxicity in aged cells'' (Poster presentation).

International congress:

- Autophagy – 40th keystone symposia season meeting. Whistler, British Columbia, Canada. 2011. ''Heterologous expression of alpha-synuclein, wild type and A53T mutant form, induces autophagy in yeast''. (Poster presentation).
- 6th International Symposium on Autophagy. Okinawa, Japan. 2012. "α-synuclein-induced toxicity is dependent on Sir2-mediated mitophagy in yeast aged cells"

## 3.1 α-Synuclein toxicity is associated with autophagy and mitophagy induction in yeast aged cells

### 3.1.1 α-Synuclein-induced toxicity is phase growth dependent

Yeast cells have been extensively used as a model to study the cytotoxic effects of α-synuclein (α-syn) [290]. Previous data showed that α-syn mediates cell death in yeast thought an age- and mitochondria-dependent process [319], which is in good agreement with the late onset of the vast majority of Parkinson disease (PD) cases. However, it is still not clear how age traits potentiate the cytotoxic effects of α-syn. To address this question, we used a previous developed yeast model [291] displaying high toxicity and that consists on the heterologous expression of the human wild type α-syn (α-syn WT) gene and the clinical A53T and A30P mutant forms under the control of a *GAL1*-inducible promoter, with two copies of the construct integrated in the genome [291]. α-Syn expression was induced in different phases of the yeast cell growth, namely exponential, diauxic and stationary, and effects on cells growth and on the chronological life span (CLS) were monitored. As expected, growth analysis demonstrated that the expression of α-syn WT or A53T in yeast cells produced a strong growth defect phenotype (Fig. 19A-C). This growth defect was dependent on the growth phase, given that a more aggravated phenotype was observed when heterologous expression of α-syn was induced in stationary phase cells (Fig. 19A-C). CLS analysis revealed that the expression of α-syn WT or the familial mutant A53T form, resulted in the CLS reduction, leading to only 10% of survival, compared with about 100% survival of cells harboring the vector control after 30 hours of induction (Fig. 20A-C). The CLS observed effects were also dependent on the growth phase, since it was revealed a more aggravated phenotype when the α-syn expression was induced in stationary phase cells (Fig. 20A-C). These negative effects on CLS were not related to a lower expression of α-syn in post-mitotic cells, since an immunoblot analysis confirmed the α-syn levels even in stationary phase cells (Fig. 20A-C). In addition, it was also possible to observe the presence of foci, disperse in the cytosol, in the stationary phase cells expressing α-syn WT or the familial A53T mutant (Fig. 21). Furthermore, the presence of foci, correlated to α-syn toxicity, was observed independently of the growth cells phase in which the expression was induced.

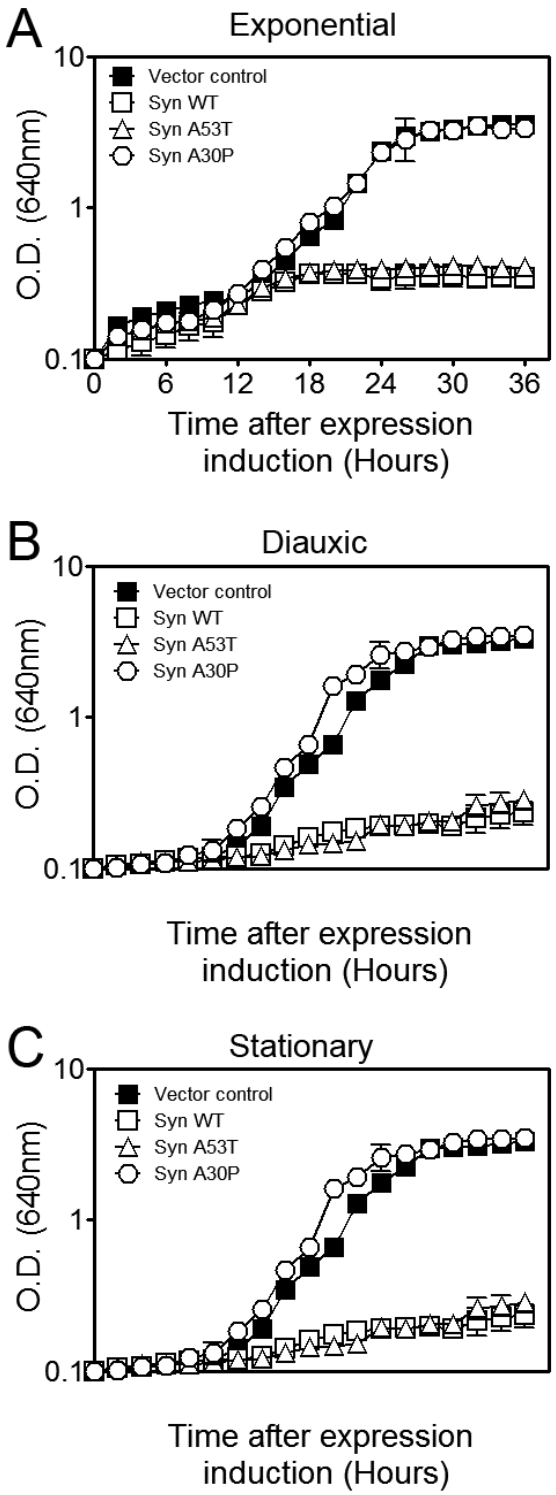


**Figure 19.** α-Syn WT and A53T induce growth inhibition dependent on the growth phase. Growth curves of wild type cells expressing α-syn WT, A53T or A30P under the control of the *GAL1* promoter or harboring the vector control. α-Syn expression was induced at (A) exponential, (B) diauxic or (C) stationary growth phases. The data represents mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM).

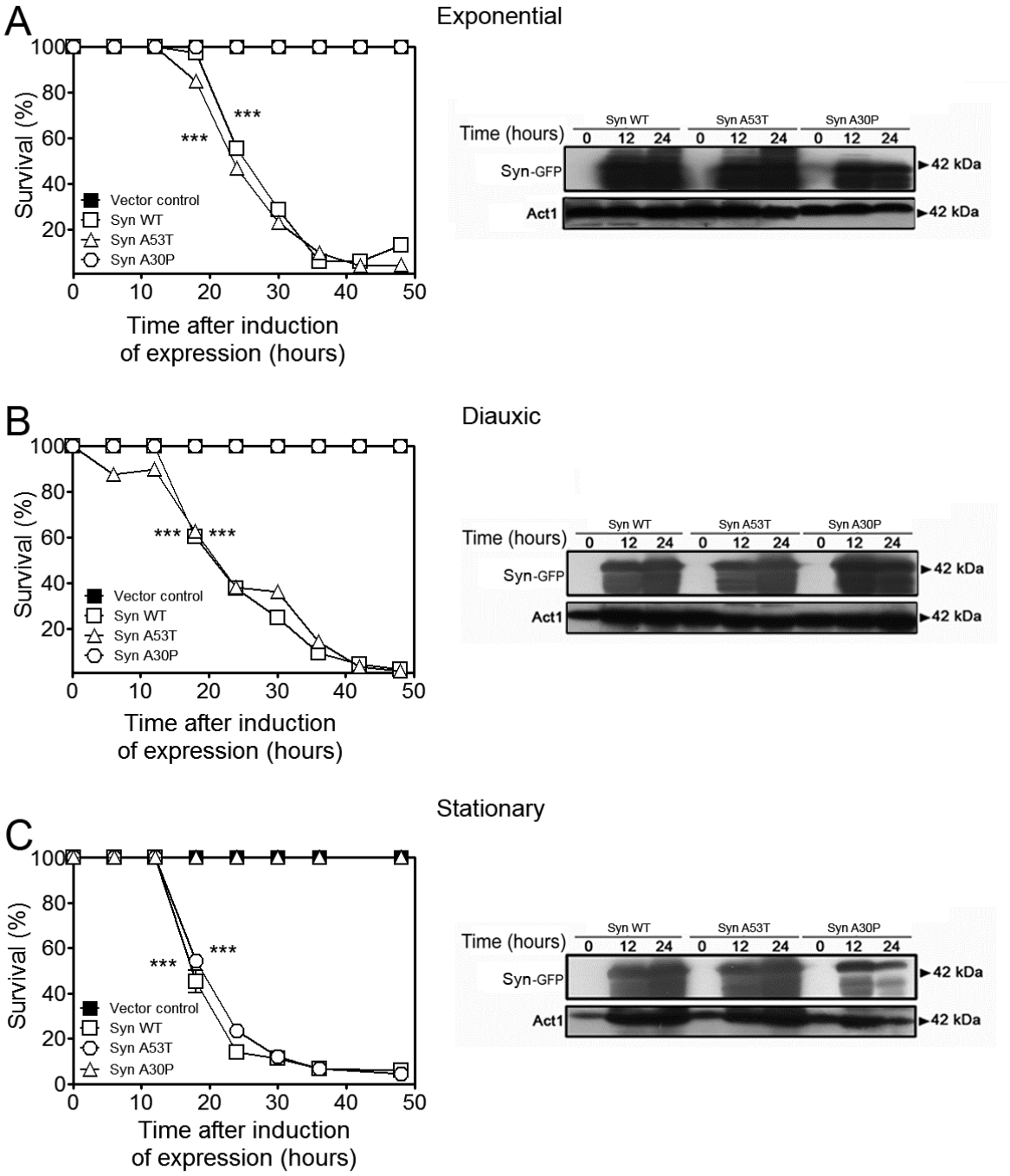


**Figure 20.** Chronological longevity of cells expressing α-syn WT or A53T is dependent on the growth cells phase at which the expression was induced. Chronological life span of wild type cells expressing α-syn WT, A53T or A30P under the control of the *GAL1* promoter or harboring the vector control. α-Syn expression was induced at (A) exponential, (B) diauxic or (C) stationary growth phases. Cell viability was measured at 6 h of intervals beginning at the time that was induced the α-syn and is expressed as % survival compared to survival at time 0 (100%). The data represents mean ± SEM of three biological independent replicas. Significance of the data was determined by two-way ANOVA (***$p<0.001$).

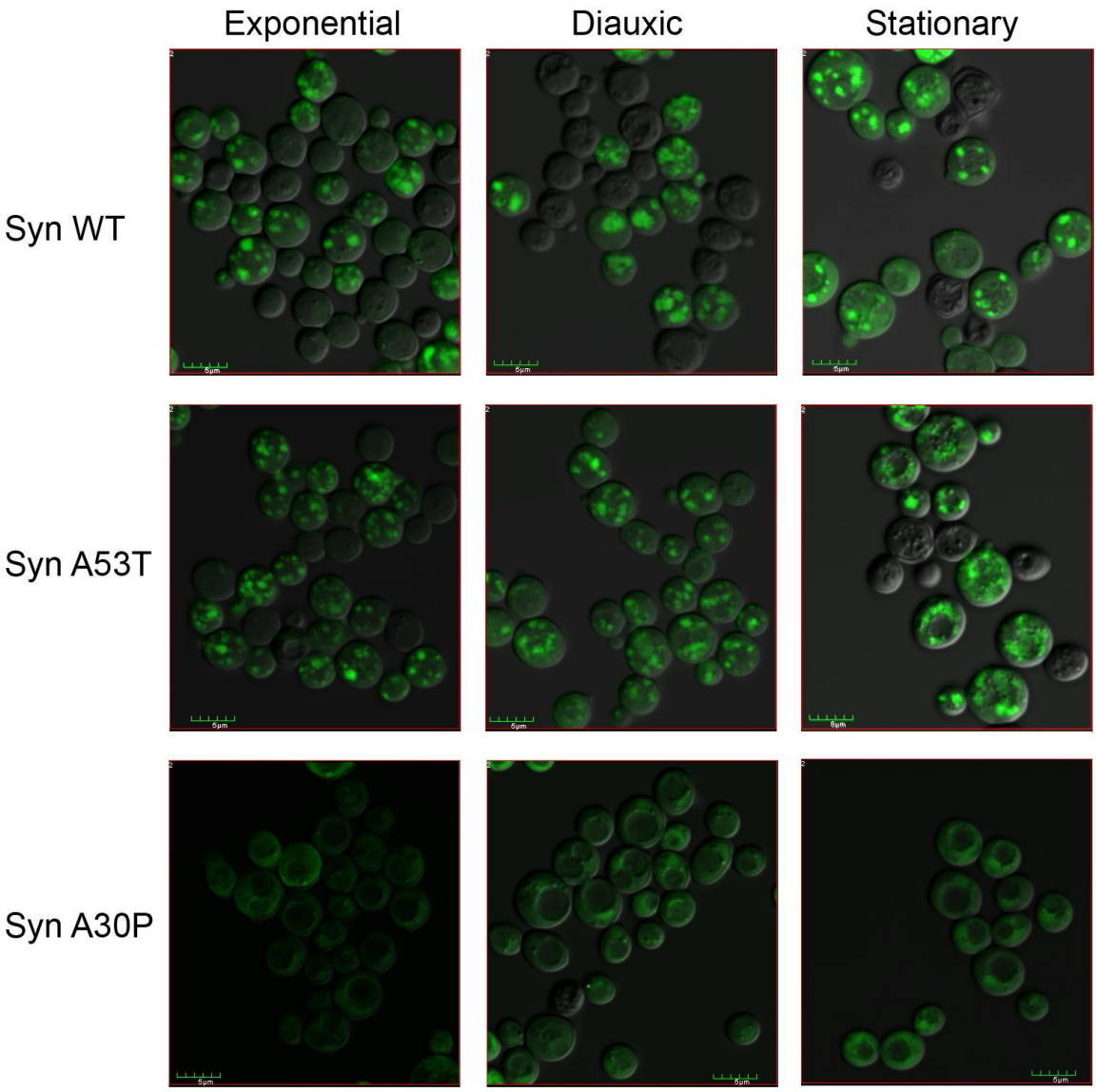


**Figure 21.** α-Syn WT and A53T expression induces foci formation independently on the growth cells phase. Image of wild type cells expressing α-syn WT-GFP, A53T-GFP or A30P-GFP under the control of *GAL1* promoter. α-Syn foci were analyzed at 24h after α-syn expression induction at exponential, diauxic or stationary growth phases. Scale bars: 5 µm.

The determination of the mean (50% survival) and maximum (10% survival) CLS of cells expressing α-syn WT and the mutant forms, induced in the different growth cells phases (Fig. 22) also corroborate the data, showing that the expression of α-syn WT or A53T in stationary phase cells promoted a statistical significant decrease of the mean and maximum life span when compared to α-syn expression in exponential or diauxic phase cells (Fig. 22A and B).

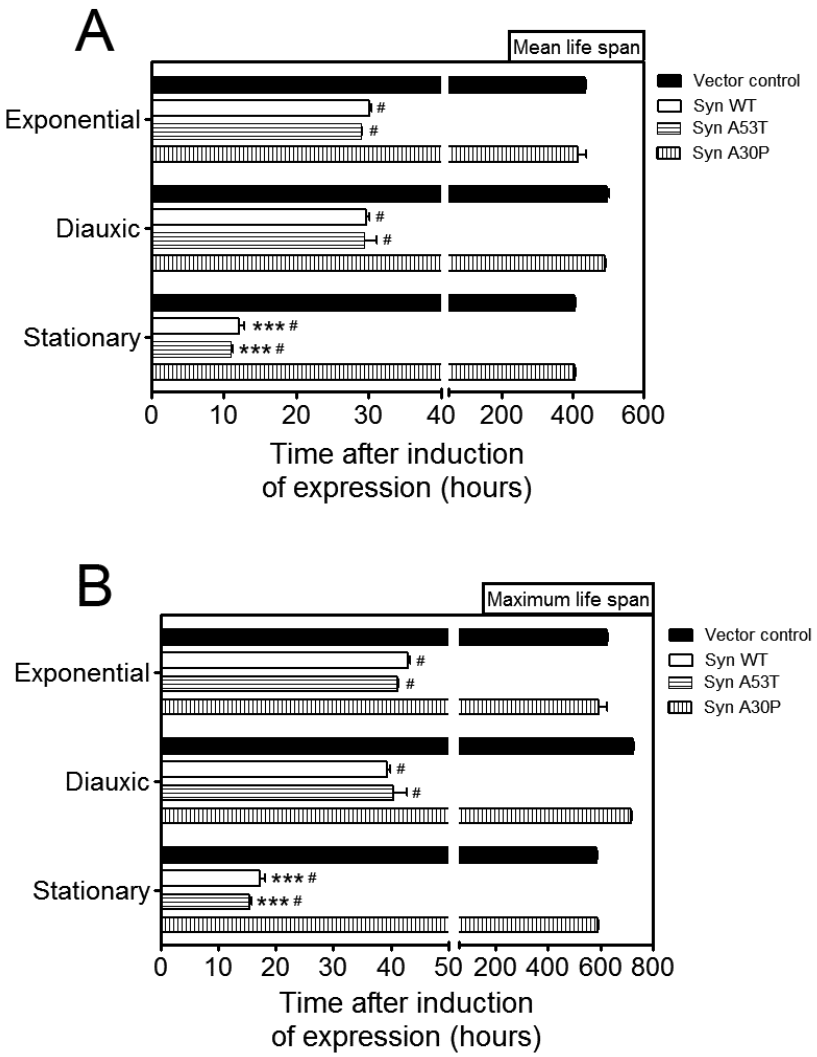


**Figure 22.** α-Syn toxic variants expression reduces the mean and maximum chronological life span (CLS) in a growth cells phase dependent fashion. (A) Mean (50% survival) and (B) maximum (10% survival) CLS were determined from curve fitting of the survival data (Fig. 18A-C) (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between exponential, diauxic or stationary cells expressing vector control or α-syn variants (*). The significance determined between cells expressing the vector control or α-syn variants within growth phase (exponential, diauxic or stationary) was also determined (#).The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (#$p<0.001$; ***$p<0.001$).

As previous described in the literature [291] the heterologous expression of the α-syn A30P mutant form resulted in a non-toxic phenotype, since the growth phenotype and the mean and maximum CLS were similar to the ones revealed by the cells harboring the vector control

(Fig. 19, 20 and 22). The reason for this phenotype is believed to be related to the fact that A30P mutation disrupts the first α-helical domain of α-syn blocking the unfolded to folded transition of α-syn that apparently is crucial for α-syn interaction with phospholipids [332]. Therefore the absence of A30P toxicity in yeast cells can be related to its inability to associate with the plasma membrane, and perhaps other membranes [291, 315]. In contrast, A30P toxicity in mammalian cells can result from a loss of function, leading to mistargeting, and possible sequestration of α-syn WT in inappropriate intracellular compartments [315].

Together, the results suggest that the expression of the toxic form of α-syn (WT and A53T mutant form) induced toxicity (growth inhibition and CLS reduction) dependent on the cell growth phase.

In this high toxicity yeast model, in few hours, the cells expressing α-syn WT or A53T mutant form lost viability (Fig. 20). Thus in an attempt to better sequentially monitor the events that occur in the cells promoted by the expression of toxic α-syn forms, we decided to develop a yeast model with lower toxicity. This low toxicity model consists in the expression of α-syn under the control of an inducible Tet-On system from a single integrated copy, since as described in literature, the toxicity of the yeast PD model is dependent on the number of gene copies inserted in the genome and also on the type of plasmid used [291] (see Material and Methods).

The expression of α-syn WT was induced at the exponential and stationary growth cell phases. The CLS analysis demonstrated that in the yeast model with low toxicity, the expression of α-syn WT or the toxic familial mutant A53T resulted in a reduction of the CLS, ranging from 25% in exponential phase cells to 41.66% in stationary phase cells (Fig. 23). The α-syn levels were also confirmed by an immunoblot assay during the CLS (Fig. 23).

The determination of the mean and maximum life span of these cells also demonstrated that the expression of the toxic α-syn variants (WT and mutant A53T form) resulted in a statistical significant reduction of the mean and maximum CLS in comparison with cells harboring the vector control (Fig. 24), being the reduction more pronounced in stationary phase cells, corroborating the results described with the high toxicity yeast model.

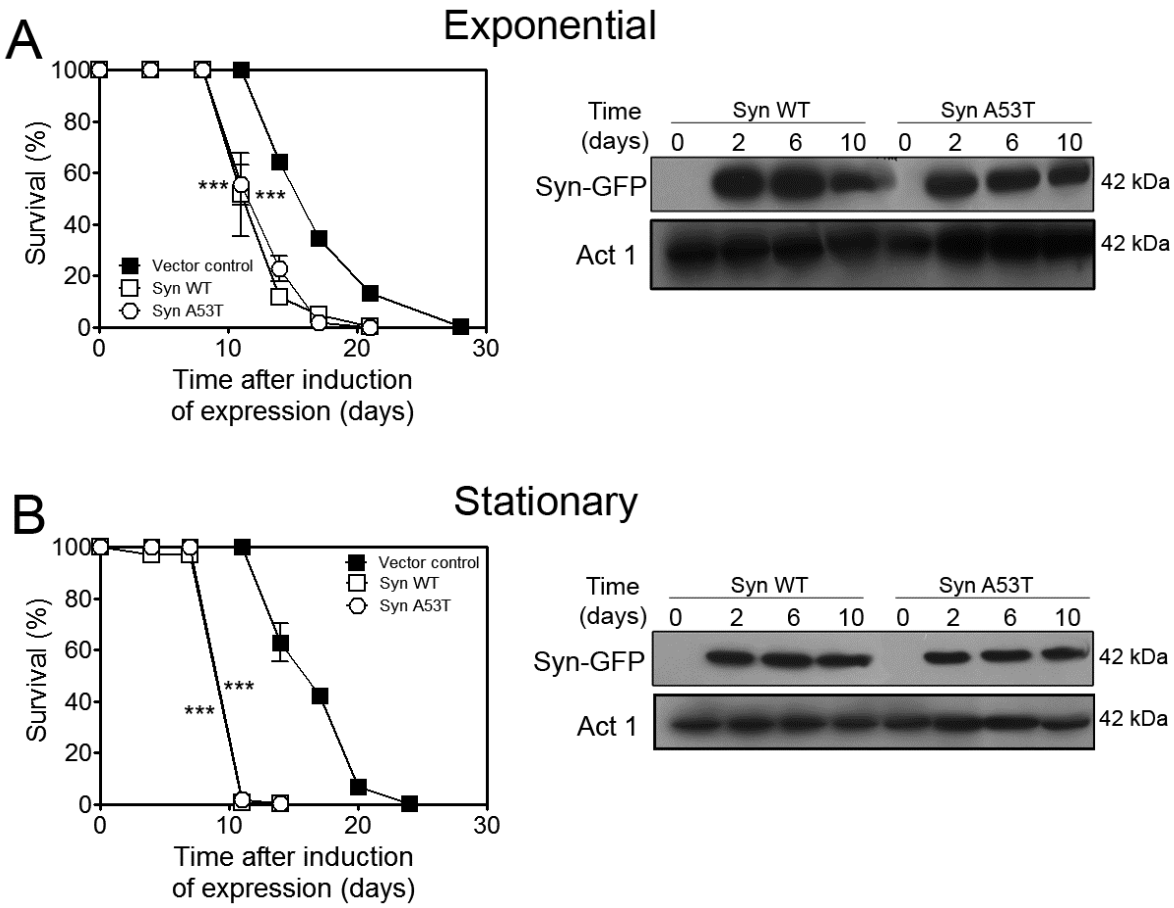


**Figure 23.** Low expression levels of α-syn WT or A53T also induce toxicity dependent on the cells growth phase. Chronological life span (CLS) of wild type cells expressing α-syn WT or A53T under the control of a Tet-On promoter or harboring the vector control. α-Syn expression was induced at (A) exponential or (B) stationary growth phases and viability was measure at 2-3 day intervals beginning at the time that α-syn expression was induced and is expressed as % survival compared to survival at time 0 (100%). α-Syn levels during CLS of cells were also monitored by immoblot analysis of α-syn. The data represents mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (***$p<0.001$).

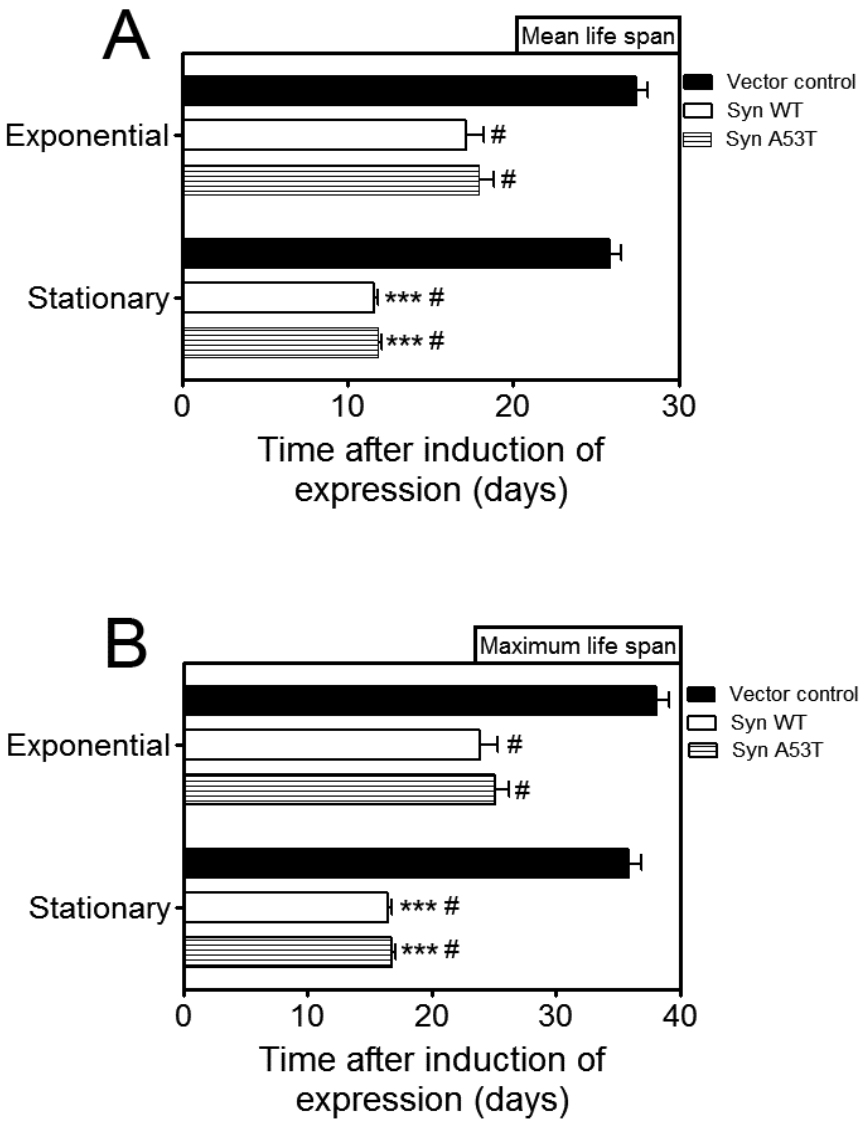


**Figure 24.** Low levels of α-syn also reduce the mean and maximum chronological life span (CLS) of cells dependent on the cells growth phase. (A) Mean (50% survival) and (B) maximum (10% survival) CLS were determined from curve fitting of the survival data (Fig. 23) (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between exponential or stationary cells expressing vector control or α-syn variants (*). The significance determined between cells expressing the vector control or α-syn variants within growth phase (exponential or stationary) was also determined (#).The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (#$p<0.001$; ***$p<0.001$).

In these assays, α-syn was expressed as C-terminally tagged GFP fusion to allow the microscopic evaluation of foci (Fig. 22). Although the new developed model has low toxicity, it

was also possible to observe the presence of foci (Fig. 25), in cells from both exponential and stationary growth phases.

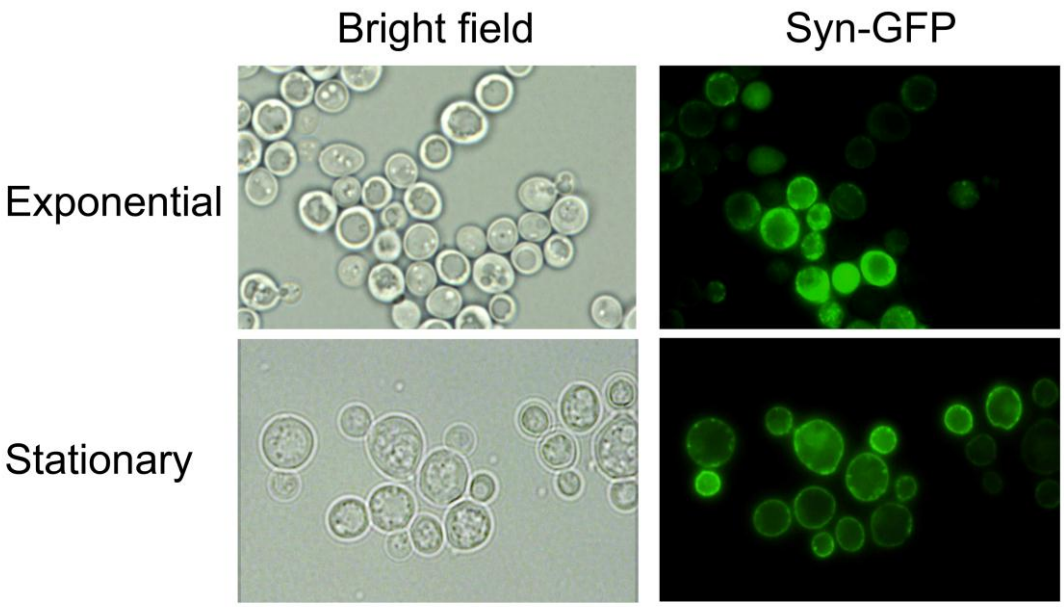


**Figure 25.** Expression of α-syn WT or A53T in yeast under the control of Tet-on promoter also induces the appearance of foci. Image of wild type cells expressing α-syn WT-GFP or A53T-GFP under the control of Tet-on promoter. α-Syn foci were analyzed at 24h after α-syn expression induction.

This new developed yeast model, with low toxicity, is extremely important because it allows the induction of α-syn expression, at any time of the cells growth phase, without the need of changing the culture medium. Additionally, with this model, it is also possible to dissect, with temporal sensibility, the α-syn cellular toxicity, due to the more extended CLS.

### 3.1.2 α-Synuclein-induced toxicity in yeast aged cells is associated with the unfolded protein response (UPR) activation

The accumulation of misfolded proteins in the endoplasmic reticulum (ER) is the main event that activates a complex intracellular signal transduction pathway called unfolded protein response (UPR) [333]. Previous findings from postmortem assessment of PD patients brains revealed that the UPR pathway is activated in nigral dopaminergic neurons in the presence of α-syn inclusions, suggesting that the activation of the UPR may be induced by the accumulation of α-syn [334]. Conversely, in a yeast model of PD, it was demonstrated that the expression of α-syn WT or the A53T mutant form causes ER stress accompanied with alteration on the ERAD pathway [335]. Besides ERAD, the cells exposed to ER stress activates the UPR [22]. In yeast, UPR activation occurs through the inositol-requiring kinase 1 (Ire1) dissociation from its ER-sensing domain allowing the cytosolic component of Ire1, with endonuclease activity, to splice an intron out of the messenger RNA coding for Hac1 (homologous to activating transcription factor (ATF)/CREB 1) (orthologue of mammalian XBP1). Translation of this mRNA produces active Hac1p, a transcriptional factor culminates in the expression of several genes [194].

To address the question of whether or not α-syn-induced toxicity activates the Ire1-Hac1 signaling pathway, we assessed the splicing activation of the *HAC1* mRNA after induction of the heterologous expression of α-syn variants under the control of a *GAL1*-inducible promoter, the high toxicity yeast model. Therefore, yeast cells were grown until the stationary phase and then the α-syn expression was induced by the presence of galactose in the culture medium. Next during specific times points of the CLS, samples were collected and the *HAC1* splicing analyzed by quantitative real-time PCR (qPCR). The data obtained revealed that yeast aged cells expressing α-syn WT or the mutant A53T variant suffered a *HAC1* splicing after 12h of expression induction, being also detected at 24h, as represented in figure 26A, by the band with a length of 687 bp, (splicing of the 252 bp intron in the *HAC1* primary transcript). In contrast, cells harboring the vector control or expressing the non-toxic α-syn variant A30P showed, at 12h, both the spliced and unspliced forms of *HAC1*, revealed by the visualization of two bands with 939 and 687 bp, probably due to an adaptation of the cells to carbon source change in the culture medium from glucose to galactose. Nevertheless, at 24h it was only observed the unspliced *HAC1* form (Fig. 26A). These results indicated that Ire1-Hac1 signaling pathway is stimulated by the presence of

the toxic α-syn forms, corroborating the evidence already observed by studies performed in animal models of PD [333, 336].

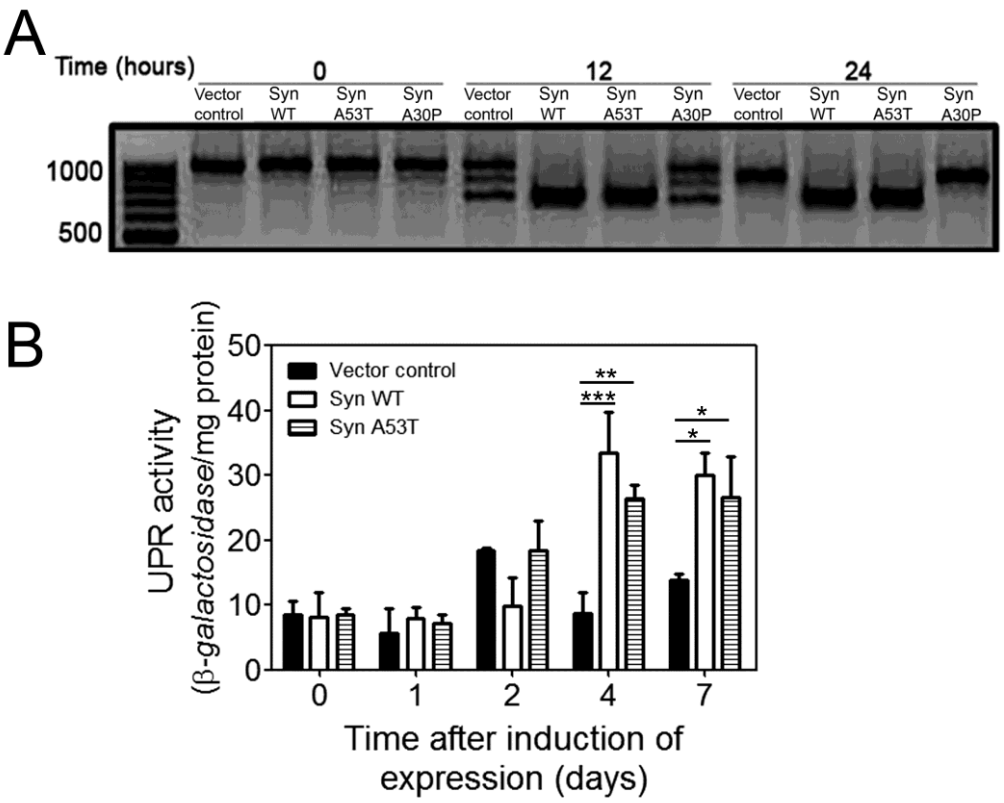


**Figure 26.** α-Syn WT and A53T expression induced a sustained unfolded protein response (UPR). (A) The splicing activation of the *HAC1* mRNA at 0, 12 and 24 h in cells expressing α-syn WT or the mutant forms under the control of the *GAL1* promoter. (B) UPR activity was measured, during chronological life span (CLS), through the β-galactosidase assay that was carried out in the stationary wild type cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the Tet-on promoter and co-harboring a plasmid containing *lacZ* under the control of the UPR element (UPRE). The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

To further confirm these data, we also used the low toxicity yeast model to evaluate the UPR activity by a different methodology. For that, yeast cells were transformed with a second plasmid containing *lacZ* under the control of the UPR element (*UPRE*), which is a consensus sequence usually present in the genes required for yeast UPR [34]. Expression of α-syn WT or

A53T mutant form resulted in the stimulation of the UPR activity during the CLS, as revealed by β-galactosidase activity, being more pronounced from day 4 of induction of α-syn expression, compared to cells harboring the vector control (Fig. 26B), consistently with the low toxicity of this yeast model.

Together, the results obtained with the two yeast models, which result in different α-syn expression levels, disclosed that the heterologous expression of α-syn WT or A53T mutant variant resulted in the stimulation of the UPR, detected by the activation of the Ire1-Hac1 signaling pathway and by the indirect measurement of the UPR activity. The results indicat that the accumulation of misfolded α-syn causes the persistent activation of the UPR system that is likely to be unable to deal with α-syn accumulation. Accordingly, in mammalian cells, α-syn also leads to the UPR pathway [333].

### 3.1.3 Autophagy modulation rescues yeast aged cells from α-synuclein-induced toxicity

As described in the above section, in yeast, the signaling pathway that mediates the UPR, which is activated in response to several physiological and pathological conditions, like the accumulation of misfolded proteins, is ultimately responsible for the activation of several genes associated with the degradation of the misfolded proteins. In fact, Hac1 is capable of inducing the expression of several *ATG* genes, like *ATG5, ATG7, ATG8*, *ATG14* and *ATG19* [194, 195]. Furthermore, the UPR is activated to reestablish the ER homeostasis by adaptive mechanisms that involve the autophagy stimulation [221]. Indeed, in yeast it is described that ER proliferation leads to the formation of autophagosome-like structures that are densely and selectively packed with membrane stacks derived from the UPR-expanded ER [155]. In this sense and given the persistent activation of the UPR in response to the α-syn toxic variants observed, we asked whether or not this UPR stimulation may be linked to an autophagy induction. For that an evaluation of key autophagic genes expression and of autophagy activity during CLS was performed.

### 3.1.3.1 Heterologous expression of α-syn WT or A53T mutant leads to increased levels of *ATG6* and *ATG8* mRNA levels

As above mentioned, the activation of the Ire1-Hac1 signaling pathway is intrinsically associated with the up-regulate expression of some genes associated with autophagy, including *ATG8* [194, 195]. Thus, the evaluation of the mRNA expression levels of some autophagy regulators was accomplished. Although, it has been suggested that autophagy is mainly regulated at the post-translational level, the identification of transcription factors involved in the regulation of autophagy genes has exposed the possible transcriptome regulation of the autophagic genes [337]. Therefore, we started with the evaluation of the levels of two relevant autophagic mediators, *ATG6* and *ATG8*. Autophagy-related protein 6 (Atg6)/vacuolar protein sorting 30 (Vps30) is a component that is common in two distinct phosphatidylinositol 3-kinase complexes. In the complex I, particularly involved in the nucleation step [54], Atg14 links Atg6 to Vps34 lipid kinase [338], while in the complex II, Vps38 links Vps30 to Vps34 and plays a role in the vacuolar protein sorting [338]. On the other hand, the *ATG8* gene encodes a protein that is predominantly associated with the autophagosome, remained attached to the autophagosomes membrane structures [118]. Apparently, *ATG8* is the main autophagic regulator showing a strong transcriptional regulation [110, 189].

To evaluate the mRNA levels, the high toxicity yeast model was used. Cells were grown until the stationary phase where the α-syn expression was induced. Samples were collected at specific time points, at 12h that represents the time at which the cells expressing the α-syn toxic variants (WT or A53T) start to lose viability and at 24h, representing the time where only about 10% of cells still survive. The *ATG6* and *ATG8* levels were evaluated by qPCR (quantitative Real Time PCR), following the standard recommendations for the qPCR method [329] and as described in Chapter 2 (Material and Methods).

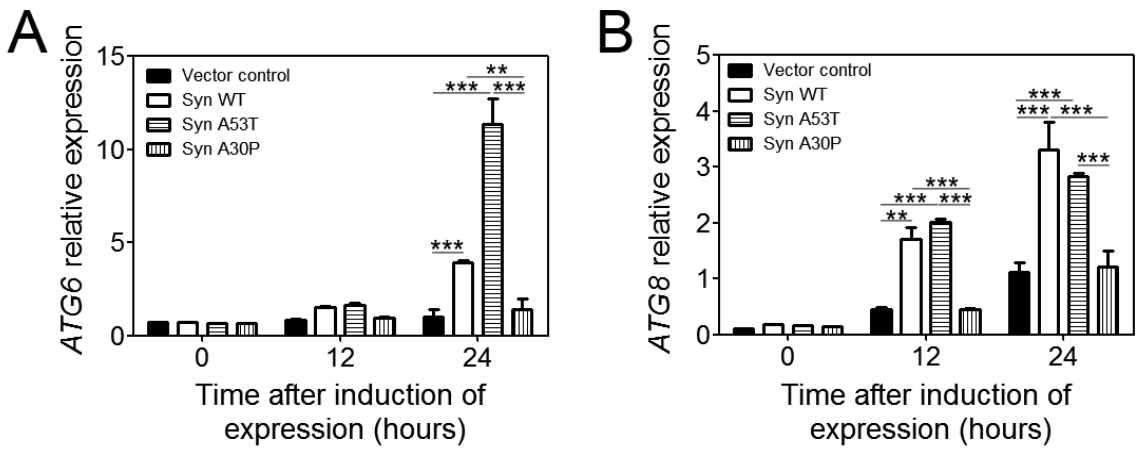


**Figure 27.** α-Syn WT and A53T lead to increased levels of *ATG6* and *ATG8* mRNA levels. (A) *ATG6* and (B) *ATG8* mRNA relative expression levels at 0, 12 and 24 h in cells expressing the α-syn WT, A53T or A30P under the control of the *GAL1* promoter or harboring the vector control. Three reference genes [*ACT1* (actin), *PDA1* (alpha subunit of pyruvate dehydrogenase) and *TDH2* (isoform 2 of glyceraldehyde-3-phosphate dehydrogenase)] were used as internal standards and for the normalization of mRNA expression levels. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (**p<0.01; ***p<0.001).

The analysis of the relative mRNA expression levels of *ATG6* revealed that only after 24h the mRNA levels were significantly increased in cells expressing α-syn WT or A53T compared with the cells expressing the non-toxic A30P variant or harboring the vector control (Fig. 27A). in contrast, the data showed that the relative *ATG8* mRNA expression levels were already 2-fold increased after 12 h of α-syn WT or A53T expression when compared to cells expressing the vector control or the non-toxic A30P (Fig. 27B). At 24 h of α-syn WT or A53T expression the *ATG8* mRNA levels were even higher (Fig. 27B). Together these results indicated that the CLS reduction (Fig. 20C) promoted by the expression of α-syn WT or the clinical A53T mutant is associated with an increase of the *ATG6* and *ATG8* mRNA levels (Fig. 27). The increase in the mRNA expression levels of the two evaluated genes showed that both are transcriptionally regulated after α-syn expression, suggesting that the sustained UPR activation might be related to autophagy induction.

### 3.1.3.2 Heterologous expression of α-syn WT or A53T mutant leads to increased levels of mitophagy specific genes

Oxidative stress and mitochondrial dysfunction are hallmarks of α-syn induced toxicity in yeast aged cells [319]. Additionally, there is also evidence that the specific mitochondria degradation by autophagy, called mitophagy, is the primary pathway to eliminate dysfunctional, aged or excessive mitochondria. Furthermore, recent evidence highlighted that two proteins encoded by genes linked to familiar PD forms, PARK2/PARKIN and PINK1, are important for the degradation of dysfunctional mitochondria (mitophagy) [306, 307]. Taking into account the mitochondrial importance on PD pathogenesis, we decided to assess the expression of three mitophagy related genes: *ATG32* and *ATG33*, mitophagy-specific genes [339], and *DNM1*, a mitochondrial dynamic-related GTPase required for mitochondrial fission, that is through to be necessary for mitophagy [340].

By using the same yeast high toxicity model and the methodology above described, it was observed that the *ATG32* normalized mRNA expression levels have a significant increase of about 2-fold, at 12 and 24 hours of induction of α-syn WT or A53T expression (Fig. 28B). Similar results were observed relatively to the *DNM1* mRNA expression levels, however just at 24 hours of α-syn expression (Fig. 28A). *ATG33* mRNA levels were increased in WT and A53T α-syn expressing cells, only at 12 hours, whereas at 24 hours the cells harboring the vector control and cells expressing α-syn WT or A53T mutant displayed similar *ATG33* mRNA levels (Fig. 28C). These data revealed that there is a genetic upregulation of the studied genes linked to mitophagy in these conditions.

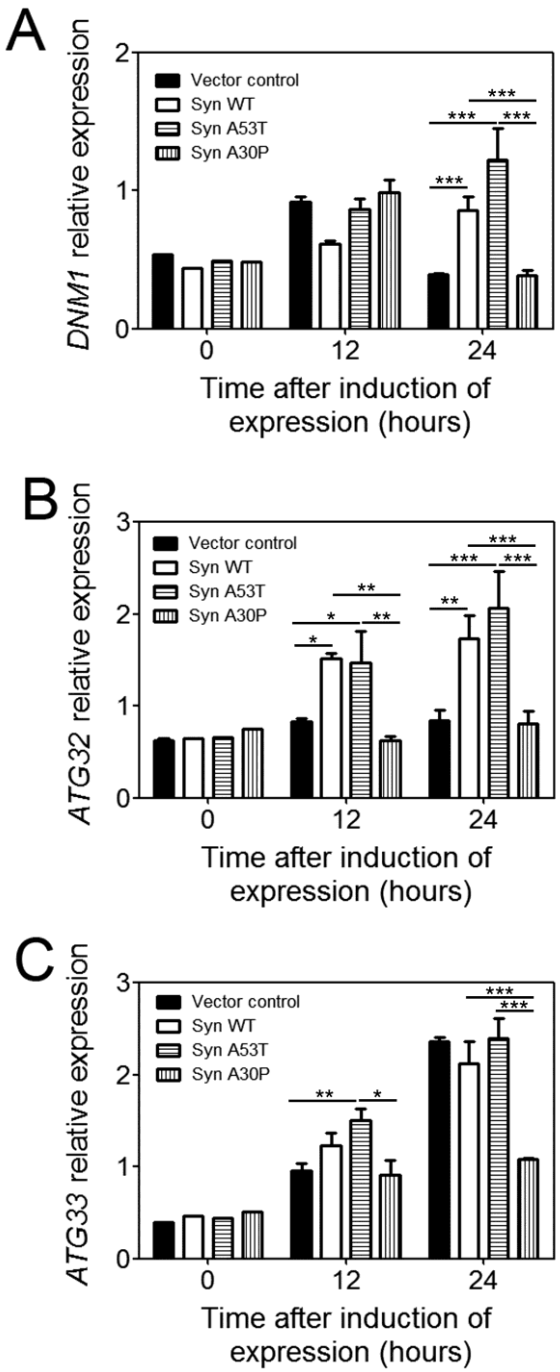


**Figure 28.** α-Syn WT and A53T lead to increased mRNA levels of proteins linked to mitophagy. (A) *DNM1*, (B) *ATG32* and (C) *ATG33* mRNA relative expression levels at 0, 12 and 24 h in cells expressing the α-syn WT, A53T or A30P under the control of the *GAL1* promoter or harboring the vector control. Three reference genes [*ACT1* (actin), *PDA1* (alpha subunit of pyruvate dehydrogenase) and *TDH2* (isoform 2 of glyceraldehyde-3-phosphate dehydrogenase)] were used as internal standards and for the normalization of mRNA expression levels. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

Mitophagy has been associated with mitochondria fission given the large size of mitochondria in comparison to autophagosome [306]. However, other authors claim that mitophagy occurs independently of the mitochondria fission [168]. Our data indicates that during CLS, cells harboring the genes that codify for the toxic α-syn variants display higher mRNA *DNM1* levels associated with elevated mRNA levels of *ATG32* and *ATG33*, supporting a link between the two processes: mitochondria fission with mitophagy. Altogether, the results suggest that the expression of α-syn toxic variants lead to mitochondria selective degradation, which is associated with the stimulation of both, UPR and autophagy.

#### 3.1.3.3 Modulation of autophagy in yeast aged cells expressing the toxic α-syn variants

The increase of mRNA levels of autophagic regulators, per se, does not prove that autophagic activity is increased, therefore to further set out whether autophagy impacts on the toxicity of α-syn in yeast aged cells, we have decided to pharmacologic inhibit the autophagy process. For such purpose, the high toxicity yeast models were used, and α-syn expression was induced in stationary cells phase, representing the day 0 of CLS, and at the same time autophagy was pharmacologically inhibited by adding 3-methyladenine (3-MA), which inhibits the phosphatidylinositol 3-kinases (PI3K), blocking the autophagosome formation [322]. The results obtained showed that autophagic inhibition in yeast aged cells completely revert the toxic α-syn phenotype as reflected by the CLS extension observed (Fig. 29A), suggesting that autophagy might play a central role in the α-syn-induced toxicity. To further confirm this data, we have also modulated autophagy in the low toxicity yeast model. The induction of autophagy was achieved through a TOR-dependent pathway, by using rapamycin [69, 73, 341-345]. For the inhibition of the autophagy, 3-MA was used, as described before, but we also used chloroquine (CQ) which raises the lysosomal/vacuole pH leading to the inhibition of lysosome/vacuole-autophagosome fusion and to lysosomal/vacuole protein degradation [229, 323-326]. To perform these assays, α-syn WT expression was induced in stationary phase cells, representing the day 0 of CLS, and at this point, autophagy was pharmacologically induced with rapamycin or inhibited by adding 3-MA or CQ.

The induction of autophagy by the presence of rapamycin in the medium resulted in a slight increase of the CLS of cells expressing the α-syn WT (Fig. 29B) as confirmed by the determination of the mean and maximum chronological life spans of cells in the presence or absence of rapamycin (Fig. 29E and F). As referred, rapamycin inhibits Tor1, and as known Tor1 inactivation results in a pleiotropic cellular response [259] that leads to a CLS extension. These reasons could explain the slight increase of CLS in the presence of rapamycin independent on autophagy.

The inhibition of the autophagy with 3-MA or CQ resulted in a CLS extension of cells (Figs. 29C and D), consistently with the results obtained with the high toxicity models in the presence of 3-MA (Fig. 29A). The determination of the mean and maximum CLS of wild type cells expressing α-syn WT with autophagy inhibition corroborated these results. In fact, the expression of α-syn WT in wild type cells in the presence of 3-MA or CQ promoted a statistical significance increase of the mean and maximum life span when compared to wild type cells expressing α-syn WT without any pharmacological treatment (Fig. 29E and F).

The observation that autophagy inhibition results in a better cell fitness accompanied by CLS extension suggests a role for autophagy in the α-syn-induced toxicity.

#### 3.1.3.4 α-Synuclein-induced toxicity in yeast aged cells is associated with autophagy induction and selective mitochondria degradation

To further elucidate the role of autophagy in α-syn-induced toxicity, a new yeast model with moderate toxicity, in which α-syn WT, A53T or the A30P mutant are constitutively expressed under the control of an endogenous triose phosphate isomerase (*TPI1*) promoter from a single integrated copy, was developed. This model was used to perform additional functional analysis, particularly the determination of the autophagy activity during CLS. For that purpose, *PHO8* mutant cells were transformed with a plasmid expressing an inactive Pho8 proenzyme targeted to the cytosol. The activation of autophagy resulted into the translocation of the inactive Pho8 proenzyme from the cytosol to the vacuole and its consequent activation, allowing the determination of the autophagy activity by the alkaline phosphatase (ALP) assay [168, 269].

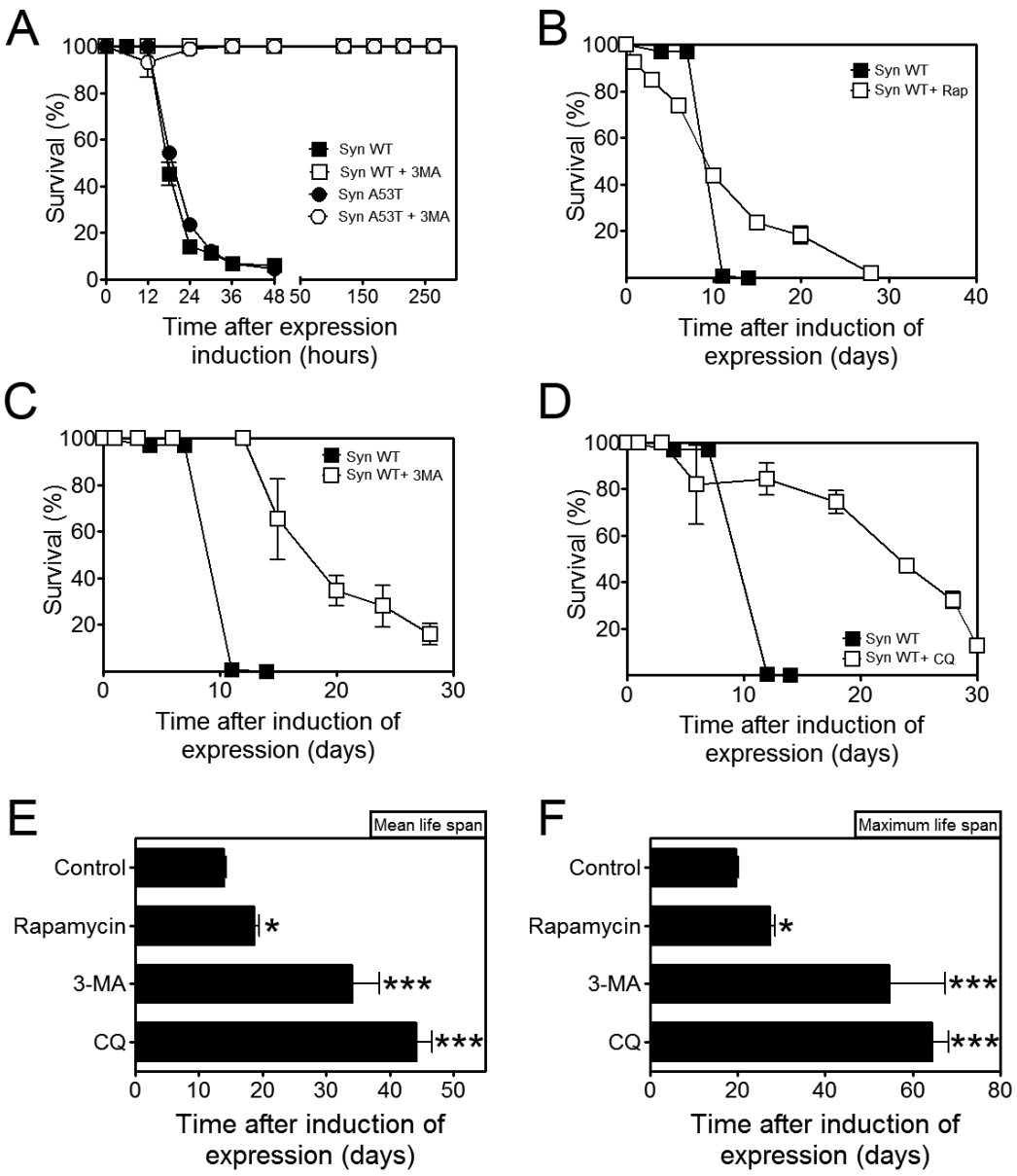


**Figure 29.** Autophagy inhibition rescues aged yeast cells from α-syn-induced toxicity. (A) Chronological life span (CLS) of stationary wild type cells expressing α-syn WT or A53T mutant under the control of the *GAL1* promoter or harboring the vector control in the presence or absence of 3-methyladenine (3-MA), an inhibitor of autophagy. CLS of stationary wild type cells expressing α-syn WT under the control of the Tet-on promoter or harboring the vector control in the presence or absence of (B) rapamycin (inducer of autophagy), (C) 3-MA or (D) chloroquine (CQ) (inhibitor of autophagy). (E and F) Mean (50% survival) and maximum (10% survival) chronological life spans were determined from curve fitting of the survival data (B, C and D) (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between wild type cells expressing α-syn WT with wild type cells expressing α-syn WT treated with 3-MA, CQ or rapamycin. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the CLS curves was determined by two-way ANOVA (*$p<0.05$; ***$p<0.001$).

As expected, with this moderate toxicity model, the expression of α-syn WT or the familial mutant α-syn A53T led to a CLS reduction when compared with the CLS of cells harboring the vector control or the α-syn A30P non-toxic mutant [291] (Fig. 30A). An immunoblot assay confirmed the expression of α-syn variants during CLS (Fig. 30B). To confirm the data obtained by the evaluation of proliferative capacity, other viability assays, namely the plasma membrane integrity, assessed by the exclusion of propidium iodide (PI), and by the metabolic activity evaluated by FUN1 were also used (Fig. 30C and D). The results obtained with PI and FUN1 assays also revealed a drastic CLS decrease in cells expressing the toxic α-syn variants, WT or mutant A53T form (Fig. 30C and D).

The results obtained with these new moderate toxicity models are in concordance with the data obtained with the other two models, with low or high toxicity levels.

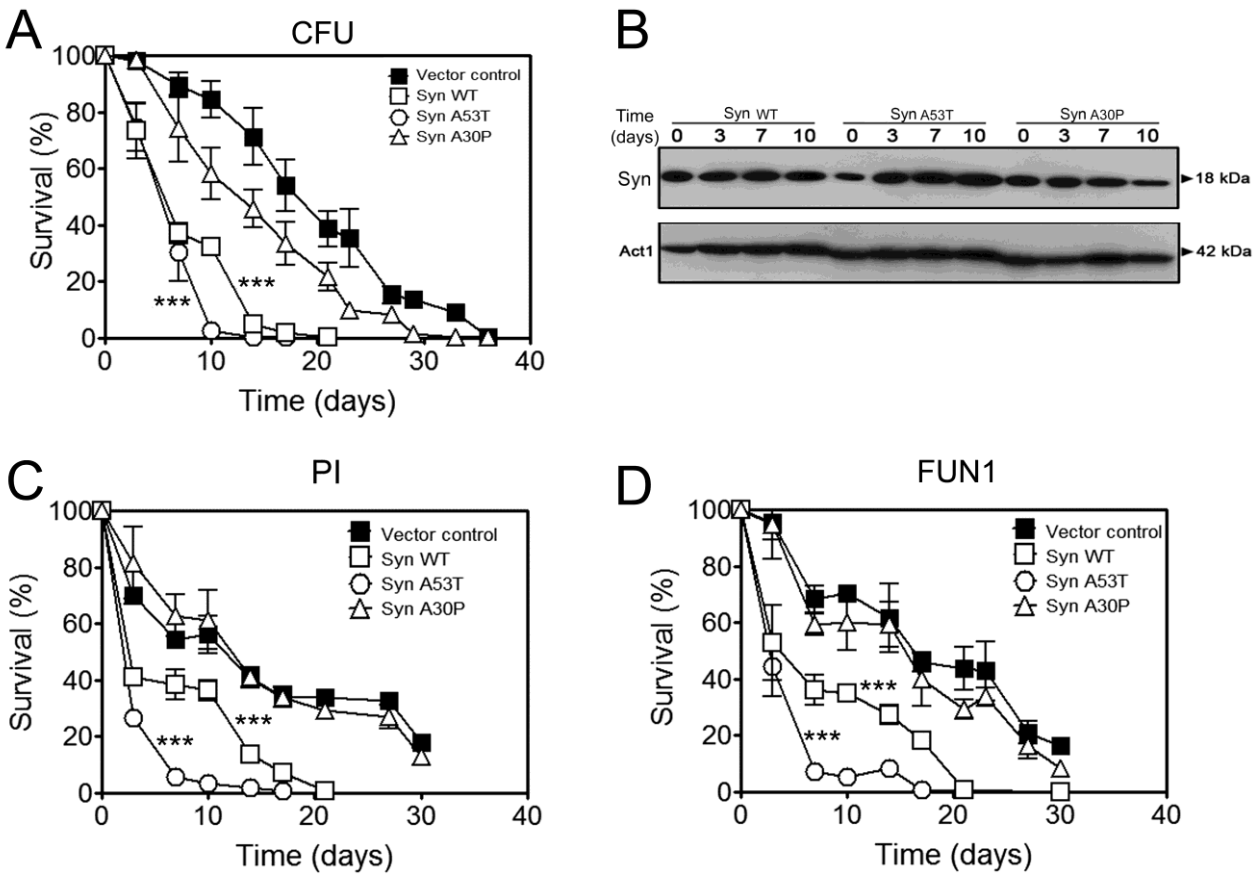


**Figure 30.** Moderate toxicity yeast model. Chronological life span measured by (A) CFU, (C) PI exclusion or (D) FUN1 metabolic activity and (B) α-syn levels of stationary wild type cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the constitutive *TPI1* promoter. The data represent mean ± SEM of six biological independent replicas. Cell viability was measured at 2-3 day intervals beginning at the day that

cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (***p<0.001).

After the establishment of these yeast models for α-syn toxicity, the autophagy activity during CLS was evaluated. The data from the ALP assay showed that the heterologous expression of α-syn WT or A53T induced a gradual increase of the autophagic activity over time, more pronounced from day 3, when compared to cells harboring the vector control or expressing the non-toxic A30P (Fig. 31A). These results corroborate the increased mRNA levels of genes associated with autophagy, *ATG8* and *ATG6* (Fig. 27), and associate α-syn toxicity to autophagy induction.

It is well established by results obtained with other animal models and also with post-mortem analysis of PD patient brains that mitochondria and oxidative stress have a central role in the PD pathogenesis. These facts associated with the data from the mRNA analysis, which revealed increased *ATG32* mRNA levels, raised the question about the role of mitophagy in the toxicity induced by the expression of α-syn toxic variants. To deal with this question, *PHO8* mutant cells were transformed with a plasmid expressing an inactive Pho8 proenzyme targeted to the mitochondrial matrix. The activation of mitophagy resulted into the translocation of the inactive Pho8 proenzyme within the mitochondria to the vacuole and its consequent activation, allowing the determination of the mitophagy activity by the ALP assay [168, 269]. The data resultant from the analysis of the mitophagy activity revealed a similar profile to the one obtained by the autophagic activity analysis, which demonstrated sustained mitophagy activation, more evident after day 3 of CLS in the cells expressing α-syn WT or the familial A53T mutant form compared with cells harboring the vector control or expressing the non-toxic A30P (Fig. 31B).

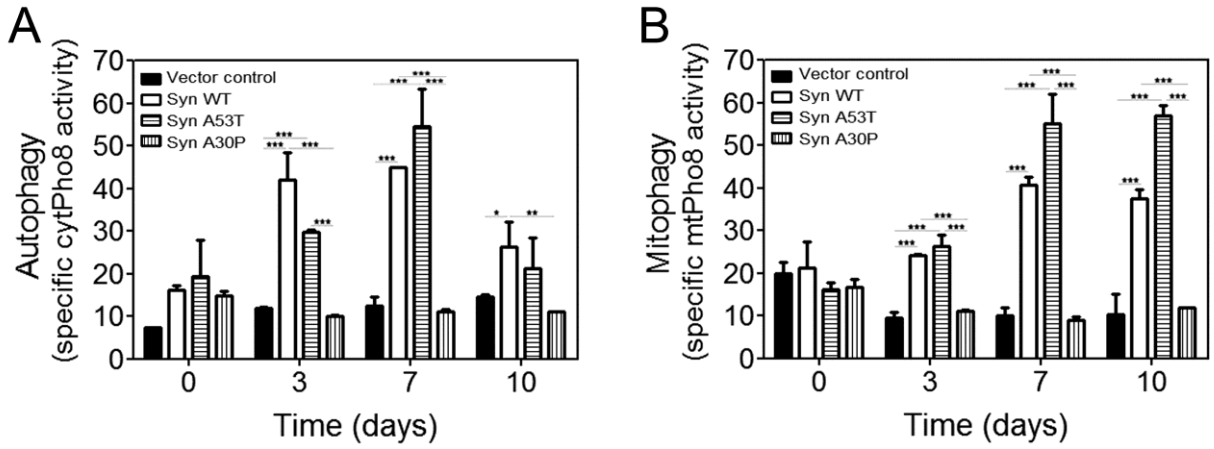


**Figure 31.** α-Syn-induced toxicity in aged yeast cells is associated with autophagy induction and selective degradation of mitochondria. Autophagy and mitophagy activity were measured through the alkaline phosphatase (ALP) assay that was carried out in the wild type cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant and co-harboring a plasmid expressing the inactive Pho8 proenzyme targeted to (A) the cytosol or (B) to the mitochondrial matrix, during chronological life span. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

The results described in this section and obtained with the different α-syn expression and toxicity systems suggest that the expression of α-syn WT or A53T variant results in a sustained UPR response due to the presence of misfolded α-syn that accumulate originating foci. This UPR response is correlated with an activation of autophagy with selective degradation of mitochondria, as demonstrated by the mitophagy ALP assay and the increased *ATG32* mRNA levels.

### 3.1.4 The contribution of mitophagy to the α-synuclein-induced toxicity

As mentioned before, mitochondria are organelles essentials for energy production, regulation of cell signaling and apoptosis [346-348]. Nevertheless, mitochondria are the major source of reactive oxygen species (ROS), which may oxidize mitochondrial lipids, proteins and DNA [349]. Due this dual function, the cell has mechanisms of mitochondrial quality control to maintain the overall fitness. In this sense, mitophagy has emerged as a key mechanism in this quality control system, responsible of the elimination of superfluous or damaged mitochondria

[350]. In fact, oxidative stress and mitochondria dysfunction have long been recognized as central players that operate in the PD pathogenesis [351]. Indeed dysfunctional mitochondria are observed in the *substantia nigra* of PD patients, and it was recently proposed that this could be a consequence of the loss of mitochondria due to the overactivation of autophagy [304]. However, little is known about the factors that predispose mitochondria for degradation by mitophagy. As referred in chapter 1, studies on genes associated with autosomal recessive forms of PD demonstrate that PINK1 and PARK2 collaborate in mitochondrial quality control systems [179]. Hence, defects in mitophagy have recently emerged as a central pathogenic hypothesis in PD [51, 352]. Nevertheless, the precise role of autophagy and mitophagy in the pathogenesis of PD still remains controversial [353].

To get new insights into the role of mitophagy in α-syn-induced toxicity we extended our analysis to the evaluation of the CLS in cells lacking genes associated with specific types of autophagy, namely, *ATG11* and *ATG32*. Atg11 is a scaffold protein that directs receptor (Atg19)-bound cargo to the phagophore assembly site and that participates in all specific types of autophagy [140]. Atg32 is an outer mitochondrial membrane protein essential for mitophagy, but not for autophagy or other types of selective autophagy. In yeast, the selective autophagy requires a receptor and an adaptor protein: Atg32 acts as a receptor protein that interacts with the adaptor protein Atg11, most likely to sequester mitochondria to the phagophore assembly site (PAS) [161, 166]. Additionally, an evolutionary conserved motif (WXXI/L) is present in Atg32, which is a pre-requisite for the interaction with Atg8. Furthermore, this interaction, Atg32 and Atg8, is essential for mitochondrial recruitment [161].

The CLS results analysis demonstrated that in *atg11*Δ cells the expression of α-syn WT or A53T no longer had an effect on longevity, since the CLS curves overlapped with the curve obtained for vector control cells (Fig. 32A and B). These results were also confirmed with different viability assays, namely the propidium iodide (PI) exclusion, and the metabolic activity evaluated by FUN1 (Fig. 32C and D).

Interestingly, expression of the non-toxic α-syn A30P induced a slight decrease of longevity, compared to *atg11Δ* cells expressing the vector control, particularly when viability was evaluated by propidium iodide exclusion or FUN1 processing (Fig. 32C and D). This most likely relates to the previously made observations that the A30P mutant is predominantly cytoplasmic [354] and subject to vacuolar degradation, in contrast to α-syn WT and A53T [355]. It is

conceivable that the increased toxicity of the A30P mutant in *ATG11* deleted cells is associated with a general defect in cargo recognition given the role of Atg11 in connecting the cargo molecules with components of the vesicle-forming machinery [91, 156].

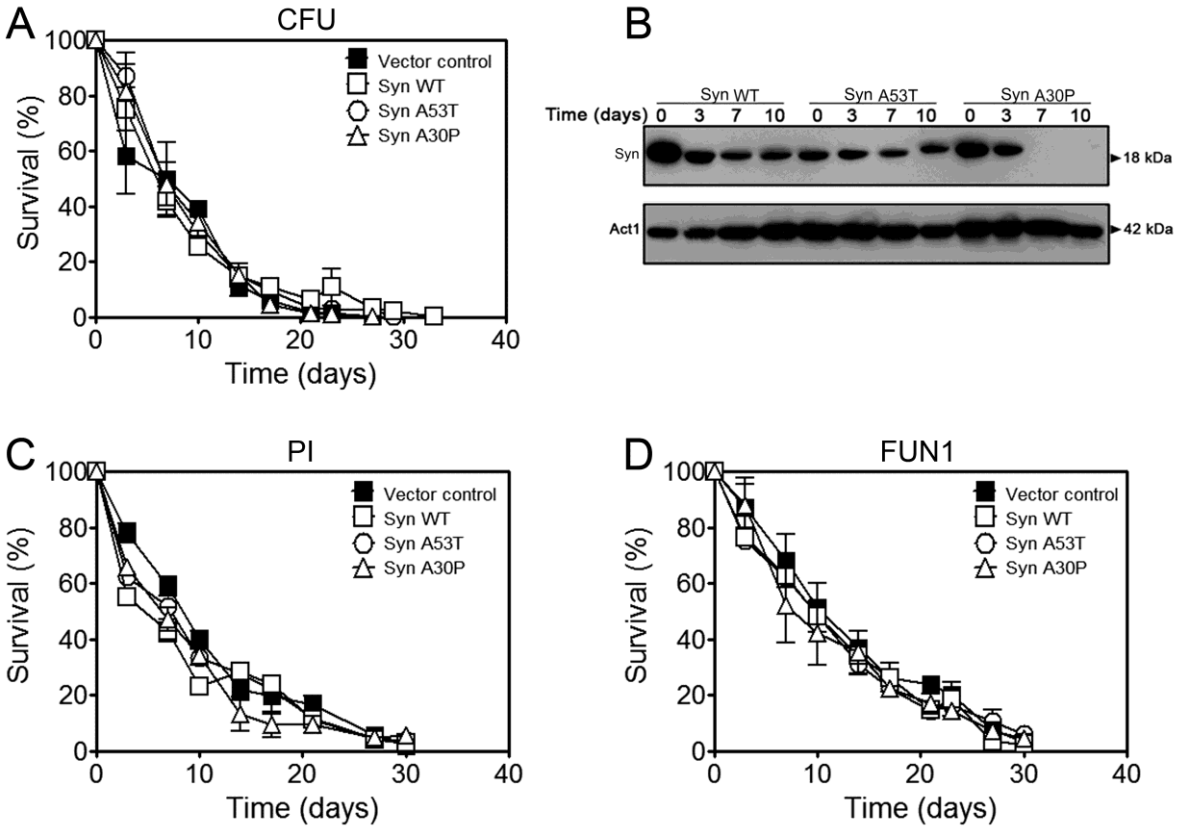


**Figure 32.** *ATG11* deletion increases longevity in aged yeast cells expressing the toxic α-syn variants. Chronological life span measure by (A) CFU, (C) PI exclusion or (D) FUN1 metabolic activity and (B) α-syn levels of stationary *atg11Δ* cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the constitutive *TPI1* promoter. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The data represents mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA.

Next, we addressed the effect of α-syn heterologous expression in the CLS of *atg32Δ* cells. Similarly to what we observed for the *atg11*Δ cells, the chronological longevity of *atg32Δ* cells harboring the vector control or expressing A30P was reduced when compared to wild type cells, and the expression of α-syn WT or A53T no longer triggered a toxic effect (Fig. 33A and B).

To confirm these results we used other different viability assays, namely the PI exclusion, and the metabolic activity evaluated by FUN1 (Fig. 33C and D). The results obtained with these assays also revealed the lack of an effect of α-syn WT or A53T expression in *atg32*Δ cell survival (Fig. 33C and D).

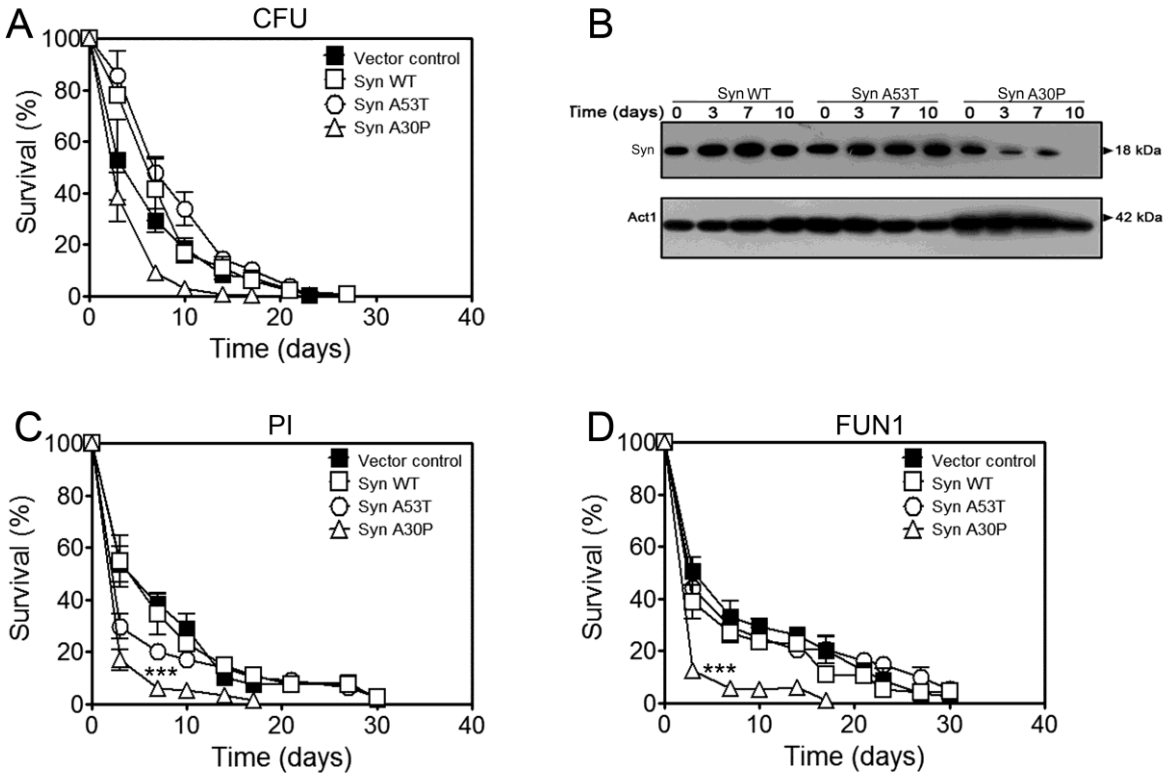


**Figure 33.** *ATG32* deletion increases longevity in aged cells expressing the toxic α-syn variants. Chronological life span measure by (A) CFU, (C) PI exclusion or (D) FUN1 metabolic activity and (B) α-syn levels of stationary *atg32Δ* cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the constitutive *TPI1* promoter. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The data represents mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (***p<0.001).

The determination of the mean (50% survival) and maximum (10% survival) chronological life spans of wild type and the mutant strains also confirmed the absence of effects on CLS by the expression of α-syn toxic variants (Fig. 34). Consistently, the results showed that the

expression of α-syn WT or A53T in *atg11*Δ and *atg32*Δ cells promoted a statistically significant increase of the mean and maximum life span when compared to wild type cells (Fig. 32A and B).

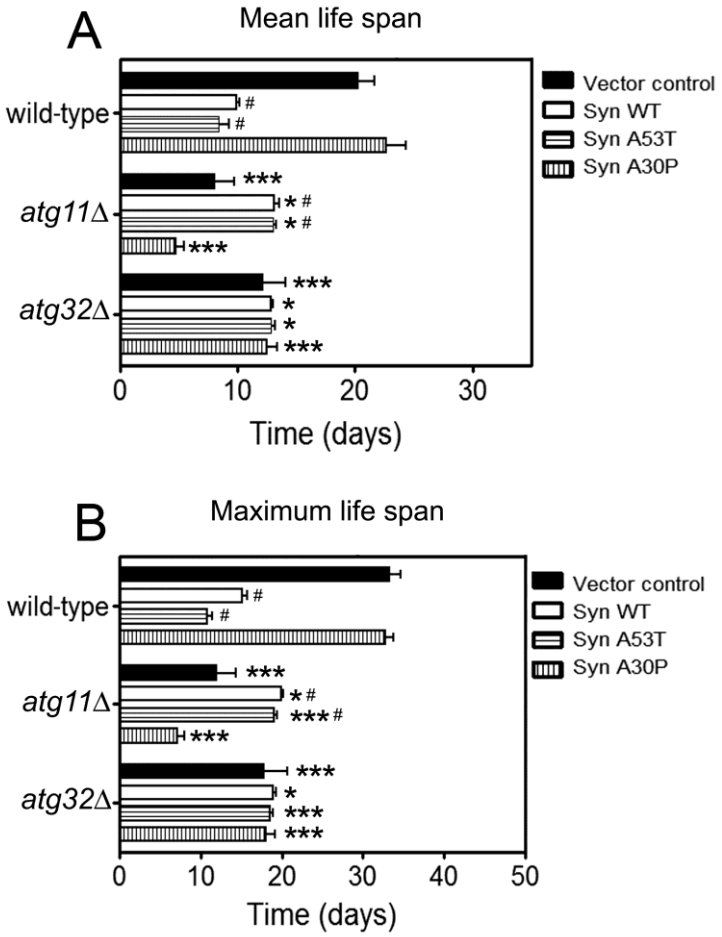


**Figure 34.** *ATG11* or *ATG32* deletion increases mean and maximum CLS of aged cells expressing the toxic α-syn variants. (A) Mean (50% survival) and (B) maximum (10% survival) chronological life spans were determined from curve fitting of the survival data (Figs. 30, 32 and 33) (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between wild type cells and *atg11Δ* or *atg32Δ* cells expressing vector control or α-syn variants (*). The significance determined between cells expressing the vector control or α-syn variants within each strain (wild type, *atg11Δ* or *atg32Δ*) was also determined (#).The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA ($^{\#}p<0.01$; $^{*}p<0.05$; $^{***}p<0.001$).

To evaluate whether or not the lack of α-syn effects on the CLS of cells with impaired mitophagy were related to lower α-syn aggregation, the foci formation was evaluated by

immunostaining against α-syn and analyzed by confocal microscopy. The analysis showed that cells expressing α-syn WT or A53T displayed the typical formation of α-syn foci (Fig. 35A), and the number of foci seemed to be independent of the abrogation of mitophagy by the deletion of the *ATG11* or *ATG32* genes, since we observed similar number of foci in wild type cells and in *atg11Δ* and *atg32Δ* cells. Therefore, the foci formation apparently is not linked to the decreased toxicity of α-syn cells in *ATG11* or *ATG32* mutant cells (Fig. 35A and B).

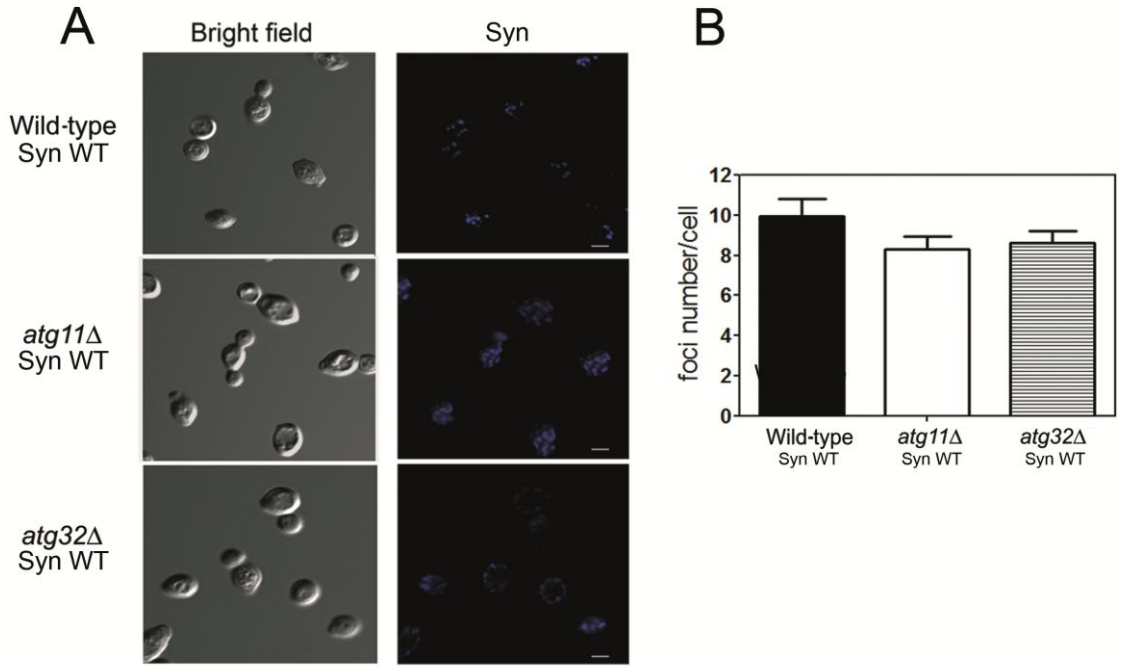


**Figure 35.** Foci formation is not linked to the abrogation of α-syn toxicity observed in *ATG11* or *ATG32* mutant cells. (A) α-Syn was analyzed after incubation with antibody against α-syn as the primary and the Pacific Blue conjugated goat anti-rabbit secondary antibody. Single confocal planes are shown. Scale bars: 5 μm. (C) Foci number was quantified in wild type, *atg11Δ* and *atg32Δ* cells expressing α-syn WT. The error bars represent the standard error of the mean (SEM).

Next the autophagy and mitophagy activities in the *atg11Δ* or *atg32Δ* mutant cells were evaluated as previously described. For that we applied the ALP assay, as described. The ALP assay indicated that *atg11Δ* cells expressing the vector control displayed slightly higher levels of autophagic activity (Fig. 36A), when compared to vector control wild type cells (Fig. 31A). Nevertheless, the observed induction of autophagy in wild type cells upon expression of the

different α-syn variants was abrogated to basal levels in *atg11Δ* cells (Fig. 36A). Importantly, mitophagy upon expression of the different α-syn variants was abrogated in *atg11Δ* cells (Fig. 36B). This clearly demonstrated that selective autophagy, the process abolished in *atg11Δ* cells, is associated with the toxicity of α-syn in aged yeast cells.

Due to the inhibition of mitophagy, *atg32*Δ cells displayed a higher autophagic activity and these levels decreased during aging both in vector control and in cells expressing the different α-syn variants (Fig. 36C). Furthermore, and as expected, the ALP assay indicated that the expression of the different α-syn variants in *atg32*Δ cells no longer induced mitophagy (Fig. 36D).

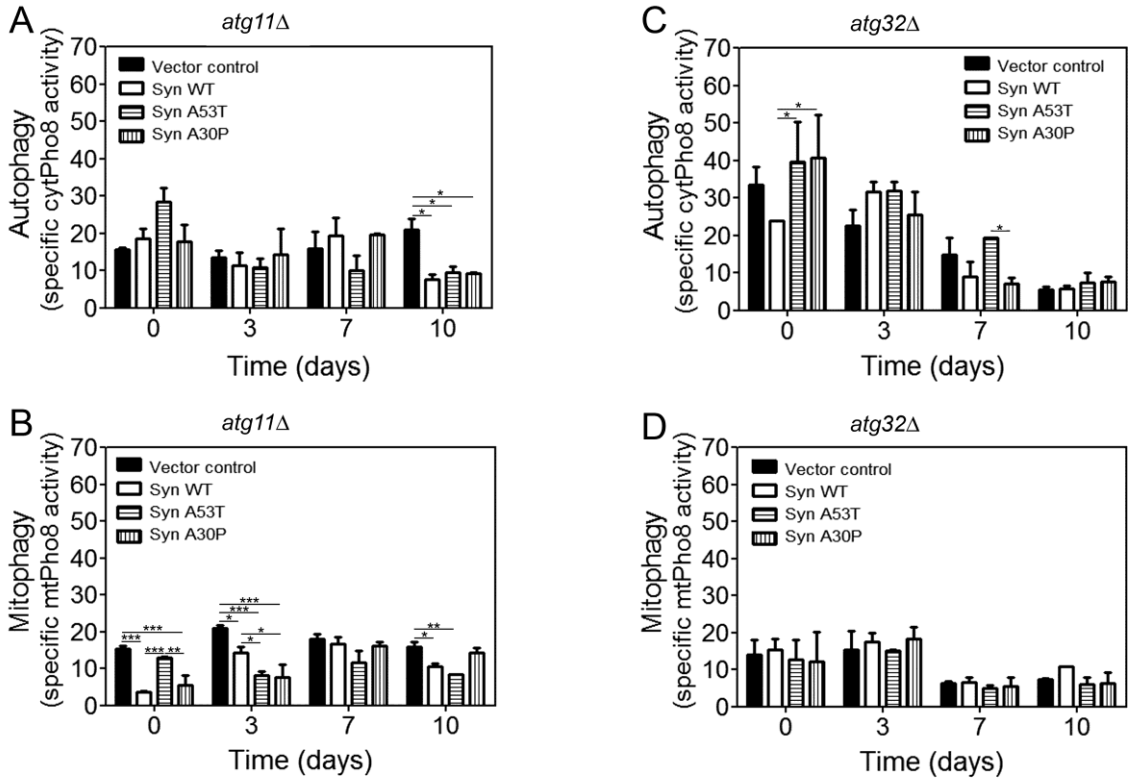


**Figure 36.** Deletion of *ATG11* or *ATG32* almost abolishes the autophagy and mitophagy induced by the expression of α-syn toxic variants. Autophagy and mitophagy activity were measured through the alkaline phosphatase (ALP) assay that was carried out to assess (A and C) autophagy and (B and D) mitophagy in *atg11Δ* or *atg32Δ* cells, respectively. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

Buttner and collaborators [319] identified that mitochondrial function and oxidative phosphorylation are key players in α-syn-induced toxicity in yeast cells, since deletion of mtDNA completely abrogates the α-syn-induced toxicity. These observations were also confirmed by us, since the expression of α-syn WT or A53T mutant variant in cells where the mtDNA was deleted, the *rho* 0 cells, promoted a CLS similar to that of cells harboring the vector control or expressing the non-toxic A30P variant (Fig. 37).

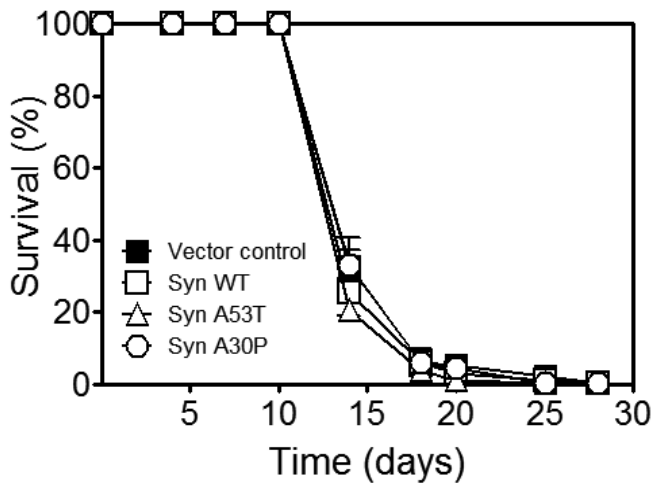


**Figure 37.** α-Syn-induced toxicity is dependent on the mitochondrial function. Chronological life span of *rho* 0 cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the constitutive *TPI1* promoter. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The data represents mean ± SEM of three biological independent replicas.

In this context, and to test the hypothesis that the lower toxicity of α-syn WT or A53T may be not detected due to an inherent mitochondrial dysfunction of *atg11*Δ and *atg32*Δ cells, we decided to determine the index of respiratory competence (IRC) [327] of these mutant cells. The IRC is defined by the percentage of yeast cells that have the ability to grow in both fermentable (glucose) and non-fermentable (glycerol or ethanol) carbon sources, and, additionally it can reflect the respiration status of mitochondria [327]. The results obtained showed that *atg32*Δ cells are able to grow on glycerol and present an IRC similar to wild type cells (Table 6). *atg11*Δ cells also displayed an IRC similar to wild type cells, nevertheless, mitochondrial functionality of

these mutant cells started to be affected at 7 days of CLS (Table 6). Moreover, the data also revealed that α-syn WT and A53T expression did not negatively affect the IRC of *atg11*Δ and *atg32*Δ cells but, instead, increased their IRC when compared to wild type cells (Table 6). Altogether, the results strongly suggest that the lack of α-syn WT and A53T toxicity is not associated with mitochondrial dysfunction of *atg11*Δ and *atg32*Δ cells.

When combined, the data described in this section confirmed that the toxicity of α-syn WT and the A53T mutant as observed in wild type cells was dependent on Atg11 and Atg32, two factors required for the induction of mitophagy. To further validate the biochemical data obtained with the ALP assay, we assessed mitophagy by microscopy visualization of the process. Wild type, *atg11Δ* and *atg32Δ* mutant cells expressing α-syn toxic forms, GFP-Atg8 and mtDsRed (mitochondrially-targeted DsRed) were analyzed by confocal microscopy. In wild type cells harboring the vector control, GFP-Atg8 was distributed in the cytosol with a punctate pattern, and mtDsRed labeled a typical mitochondrial network (Fig. 38). The expression of α-syn WT resulted in the accumulation of GFP in the vacuole in the vast majority of the cells, indicating that GFP-Atg8 was shuttled to the vacuole and that autophagy was induced. Furthermore, the vast majority of the cells did not present the mitochondrial mtDsRed signal, and in those cases where red fluorescence was observed, the typical mitochondrial network was abolished (Fig. 38). In contrast, in *atg11*Δ and *atg32*Δ cells expressing the α-syn toxic forms, the GFP-Atg8 was essentially distributed in the cytosol, and the mitochondrial network remained detectable although we observed some mitochondrial fragmentation (Fig. 38). The same results were obtained for yeast cells expressing the A53T mutant (data not shown). The cells expressing the non-toxic A30P mutant produced similar results as those obtained with the cells harboring the vector control (data no shown).

**Table 6.** Index of respiratory competence (IRC) of stationary phase wild type, *ATG11* and *ATG32* mutant cells during chronological lifespan. Wild type and mutants cells were grown in selective SC medium and at the indicated time points cells were plated onto YPG (Yeast Peptone with 2% Glycerol) and also on YEPD (Yeast Peptone with 2% Glucose) plates. The IRC was calculated as the number of colonies formed on YPG divided by the number of colonies formed on YEPD times 100%. Data represents mean ± SEM of three independent samples. Significance was determined between wild type cells and *atg11Δ* or *atg32Δ* cells expressing vector control or α-syn variants by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001.

| | Time (days) | Vector Control | Syn WT | Syn A53T | Syn A30P |
|---|---|---|---|---|---|
| **Wild type** | **0** | 83.2±4.99 | 74.6±5.49 | 76.4±10.51 | 75.9±2.26 |
| | **3** | 77.1±4.91 | 99.1±14.36 | 66.5±3.76 | 76.6±4.93 |
| | **7** | 61.3±6.58 | 23.8±1.19 | 14.6±3.49 | 73.4±6.34 |
| | **10** | 81.40±9.8 | 10.3±6.34 | 3.25±0.86 | 91.9±6.91 |
| ***atg11Δ*** | **0** | 87.1±8.62 | 91.9±6.01 | 98.5±2.37 *** | 87.9±9.01 |
| | **3** | 84.8±5.59 | 65.6±1.03 * | 97.9±2.02 *** | 85.8±9.32 |
| | **7** | 20.4±5.71 *** | 8.6±4.33 ** | 53.4±6.51 *** | 61.2±0.31 * |
| | **10** | 26.7±6.78 *** | 1.31±0.30 ** | 24.3±7.79 * | 31.9±4.47 *** |
| ***atg32Δ*** | **0** | 74.24±3.97 | 93.6±7.86 | 94.8±6.93 *** | 82.7±3.84 |
| | **3** | 95.6±5.83 ** | 99.2±9.51 | 74.5±2.38 *** | 91.3±5.48 |
| | **7** | 83.2±4.28 | 75.2±0.96 ** | 75.1±4.62 *** | 84.4±2.00 |
| | **10** | 95.5±3.98 | 96.9±13.42 *** | 91.7±10.48 *** | 60.1±5.48 * |

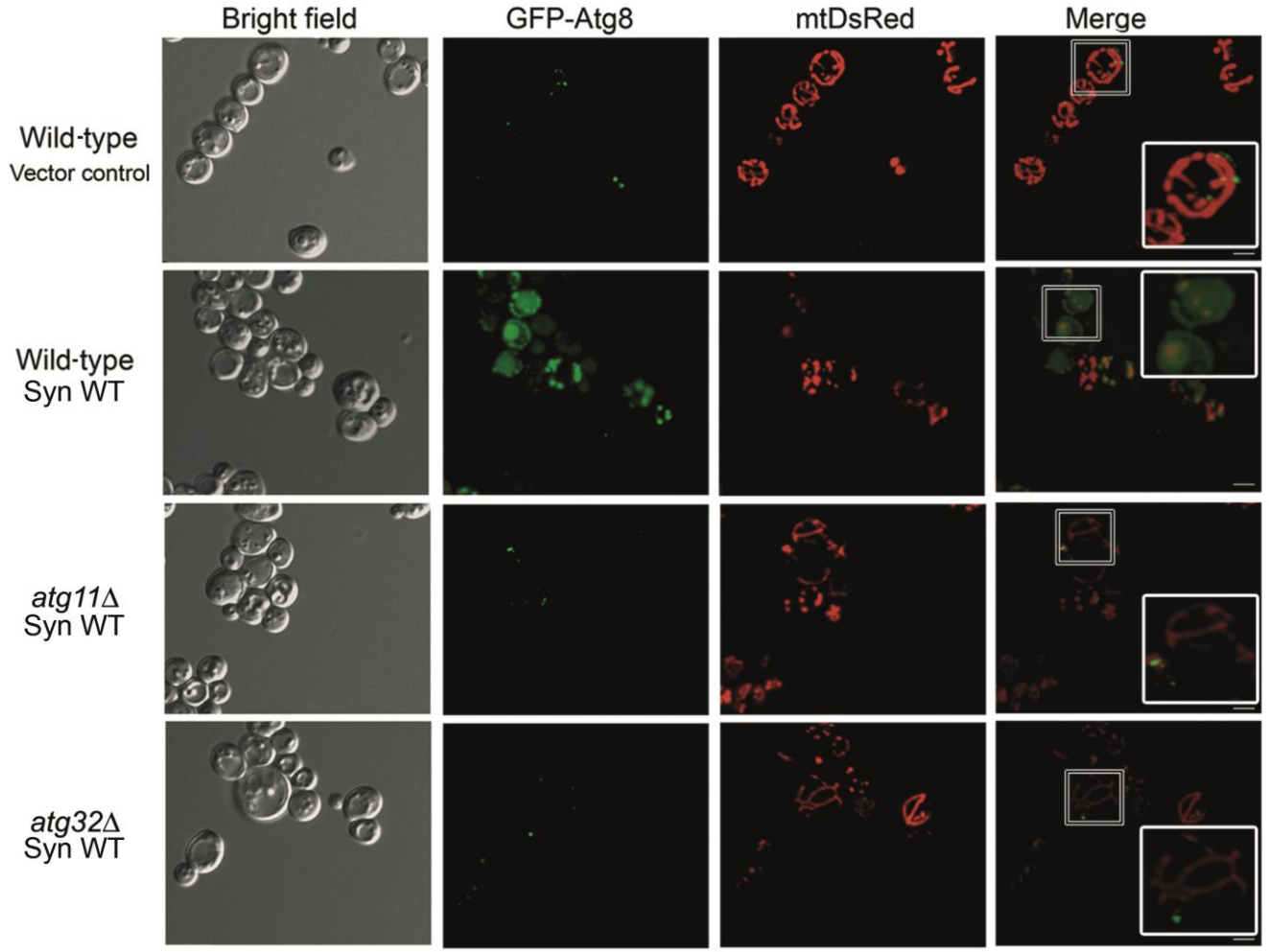


**Figure 38.** α-Syn-induced toxicity is associated with mitophagy induction. Wild type, *atg11Δ* and *atg32Δ* cells expressing, mitochondrially-targeted DsRed (mtDsRed) and GFP-Atg8. Single confocal planes are shown. Scale bars: 5 μm.

Hence, the results indicate that in control conditions mitophagy is important to maintain the homeostasis of aged cells. In contrast, under conditions of α-syn toxicity, mitophagy appears to result in increased toxicity rather than in cellular protection for the aged cells. Thus, mitophagy mediates α-syn-induced toxicity contributing to the reduction of CLS, which could be related with the fact that aged cells have a reduced ability to simultaneously upregulate anabolic processes in order to compensate for the loss of cellular material by increased mitophagy. On the other hand, it is possible that the selectivity of various types of autophagy, like mitophagy, is lost some factors, commonly required for various types of autophagy, may become limiting when mitophagy is upregulated. Additionally, the loss of selectivity could also result in the degradation of functional competent mitochondria, crucial to sustain cell survival of post-mitotic cells, contributing to the toxicity observed. The question that arises is how α-syn is stimulating autophagy/mitophagy?

ROS, particularly superoxide anions, are crucial signaling molecules implicated in the control and regulation of autophagy and aging progression [225]. Additionally, both mammalian SIRT1 and its yeast homologue sirtuins 2 (Sir2) were recently described as inducers of autophagy under starvation conditions [356], and indeed, Sir2 was already associated with α-syn-induced toxicity [320]. Therefore, the next steps in this study were to evaluate the contribution of ROS and Sir2 to the induction of autophagy/mitophagy under α-syn-induced toxicity.

### 3.1.5 Superoxide anion accumulation under α-synuclein-induced toxicity is decreased in cells with impaired mitophagy

In yeast models, it was also shown that α-syn expression triggers intracellular accumulation of ROS and mitochondrial dysfunction [319]. Recent findings established a role for superoxide anion ($O_2^{2-}$), in the regulation of autophagy [349], particularly in conditions of prolonged starvation [225].

In order to unravel whether ROS may mediate the induction of autophagy/mitophagy, due to expression of α-syn variants, we examined the accumulation of ROS, the $O_2^{2-}$ levels and total ROS levels, particularly hydrogen peroxide ($H_2O_2$), during cells CLS. The accumulation of $O_2^{2-}$ was assessed by the fluorescent probe dihydroethidium (DHE) [357] and the accumulation of

total ROS levels by the use of the fluorescent probe dihydrorhodamine 123 (DHR) [358]. Flow cytometry analysis revealed that wild type cells expressing α-syn WT or A53T mutant form displayed a significant increase of DHE fluorescence intensity over time, as compared to the wild type cells harboring the vector control or expressing the non-toxic A30P mutant (Fig. 39A). In contrast, the expression of α-syn WT or A53T in cells lacking either the *ATG11* or *ATG32* gene did not result in such increased accumulation of $O_2^{2-}$ (Fig. 39B and C). The same results were observed when the accumulation of total ROS levels, by DHR, was analyzed (Fig. 39D-F). In fact, the accumulation of ROS was higher in wild type cells expressing α-syn WT or A53T mutant form compared with the same cells harboring the vector control or expressing the non-toxic A30P mutant (Fig. 39D). Consistently, the absence of the *ATG11* or *ATG32* gene abrogated the accumulation of total ROS levels under the expression of α-syn WT or A53T (Fig. 39E and F).

High levels of DHE and DHR fluorescence were observed in the *atg11Δ* mutant cells expressing the non-toxic A30P (Fig. 39B and E), in accordance with the reduced CLS of these cells (Fig. 32). The data also showed that independently of background, wild type, *atg11Δ* or *atg32Δ*, vector control cells, grown in 2% glucose, a condition known to induce the accumulation of $O_2^{2-}$, resulted in increased accumulation of $O_2^{2-}$, as expected.

To further explore the reasons underlying the decrease in $O_2^{2-}$ and $H_2O_2$ accumulation displayed by cells with an impairment of mitophagy expressing toxic α-syn, we have determined the activity of superoxide dismutases, both cytosolic Cu/Zn-dependent (Sod1) and the mitochondrial Mn-dependent superoxide dismutases (Sod2), known to inhibit the accumulation of ROS.

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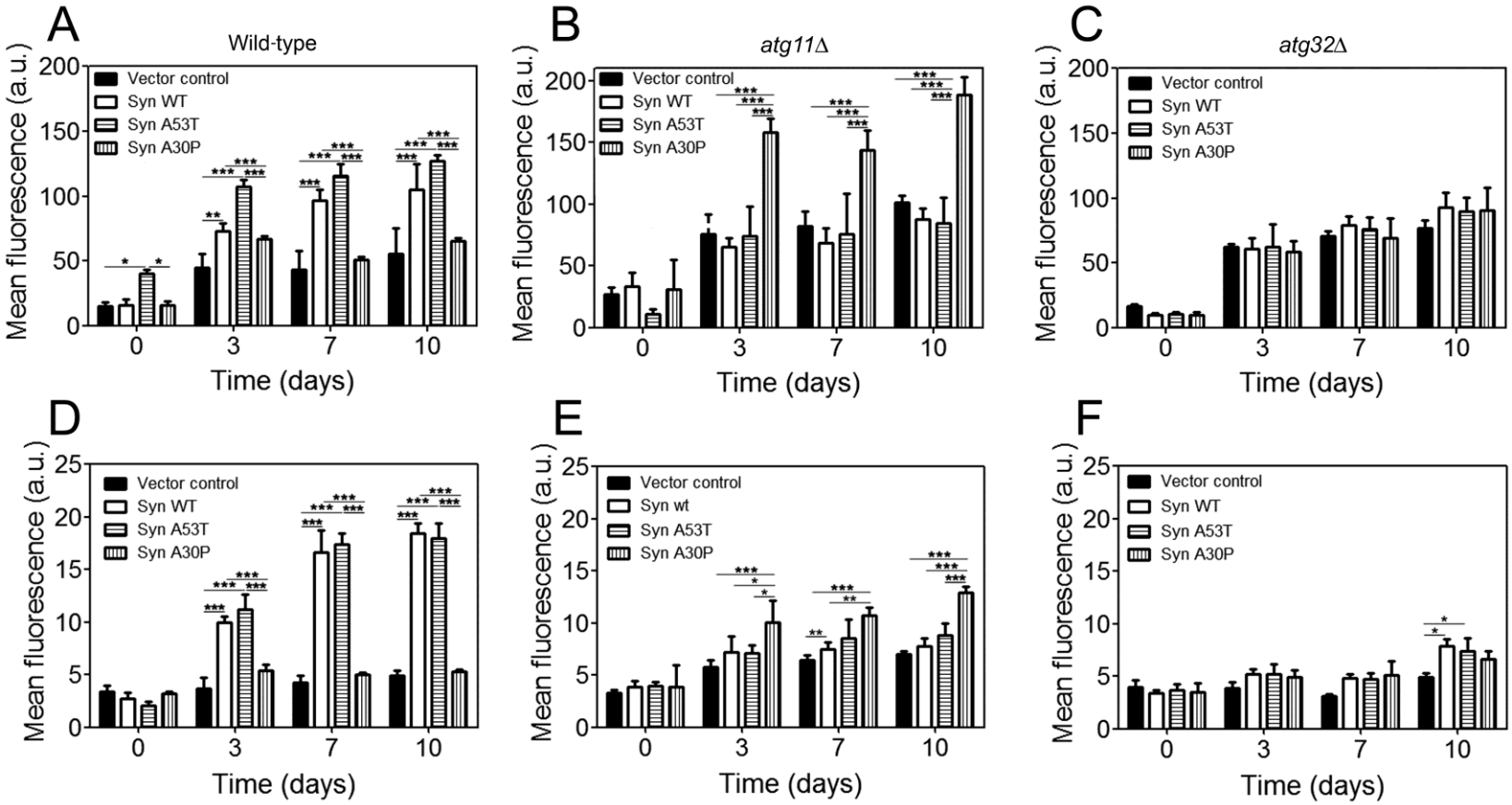


**Figure 39.** Reactive oxygen species (ROS) accumulation under α-syn-induced toxicity is decreased in cells with impaired mitophagy. During chronological life span of (A and D) wild type, (B and E) *atg11Δ* and (C and F) *atg32Δ* cells, expressing the vector control, the α-syn WT, the A53T or the A30P mutant forms under the control of the constitutive *TPI1* promoter, the accumulation of superoxide anion and hydrogen peroxide were evaluated by flow cytometry, using the fluorescent probes (A-C) dihydroethidium (DHE) and (D-F) dihydrorhodamine 123 (DHR), respectively. The error bars represent the standard error of the mean (SEM). Values indicate mean ± SEM from three independent experiments. Significance of the values between yeast strains was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

The results showed that while *atg11*Δ cells expressing the vector control displayed Sod1 activity comparable to wild type cells, *atg32*Δ cells presented a significant increase in Sod1 activity (Fig. 40A). Nevertheless, both *atg11*Δ and *atg32*Δ cells had lower basal levels of Sod2 activity, which do not increase over the CLS (Fig. 40B). The expression of α-syn A53T in wild type cells led to a decrease of Sod1 and a maintenance of Sod2 activity over the CLS time evaluated (Fig. 40C and D). In contrast, the expression of α-syn A53T did not affect the activity of Sod1 in *atg32*Δ cells, and promoted a slight increase of this activity in *atg11*Δ cells, while it increased the Sod2 activity (Fig. 40D). Hence, the ability of *atg11*Δ and *atg32*Δ cells to maintain Sod1 activity and to increase Sod2 over the CLS when expressing α-syn WT (data not shown) or A53T (Fig. 40C and D) could be responsible for the decreased accumulation of ROS observed, since it is described that reduction of $O_2^{2-}$ is controlled by the SODs [359].

The maintenance and increased in the Sods activities observed over CLS in *atg11Δ* or *atg32Δ* mutant cells could be the mechanism behind the decreased superoxide anion levels displayed by these cells. Additionally, these data on the oxidative status these mutant cells are in agreement with the respiratory competence displayed by these cells (Table 6). Furthermore, the ROS levels apparently are also correlated with the stimulation of autophagy. In conditions of starvation, the ROS accumulation is induced and consequently leads to induction of autophagy due to the interaction with Atg4, which enables the conversion of Atg8 to Atg8-PE and vice versa [224]. Probably, in wild type cells expressing α-syn WT or A53T that accumulate elevated ROS levels, the autophagy will be partially stimulated.

Thus, our work does not rule out superoxide anions as inducers of autophagy/mitophagy due to the α-syn expression of the toxic variants [360]. Nevertheless, we decided to study other molecules that might be implicated in autophagy stimulation.

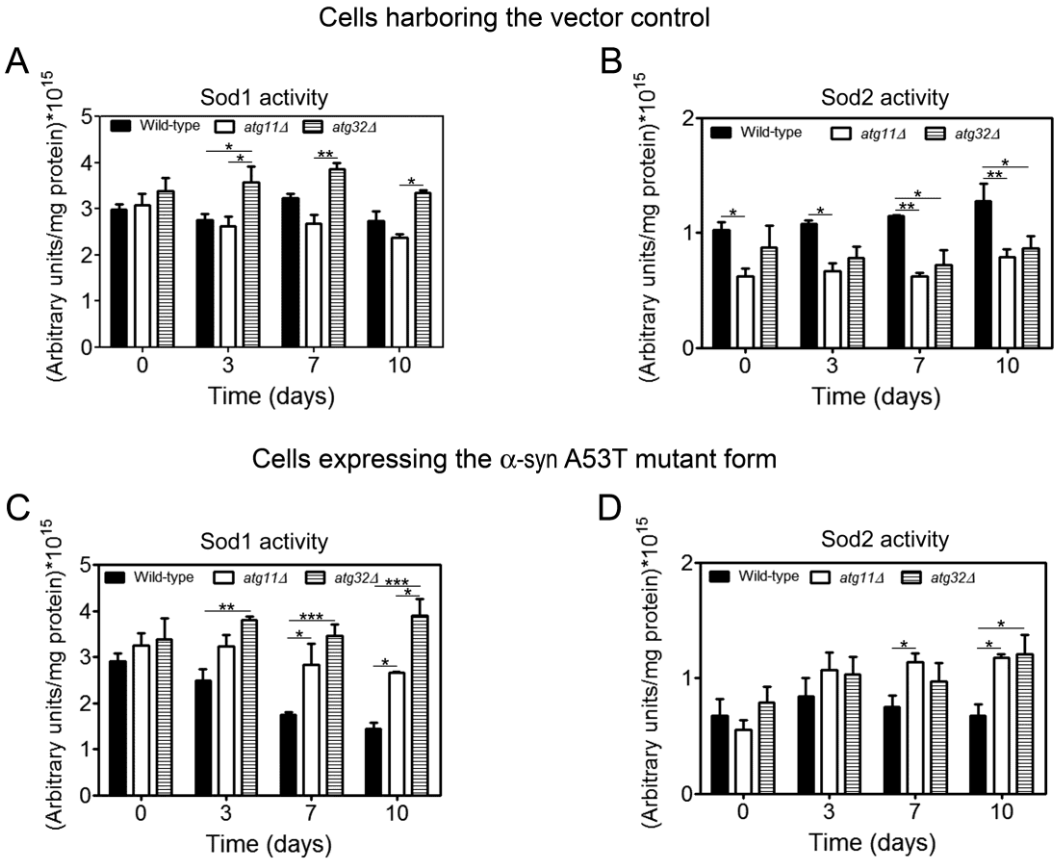


**Figure 40.** Superoxide dismutases (SODs) activity under α-syn-induced toxicity is increased in cells with impaired mitophagy. Bands corresponding to Sod1 activity of cells expressing vector control (A) or α-syn A53T mutant (C) and Sod2 activity of cells expressing vector control (B) or α-syn A53T mutant (D) were measured with a densitometer and the relative intensities calculated (Quantitaty One software, BioRad). Values indicate mean ± SEM from three independent experiments. Significance of the values between yeast strains was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

### 3.1.6 SIR2 mediates the induction of autophagy/mitophagy under α-synuclein-induced toxicity

Autophagy induction can be mediated by several pathways. Recently, it was demonstrated the ability of the human homologue of yeast sirtuin 2 (Sir2), SIRT1, to induce autophagy [202]. In fact, different studies confirmed the induction of autophagy by SIRT1 in different models and in both normal growth and starvation conditions [201]. Furthermore, SIRT1, is required to sustain autophagy in response to nutrient starvation through deacetylation of autophagic regulators, including *ATG5, ATG7*, *ATG8* and *ATG12*, suggesting that SIRT1 can

adjust autophagy activity to match the metabolic status [55, 201]. In addition, recent studies have also shown that in yeast cells, α-syn toxicity is Sir2 dependent [320]. Altogether, these findings led us to hypothesize a possible role of Sir2 in the induction of autophagy/mitophagy in response to α-syn toxicity.

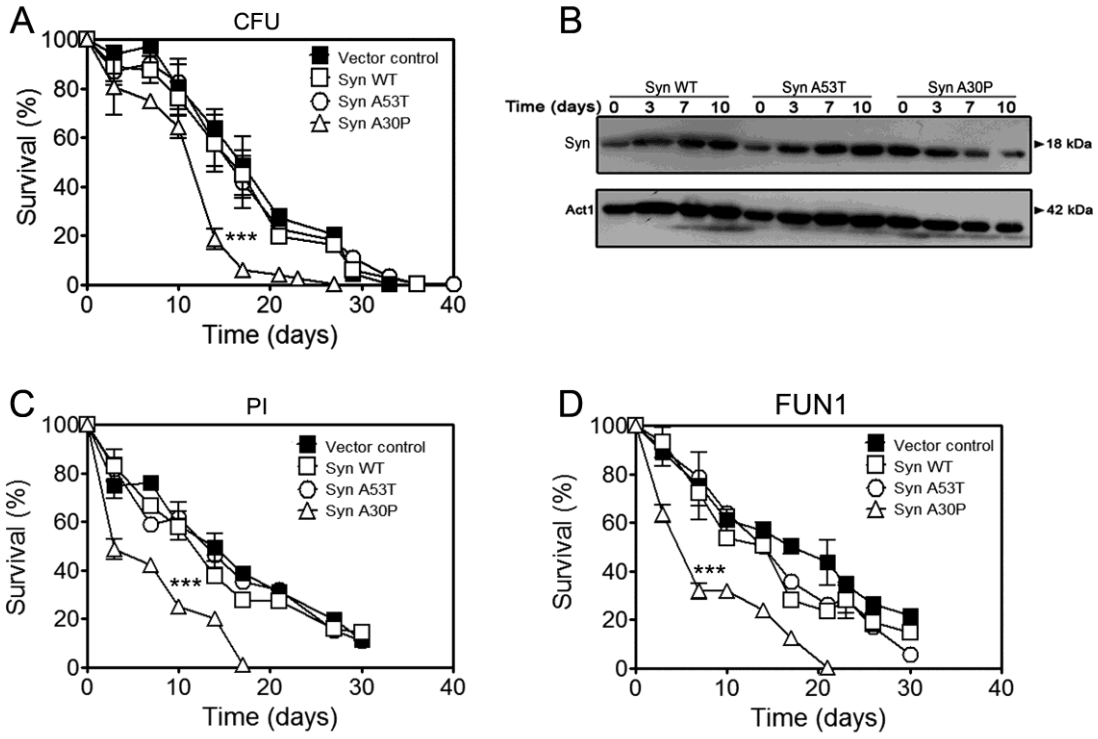


**Figure 41.** Abrogation of *SIR2* reduces the α-syn toxicity in yeast aged cells. Chronological life span measure by (A) CFU, (C) PI exclusion or (D) FUN1 metabolic activity and (B) α-syn levels of stationary *sir2Δ* cells expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the constitutive *TPI1* promoter. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The data represents mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (***p<0.001).

Thereby, using the moderate α-syn toxicity model, we expressed the human α-syn WT, the clinical A53T or A30P mutants in *SIR2Δ* cells, and grown the cultures until the stationary phase, about 2 days, representing this time the day 0 of CLS, and then the CLS and the autophagic activity were evaluated. In agreement with observations previously made [320], the

results showed a CLS extension of *sir2Δ* cells expressing α-syn WT or A53T as compared to wild type cells expressing the same proteins (Fig. 41A and B). The CLS extension of *sir2Δ* cells expressing α-syn WT or A53T was also validated by the analysis of cells viability by PI exclusion and FUN1 metabolic processing (Fig. 41C and D). Interestingly, the deletion of *SIR2* in cells expressing the A30P non-toxic mutant resulted in a shorter CLS, which is in accordance with the data obtained with the *atg11Δ* cells. For the cells carrying the vector control, the *SIR2* deletion did not alter the CLS in comparison to the wild type cells. These results are in agreement with the previous report showing that *SIR2* deletion suppresses the toxicity induced by α-syn [320]. Consistently, this data was corroborated by the determined mean and maximum CLS (Fig. 42).

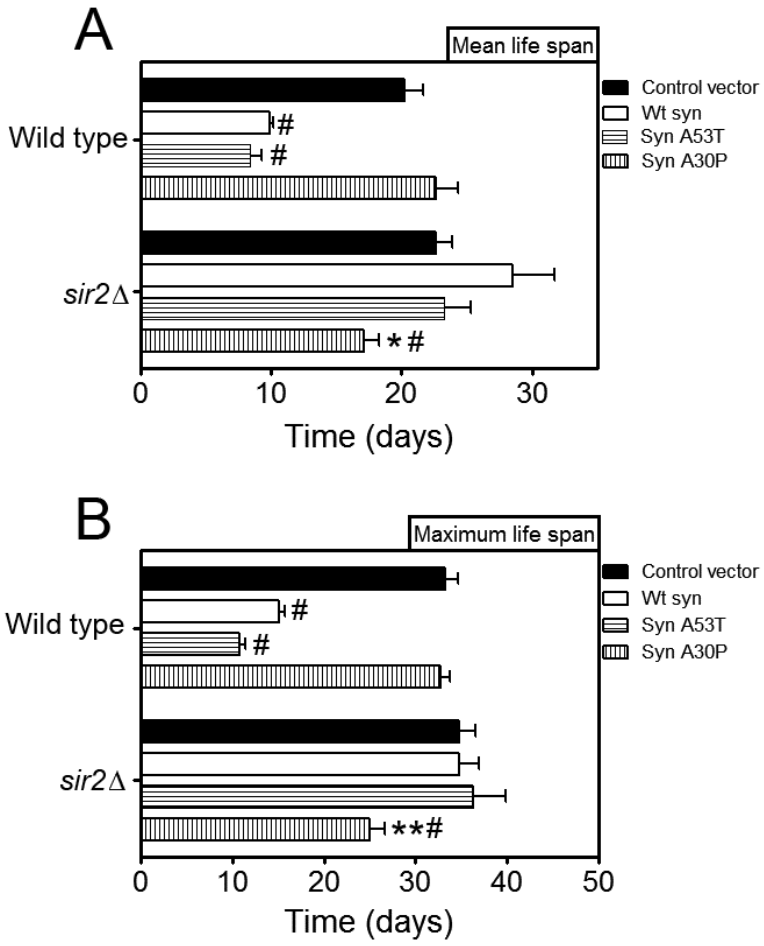


**Figure 42.** *SIR2* deletion increases mean and maximum CLS of aged cells expressing the toxic α-syn variants. (A) Mean (50% survival) and (B) maximum (10% survival) chronological life spans were determined from curve fitting of the survival data (Figs. 30 and 41) (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between wild type cells and *sir2Δ* cells expressing vector control

or α-syn variants (*). The significance determined between cells expressing the vector control or α-syn variants within each strain (wild type, *sir211Δ*) was also determined (#).The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA ($^{\#}$p<0.05; *p<0.05; **p<0.01; ***p<0.001).

The reduction of the α-syn toxicity by the abrogation of the *SIR2* gene supports a role for Sir2 in controlling autophagy/mitophagy induction upon α-syn expression. To elucidate the validity of this hypothesis, the autophagic and mitophagic activities were evaluated by the ALP assay, above described, during CLS. The results showed that *SIR2* deletion inhibits the autophagy and mitophagy activities in cells expressing the α-syn toxic variants, as reflected by the abolishment of the ALP activity in *sir2Δ* cells (Fig. 43A and B). Once again, these results support the hypothesis that α-syn toxicity is dependent of the autophagy and mitophagy sustained induction, which in turn seems to be mainly regulated by Sir2.

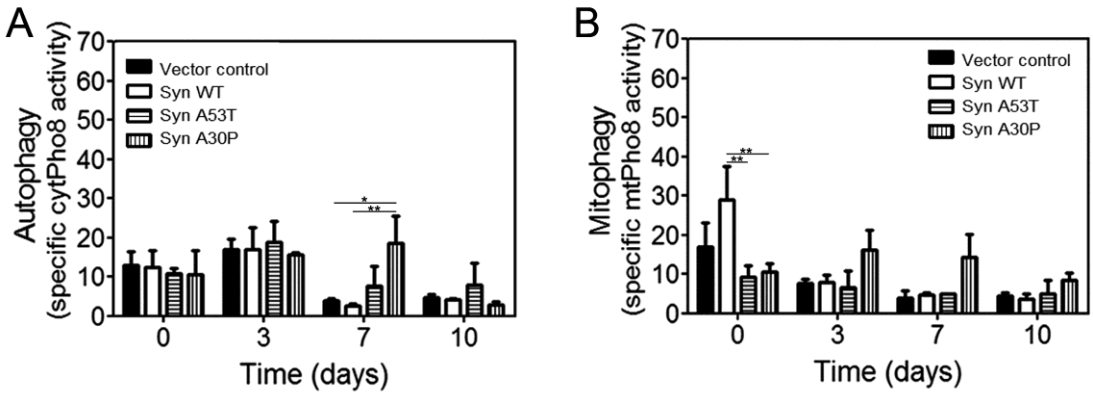


**Figure 43.** Sir2 is associated with autophagy induction and selective degradation of mitochondria under α-syn-induced toxicity. Autophagy (A) and mitophagy (B) activities were measured through the alkaline phosphatase (ALP) assay, in *sir2Δ* cells. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01).

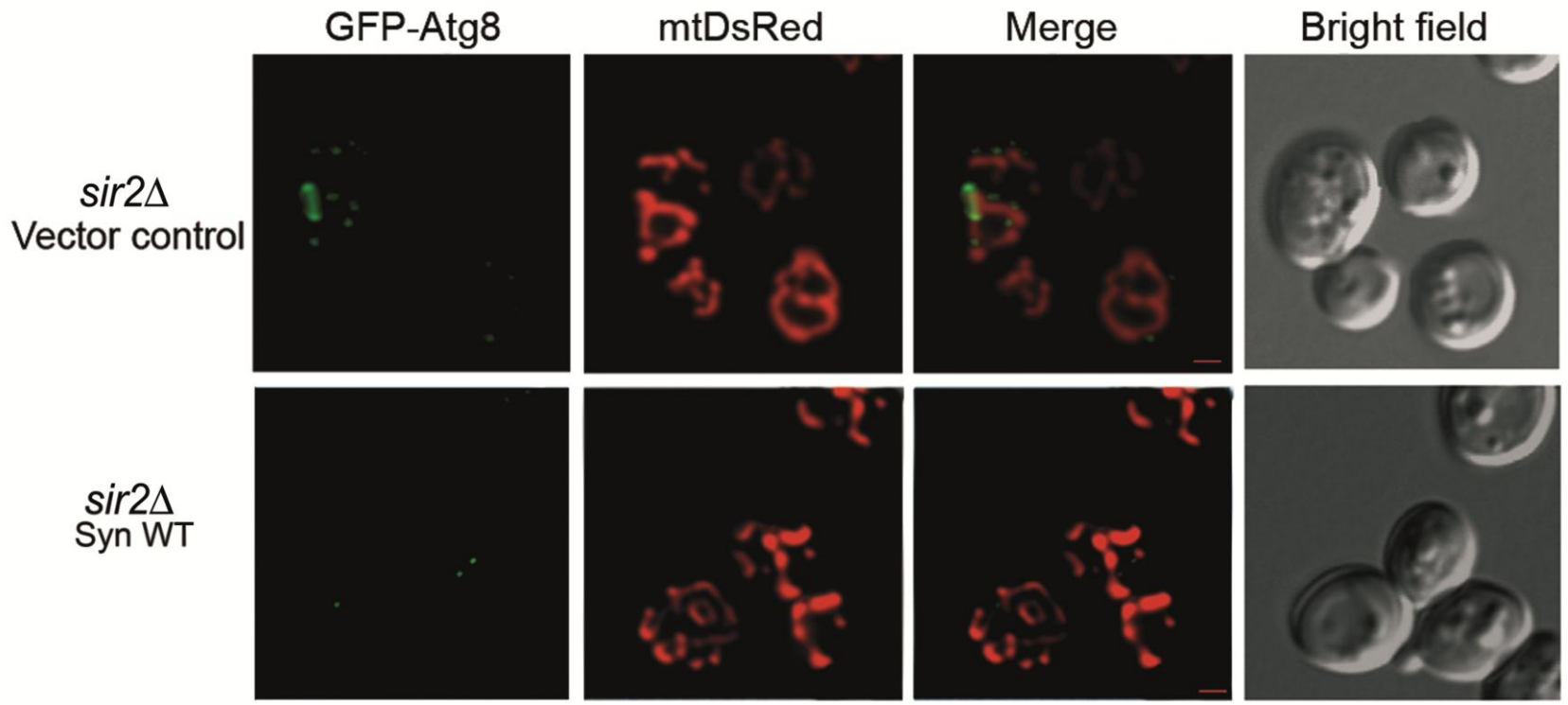


**Figure 44.** Sir2 abrogation reduces mitophagy induced by α-syn expression. Wild type and *sir2Δ* cells expressing, mitochondrially-targeted DsRed (mtDsRed) and GFP-Atg8. Single confocal planes are shown. Scale bars: 5 μm

Next, to further corroborate the biochemical data obtained with the ALP assay, we assessed the mitophagy by microscopic visualization of the process. Therefore, in wild type and *sir2Δ* cells expressing α-syn toxic forms, the GFP-Atg8 and mtDsRed (mitochondrially-targeted DsRed) were expressed. The cell visualization by confocal microscopy revealed that in *sir2Δ* cells the GFP-Atg8 was essentially found distributed in the cytosol independently of the expression of α-syn variants. In addition, mitochondrial mtDsRed marked a mitochondrial network (Fig. 44) suggesting the abolishment of the mitophagy as revealed in wild type cells expressing α-syn WT, corroborating the absence of mitophagy activity determined in *sir2Δ* cells.

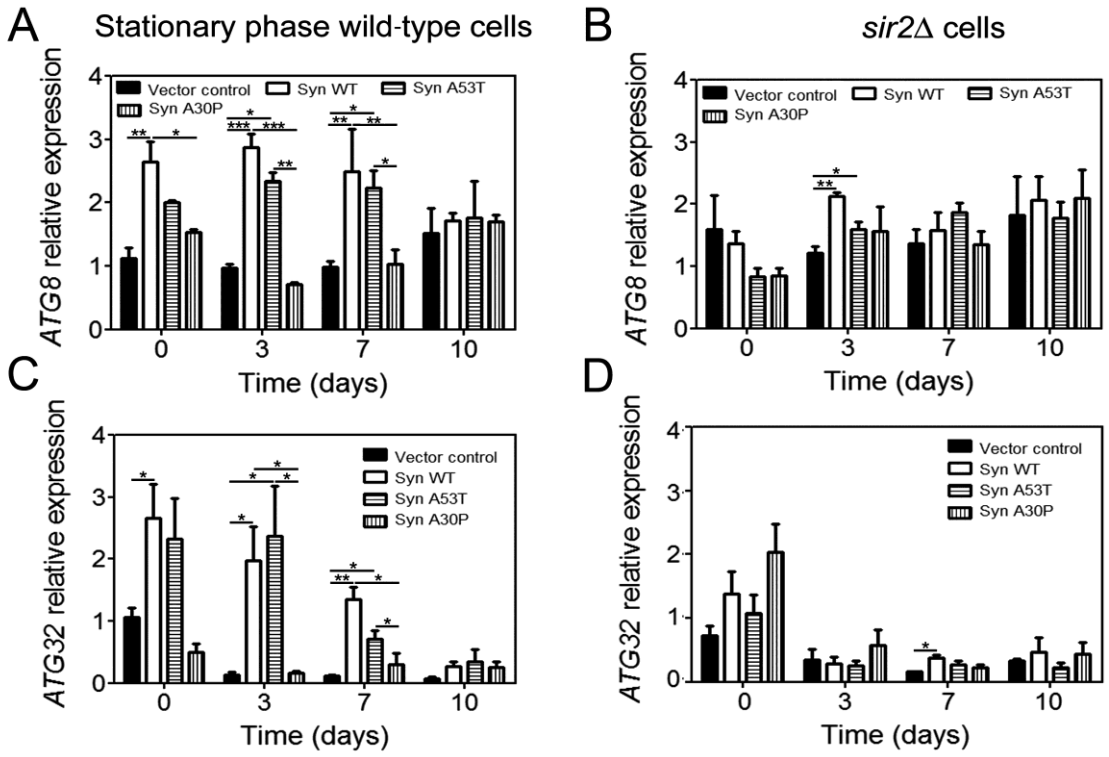


**Figure 45.** Sir2 regulates the mRNA levels of *ATG8* and *ATG32* in stationary phase cells expressing α-syn toxic variants. Relative *ATG8* and *ATG32* mRNA levels in stationary phase wild type and *sir2Δ* cells expressing α-syn at day 0, 3, 7 and 10. Three reference genes [*ACT1* (actin), *PDA1* (alpha subunit of pyruvate dehydrogenase) and *TDH2* (isoform 2 of glyceraldehyde-3-phosphate dehydrogenase)] were used as internal standards and for the normalization of mRNA expression levels. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

As described before, in wild type cells the expression of α-syn WT or A53T mutant is responsible for the induction of higher mRNA levels of *ATG8* and *ATG32* (Fig. 27B and 28B), and consequently associated with the autophagy/mitophagy stimulation. In this sense, since we observed an abrogation of the autophagy and mitophagy activities in *sir2Δ* cells, we decided to perform the analysis of the *ATG8* and *ATG32* relative mRNA levels in *sir2Δ* cells, expressing α-syn WT or A53T, during CLS. The mRNA analysis revealed that the expression of *ATG8* (Fig. 45A and B) and *ATG32* (Fig. 45C and D) is strongly dependent on Sir2 in stationary phase cells under α-syn toxic conditions, particularly the expression of *ATG32*, since the deletion of *SIR2* gene strongly reduces the *ATG32* mRNA levels (Fig. 45).

Recently it was demonstrated that the mammalian SIRT1 has the capacity to induce autophagy, in both normal and starved conditions (reviewed in [202]), however to the yeast SIRT1 homologue, Sir2, this link to autophagy was never showed. This work clearly proved that, during the yeast CLS, Sir2 is involved not only in the modulation of autophagy but also in the regulation of mitophagy.

Thus, our data strongly supports the hypothesis that α-syn toxicity is dependent on Sir2, an essential mediator of autophagy/mitophagy through the transcriptional regulation of *ATG8* and *ATG32* in stationary phase cells expressing α-syn toxic variants, during aging. Altogether, these innovative findings open new insights to the study of the mechanism associated with PD pathogenesis in higher eukaryote organisms.

## Section 3.2

# Caloric restriction mitigates α-synuclein toxicity aged cells

***Part of the results described in this chapter were published as follow:***

Sampaio-Marques, B. and Ludovico, P. Caloric restriction regulates autophagy in yeast aged cells in response to proteotoxicity. (Manuscript in preparation).

***The results described in this chapter were presented in the following national or international congresses:***

National congresses:

- XIX Jornadas de Biologia de Leveduras Prof. Nicolau van Uden. Lisboa, Portugal. (2012) "Autophagy and aged yeast cells: response to proteotoxic stress under caloric restriction". (Oral communication).

International congress:

- 9th IMYA – International Meeting on Yeast Apoptosis. Rome, Italy. (2012) "Caloric restriction regulates autophagy in yeast aged cells in response to proteotoxicity". (Poster presentation).

### 3.2 Caloric restriction mitigates α-synuclein toxicity in yeast aged cells

Caloric restriction (CR) increases life span in multiple organisms including yeast, worm, flies and mammalian, nevertheless, the molecular mechanisms by which CR slows aging remains yet poorly understood. CR promotes several physiological changes that may underlie its effects on longevity, including decreased activity of nutrient and signaling pathways, increased resistance to stress, altered translation and ribosome biogenesis, and increased autophagy. In this sense CR is a promising intervention that is able to attenuate aging and ultimately to decrease the risk of age-related neurodegenerative disorders [361]. Based on the perspective that CR is able to delay aging, age-related neurodegenerative disorders, and to modulate autophagy, our aim in this section was to contribute for a better comprehension of the effects of CR on α-syn toxicity and correlate them with the autophagy/mitophagy activity during aging. For that, we tested whether reducing the glucose concentration in the medium (CR) impacts on the autophagy/mitophagy in aged cells expressing α-syn.

#### 3.2.1 Reduced glucose uptake extends CLS of yeast cells expressing α-synuclein

Although CR in worms, flies and mammals usually requires a complex food source limitation, in yeast, moderate CR is commonly achived by reducing the glucose concentration in the culture medium from 2% to 0.5% [239, 362, 363]. In this study, we used the moderate toxicity yeast model, consisting in the constitutive expression of α-syn WT, A53T or the A30P mutant under the control of the endogenous *TPI1* promoter. Therefore, cells were grown under CR, specifically, in culture medium with 0.5% of glucose, until the stationary phase, representing the day 0 of the CLS. As expected the CLS, measured by the survival of non-dividing stationary phase cells harboring the vector control and expressing the non-toxic mutant α-syn A30P was clearly extended in comparison with the same cells under non-CR conditions (2% glucose)(Fig. 46). Cells expressing the toxic α-syn variants (WT or A53T mutant form) grown under CR condition also displayed CLS extension of two times comparatively to non-CR conditions (Fig. 46A and B ). Nevertheless, α-syn WT or A53T mutant form expression also induced a shortening of CLS when cells were under CR conditions in comparison with the cells harboring the vector

control or expressing the non-toxic mutant α-syn A30P under the same conditions (Fig. 46B). These results showed that although CR increases CLS of cells expressing α-syn toxic variants, it does not abolished α-syn toxicity, it only ameliorates the toxicity in comparison with non-CR conditions.

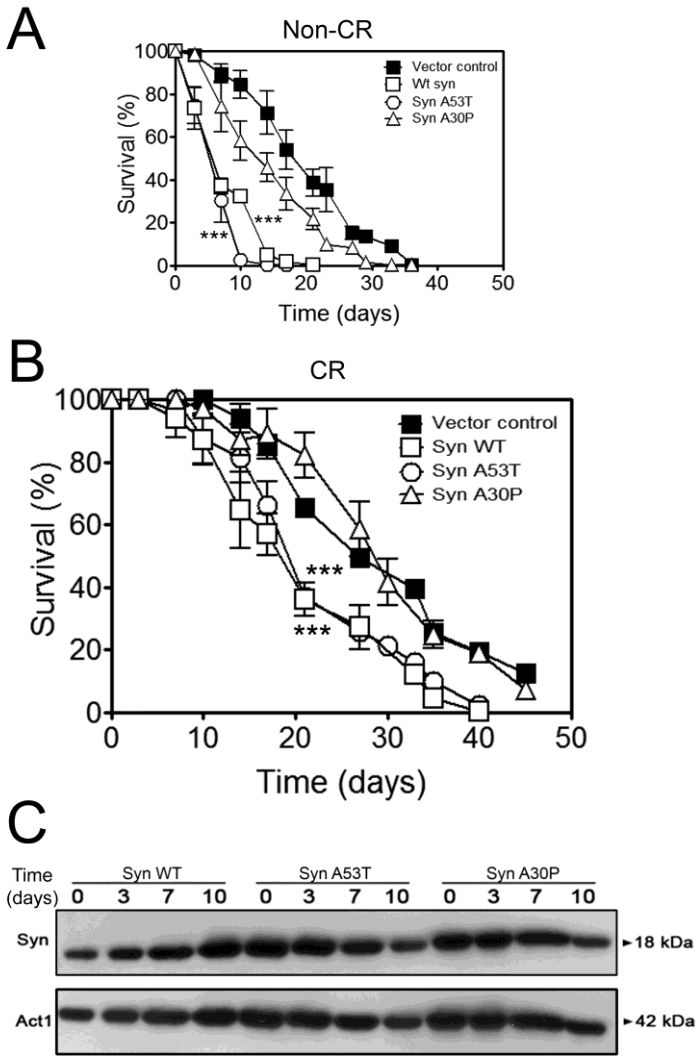


**Figure 46.** Caloric restriction (CR) ameliorates α-syn-induced toxicity in aged yeast cells. Chronological life span of (A) non-CR (Fig. 30A), (B) CR and (C) CR-α-syn levels of stationary wild type cells expressing the vector control, α-syn WT, A53T or A30P mutant forms under the control of the constitutive *TPI1* promoter, a moderate toxicity model. The data represent mean ± SEM of three biological independent replicas. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (***p<0.001).

The analysis of the mean and maximum CLS of control cells and cells expressing the α-syn variants under CR intervention revealed a significant increase (about two folds) of mean and maximum CLS when compared with the same cells grown under 2% glucose (non-CR conditions) (Fig. 47). Summing up, our data showed that CR intervention alleviates the α-syn-induced toxicity, allowing an extension of CLS.

As above described, one of the physiological changes that underlie the chronological longevity promoted by CR is based on the stimulation of autophagy [364]. Thus, whether the capacity of CR to ameliorate the α-syn-induced toxicity is associated with the regulation of autophagy was investigated.

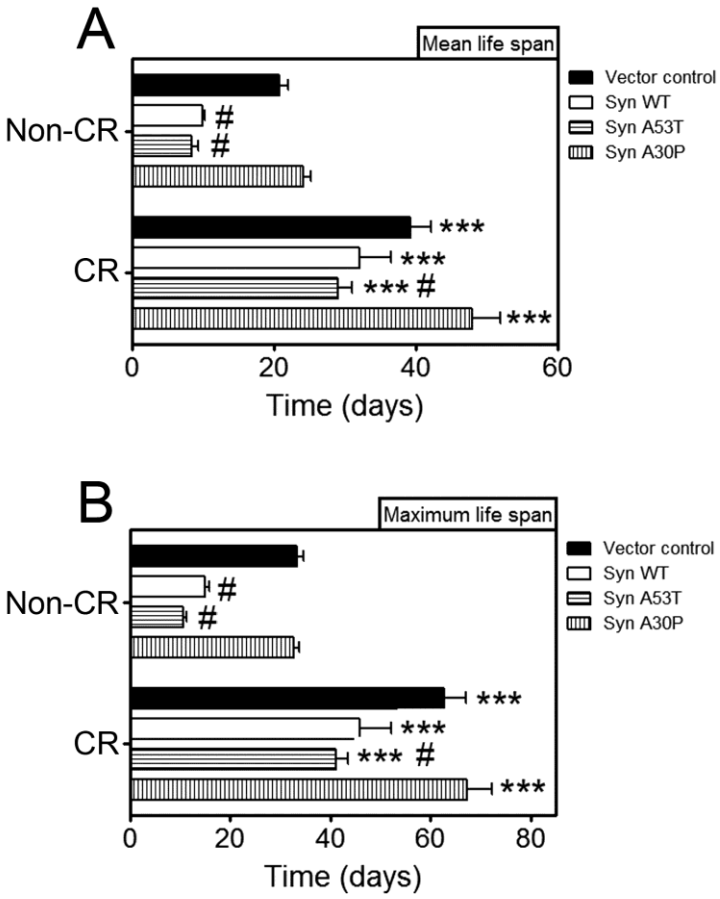


**Figure 47.** Caloric restriction (CR) increases mean and maximum CLS of wild type cells expressing α-syn toxic variants. (A) Mean (50% survival) and (B) maximum (10% survival) chronological life spans were determined from curve fitting of the survival data from (Figure 46A and B). Significance was determined between wild type cells grown under non-CR or CR conditions expressing vector control or α-syn variants (*).The significance determined between

cells expressing the vector control or α -syn variants within each condition (normal or CR conditions) was also determined (#). The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the chronological life span curves was determined by two-way ANOVA ($^{\#}p<0.001$; ***$p<0.001$).

The determination of autophagy activity, by the ALP assay (as already described), during CLS under CR conditions was performed. The data showed that CR statistically significantly stimulates autophagy in cells harboring the vector control in comparison with the same cells grown under non-CR conditions (Fig. 48A and C). The increase in autophagy activity of CR cell versus non-CR cells expressing vector control was found to be statistical significant by two-way ANOVA (**$p<0.01$; ***$p<0.001$; $^{\S}$ $p<0.001$). Furthermore, in cells harboring the vector control under CR, a regulated increased of the autophagic activity, which was maintained at similar levels during CLS, in comparison with the levels observed under non-CR conditions. The same profile was also observed for the mitophagy activity, nevertheless, the statistical significance induction was only observed at 10 days of CLS (Fig. 48B and D). The heterologous expression of α-syn WT or the A53T mutant form in wild type cells grown under CR conditions resulted in autophagy levels similar to the ones presented by the cells harboring the vector control, and significantly lower than the levels presented by the cells expressing the α-syn toxic variants when grown under non-CR conditions (Fig. 48A and C). The same results were observed for mitophagy activity in cells expressing α-syn WT or the A53T mutant form (Fig. 48B and D). The data suggests that the physiological CR intervention results in the induction of autophagy/mitophagy, but maintains the autophagy/mitophagy levels at a physiological threshold in cells expressing the toxic variants of α-syn.

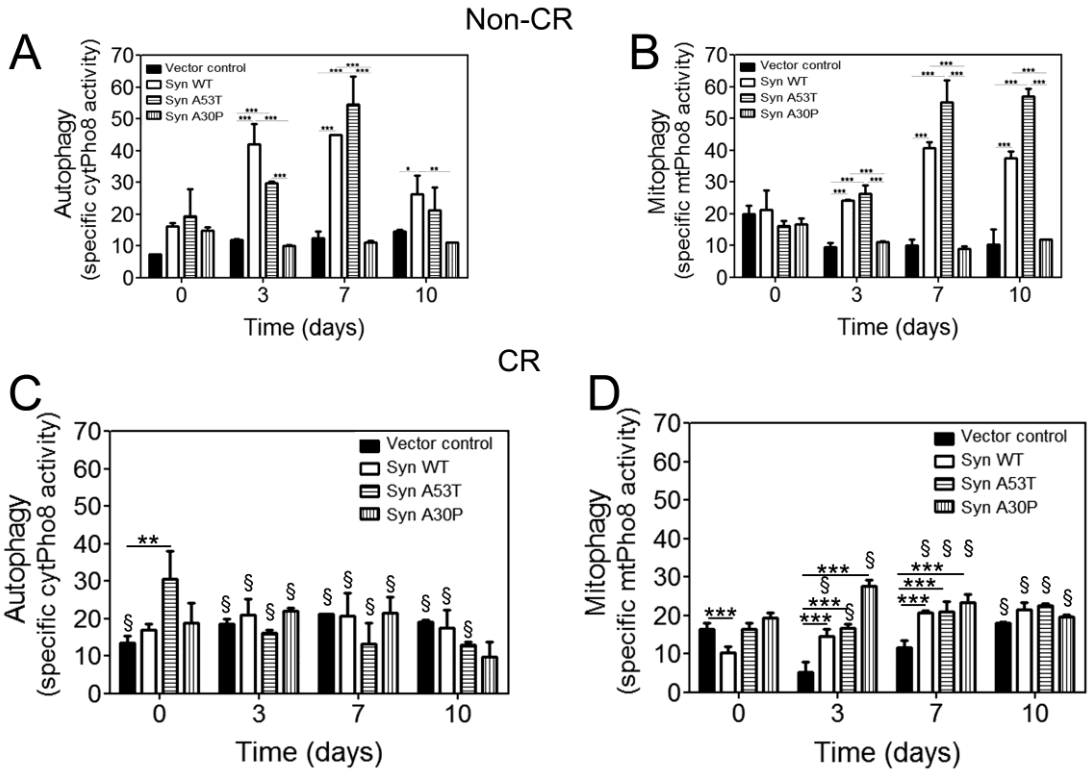


**Figure 48.** Caloric restriction (CR) regulates the autophagy and mitophagy activities. (A, C) Autophagic and (B, D) mitophagic activities were measured through the alkaline phosphatase (ALP) assay, during the chronological life span of wild type cells grown under non-CR (Fig. 31) and CR conditions, respectively. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance was determined between wild type cells expressing the vector control or α-syn variants within each growth condition (non-CR or CR) (*). The significance determined between wild type cells grown under non-CR or CR conditions expressing the vector control or α-syn variants (§). Significance of the data was determined by two-way ANOVA (**p<0.01; ***p<0.001; § p<0.001).

### 3.2.2 Caloric restriction increases chronological life span (CLS) of α-syn expressing cells independently of mitophagy

As described in previous sections, the expression of α-syn WT or the A53T mutant form induces an increase of autophagy/mitophagy, and apparently CR manipulation attenuates these activities to a more physiological threshold, avoiding the generation of autophagic stress. To further elucidate the effects of CR in preventing α-syn toxicity, we decided to evaluate if CR also extends the CLS of cells with impaired mitophagy, namely in *atg11Δ* and *atg32Δ* cells.

The CLS data demonstrated that *atg11Δ* cells harboring the vector control submitted to CR presented an extension of the longevity compared to the same cells grown in non-CR conditions (Fig. 49A and B). Expression of α-syn toxic variants in *atg11Δ* cells submitted to CR conditions resulted in CLS extension, either when compared to non-CR cells expressing the α-syn toxic variants (Fig 49A and B) or to non-CR cells harboring the vector control (Fig. 49B).

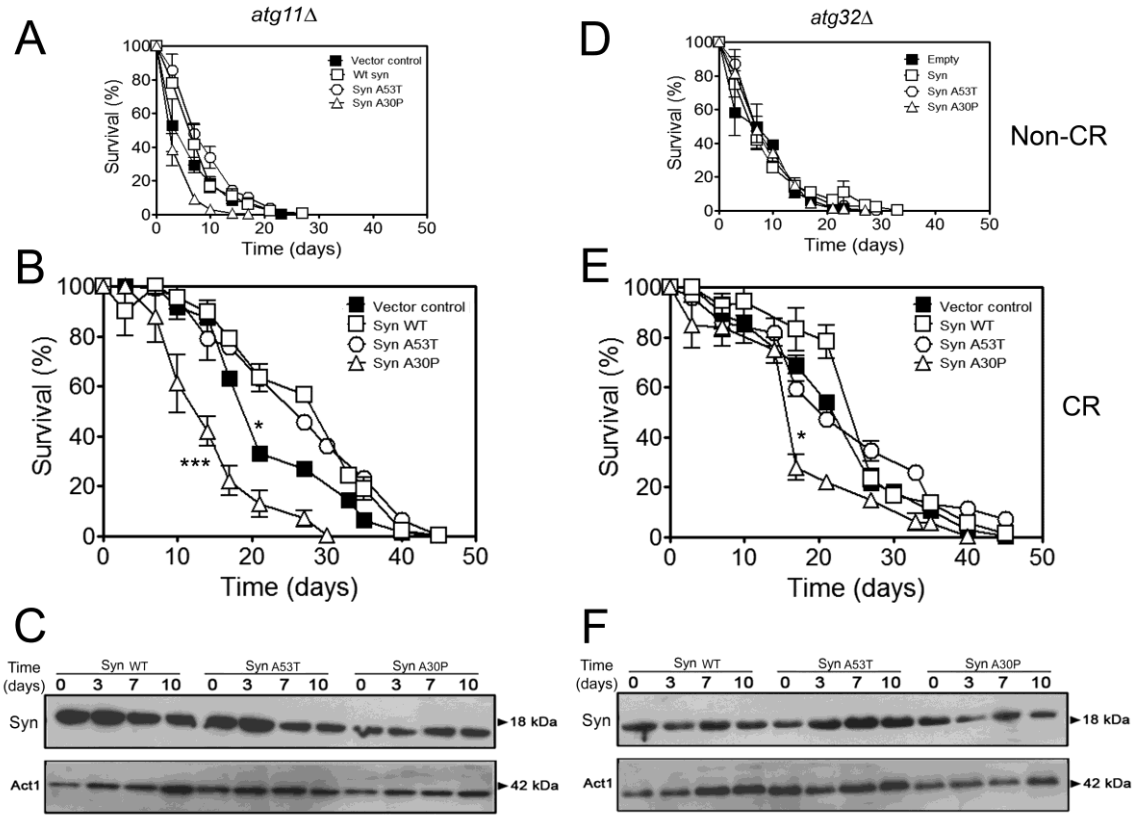


**Figure 49.** Caloric restriction (CR) promotes a CLS extension in cells with impaired mitophagy. Chronological life span of (A, D) non-CR (Fig. 32A and Fig. 33A), (B, E) CR interventions and (C, F) CR-α-syn levels of *atg11Δ* and *atg32Δ* cells, respectively, expressing the vector control, the α-syn WT, the A53T or the A30P mutant under the control of the constitutive *TPI1* promoter. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The data represents mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; ***p<0.001).

*ATG32* deleted cells harboring the vector control and under CR also displayed an extension of the chronological longevity, which was even higher in the cells expressing the toxic α-syn variants (Fig. 49D and E). Summing up, in all the studied conditions, the cells with impaired mitophagy submitted to CR revealed a chronological longevity extension, independently of the α-syn expression, in comparison to the same cells grown in non-CR conditions (Fig. 49A-F).

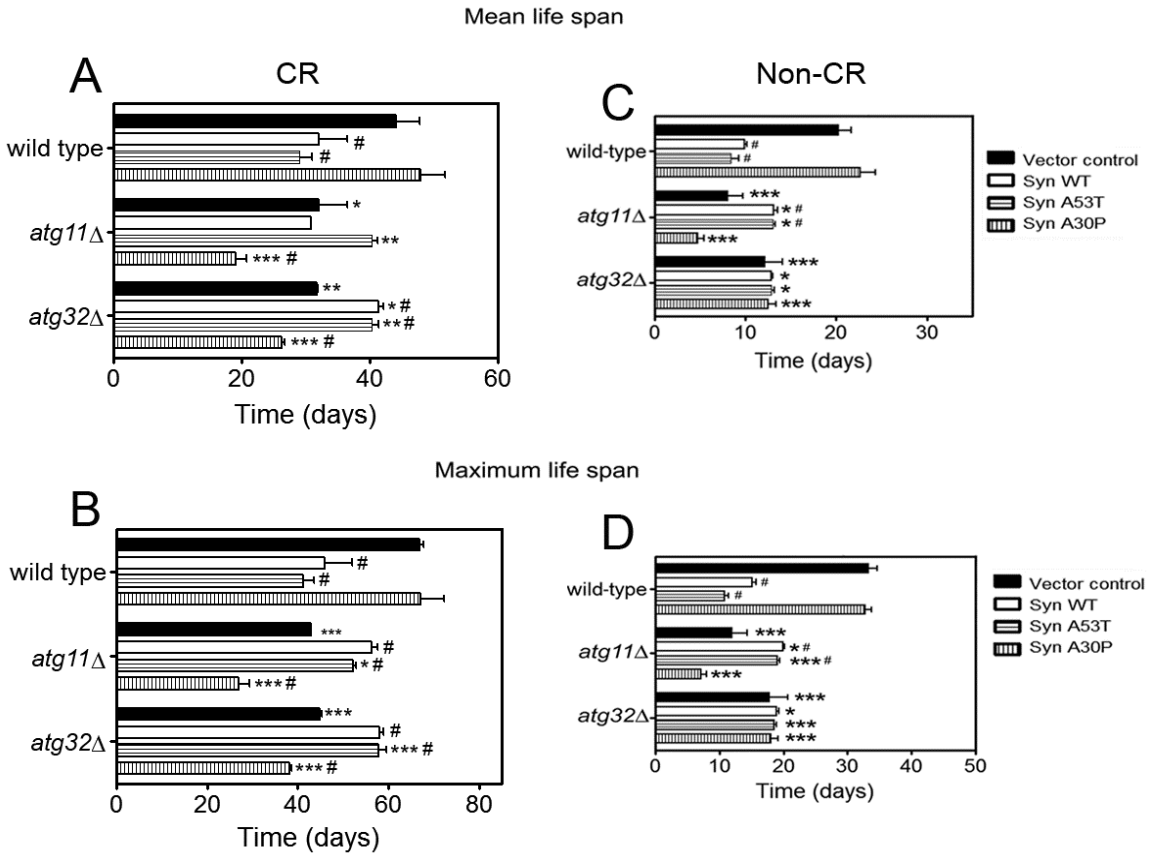


**Figure 50.** Caloric restriction (CR) increases mean and maximum CLS of cells with impaired mitophagy. (A, C) Mean (50% survival) and (B, D) maximum (10% survival) chronological life spans were determined from curve fitting of the survival data (Figure 46 and 49) of cells gown under CR or non-CR (Fig. 34) conditions, respectively (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between wild type cells and *atg11Δ* or *atg32Δ* cells expressing vector control or α-syn variants (*). The significance determined between cells expressing the vector control or α-syn variants within each strain (wild type, *atg11Δ* or *atg32Δ*) was also determined (#). (#p<0.001; *p<0.05; **p<0.01; ***p<0.001).

The determination of mean and maximum CLS also supports the CLS extension and the loss of toxicity by the expression of α-syn WT or the A53T in cells where mitophagy was abrogated and submitted to CR. The generated data showed that CR promoted a statistically significant increase of the mean and maximum CLS of wild type cells, *atg11Δ* and *atg32Δ* cells expressing the α-syn variants when compared with cells cultivated under non-CR conditions (Fig. 50). The CR was unable to overpass the decreased longevity experienced by the *atg11Δ* cells expressing the non-toxic α-syn A30P, although the cells grown under CR presented a slight extension of CLS (Fig. 50).

Given the previous results showing that CR mediates CLS extension of cells expressing α-syn toxic variants by regulating autophagy/mitophagy to physiological levels below the ones observed in non-CR cells, we decided to evaluate autophagy/mitophagy activities in the *ATG11* and *ATG32* mutant cells grown under CR conditions (Fig. 51). The ALP assay indicated that the mutant cells display autophagy/mitophagy levels similar to the ones presented by wild type cells under CR conditions (Fig. 48), although, as expected, the *atg32Δ* cells presented lower mitophagy levels (Fig. 51D). This data showed that CR-mediated life span extension of cells expressing α-syn toxic variants is independent of selective autophagy, particularly mitophagy.

The data presented in this section indicates that in cells with impaired mitophagy expressing the α-syn toxic variants, CR increases CLS by pathways independent, or at least partially independent, of autophagy/mitophagy. Nevertheless, CR is also able to regulate autophagy/mitophagy, and consequently this regulation is ameliorating the α-syn-induced toxicity as demonstrated in wild type cells subjected to CR conditions.

CR promotion of longevity extension is associated with decreased mitochondrial free radical generation and Sir2 activation, however the role of sir2 in CLS is still matter of debate [365]. In fact, during yeast replicative life span (RLS), CR induces the activation of Sir2 leading to a RLS extension. In contrast, during chronological longevity, overexpression or deletion of *SIR2* has no effect on the CLS, except when deleted cells are submitted to severe CR, such as incubation in water [257]. Given the identified role of Sir2 on autophagy/mitophagy regulation under proteotoxic stress created by α-syn expression, we decided to evaluate the contributions of ROS accumulation and Sir2 in the CR amelioration of α-syn-induced toxicity, during CLS.

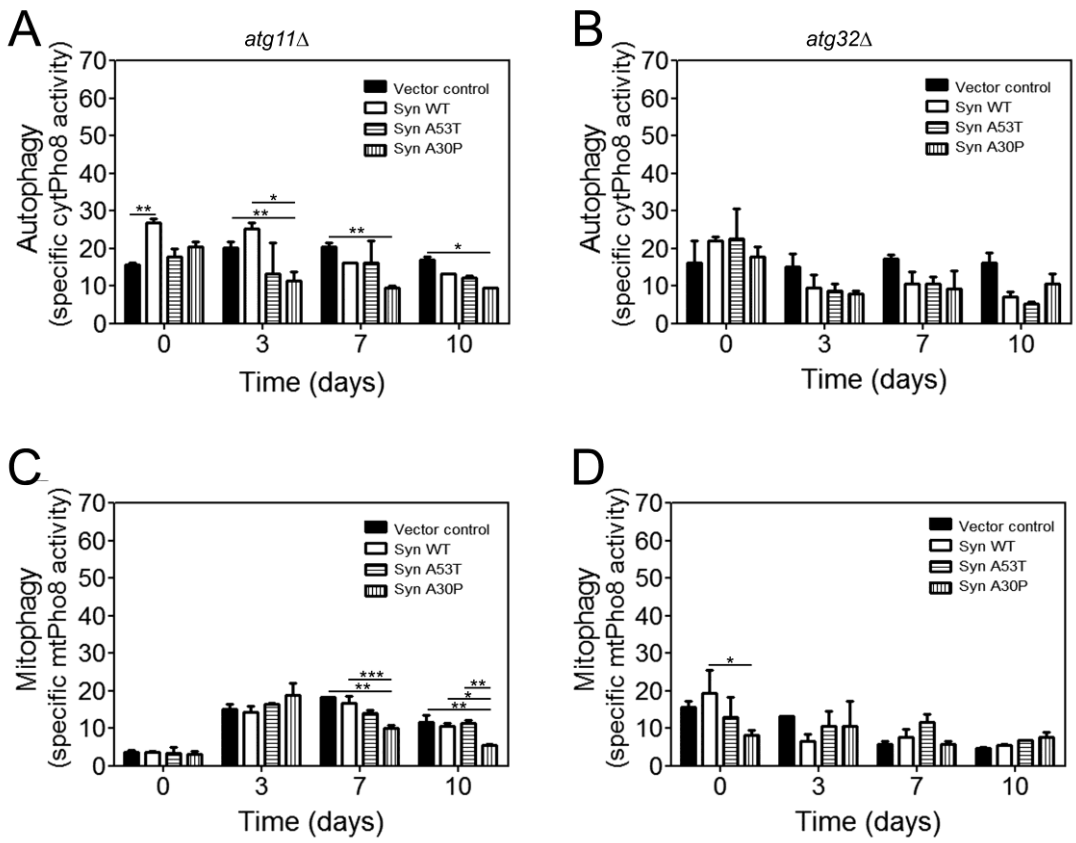


**Figure 51.** Caloric restriction (CR) does not affect autophagy and mitophagy activities in aged *atg11Δ* or *atg32Δ* cells under α-syn-induced toxicity. Autophagy and mitophagy activities were measured through the alkaline phosphatase (ALP) assay that was carried out to assess (A and B) autophagy or (C and D) mitophagy in *atg11Δ* or *atg32Δ* cells, respectively, grown under CR conditions. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p<0.05; **p<0.01; ***p<0.001).

### 3.2.3 Caloric restriction regulates superoxide anion levels in aged yeast cells expressing α-syn toxic variants

As above described, in an attempt of understand the CR extension of cells under conditions of α-syn-induced toxicity, the ROS levels were measured [358]. The accumulation of superoxide anion ($O_2^{2-}$) and total ROS levels during CLS was evaluated by flow cytometry, using the fluorescent probes DHE and DHR, respectively. As expected, the flow cytometry analysis revealed that CR resulted in the decrease of the $O_2^{2-}$ levels in wild type cells harboring the vector control (Fig. 52A). The same phenotype was observed in cells expressing the toxic α-syn variants,

since these cells displayed a significant decrease in the DHE fluorescence intensity over time, as compared to the wild type cells under non-CR conditions (Fig. 52A shadow bars versus bars and Fig. 39A). Conversely, the expression of α-syn WT or A53T in cells lacking either the *ATG11* or *ATG32* genes grown under CR conditions (Fig. 52B and C shadow bars versus bars) presented similar levels of $O_2^{2-}$ in comparison with the wild type cells in same conditions (Fig. 37A-C). Nevertheless, those mutant cells showed a lower DHE fluorescence intensity over time in comparison with the same cells submitted to non-CR conditions (Fig. 52B and C shadow bars versus bars and Fig. 39B and C). Striking, expression of the non-toxic α-syn A30P mutant form led to increased accumulation of $O_2^{2-}$ levels in *ATG11* mutant cells (Fig. 52B shadow bars versus bars), consistently with the shorter CLS observed even under non-CR conditions (Fig. 49B).

The analysis of the accumulation of total ROS levels, particularly $H_2O_2$, showed that wild type cells (Fig. 53A bars versus non-shadow bars) displayed higher total ROS levels in comparison with the *atg11Δ* and *atg32Δ* cells (Fig. 53B and C bars versus non-shadow bars), independently of the expression of toxic α-syn variants. In addition, the total ROS levels under CR conditions were higher than under non-CR conditions (Fig. 52 bars versus non-shadow bars and Fig. 37D-F). This data is in agreement with our previous report study on the pro-longevity effects of $H_2O_2$ [358]. Thus under CR conditions the mutant cells presented similar levels of ROS independently of the proteotoxic stress promoted by the expression of α-syn WT or A53T (Fig. 53B and C bars versus non-shadow bars).

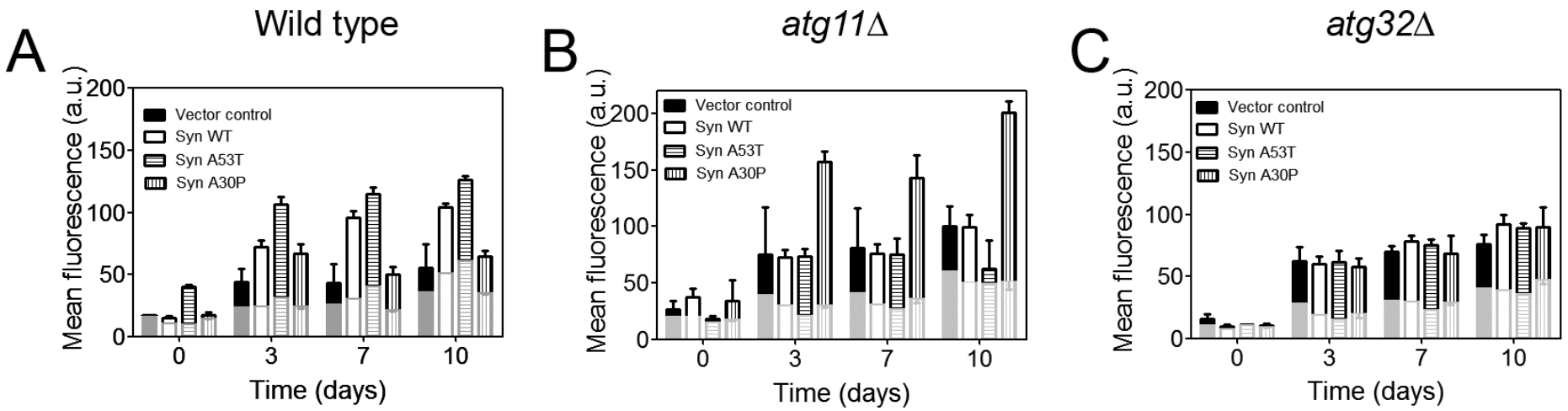


**Figure 52.** Caloric restriction (CR) decreases $O_2^{2-}$ accumulation. During chronological life span of (A) wild type, (B) *atg11Δ* and (C) *atg32Δ* cells, expressing the vector control, the α-syn WT, the A53T or the A30P mutant forms under the control of the constitutive *TPI1* promoter. The accumulation of superoxide anion was evaluated by flow cytometry, using the fluorescent probe dihydroethidium. Shadows on the bars represent the values obtained for cells submitted to CR conditions. Bars represent cells submitted to non-CR conditions. The error bars represent the standard error of the mean (SEM). Values indicate mean ± SEM from three independent experiments. Significance of the values between yeast strains only submitted to CR conditions was determined by two-way ANOVA.

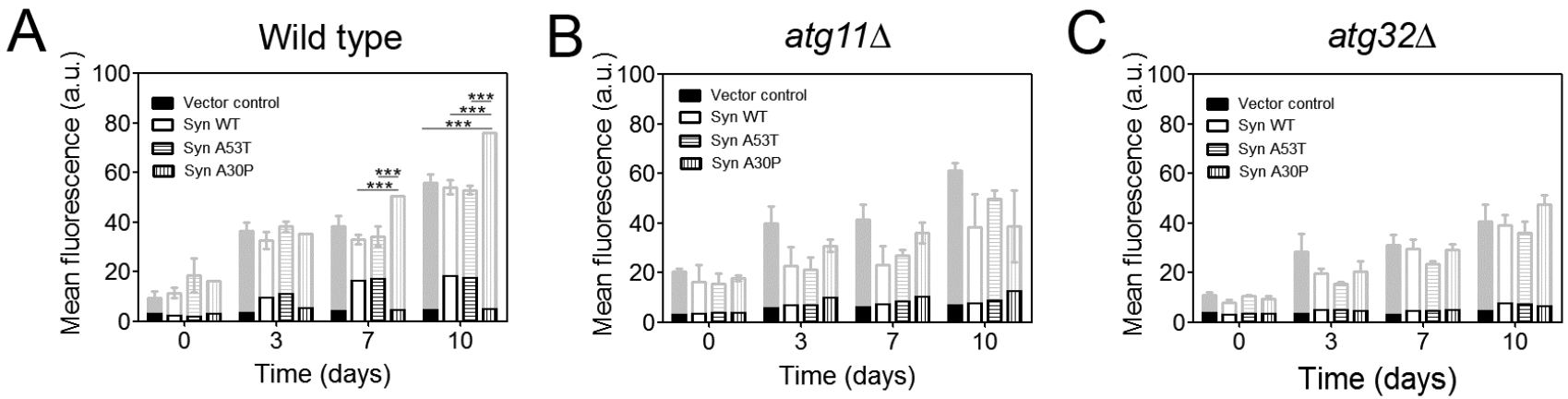


**Figure 53.** Caloric restriction (CR) increases accumulation of total ROS levels. During chronological life span of (A) wild type, (B) *atg11Δ* and (C) *atg32Δ* cells, expressing the vector control, the α-syn WT, the A53T or the A30P mutant forms under the control of the constitutive *TPI1* promoter. The accumulation of total ROS levels, particularly hydrogen peroxide, was evaluated by flow cytometry, using the fluorescent probe dihydrorhodamine 123 (DHR). Bars represent cells submitted to CR conditions. Non-shadow bars represent the cells submitted to non-CR conditions. The error bars represent the standard error of the mean (SEM). Values indicate mean ± SEM from three independent experiments. Significance of the values between yeast strains only submitted to CR conditions was determined by two-way ANOVA.

α-Syn toxic variants expression triggers intracellular accumulation of ROS and mitochondrial dysfunction in yeast [319]. Nevertheless, under CR conditions a decrease in the accumulation of $O_2^{2-}$ associated with the regulation/decrease of autophagy/mitophagy activity and lack of α-syn toxicity was observed. Furthermore, the data showed that even under proteotoxic stress, CR is able to activate a pathway dependent on $H_2O_2$ and independent of Rim 15 [358, 366]. These conclusions are supported by the increase DHR fluorescence levels of CR cells (Fig. 53).

### 3.2.4 Caloric restriction (CR)-increased chronological life span (CLS) extension is independent of Sir2 under proteotoxic stress

Given the results obtained (section 3.1) concerning the crucial role of Sir2 on the transcriptional regulation of autophagy, particularly mitophagy in cells expressing toxic α-syn variants under non-CR conditions, we decided to evaluate if the same mechanism is operating under CR conditions. Furthermore, we wondered if the regulation/decrease of autophagy/mitophagy observed in wild type cells expressing the toxic α-syn variants under CR conditions was due to Sir2 functions. For that, CLS of *sir2Δ* cells submitted to CR was evaluated and compared with wild type cells submitted to CR and non-CR conditions.

*SIR2* deleted cells harboring the vector control cultivated in CR conditions presented a CLS extension in comparison with the same cells grown in non-CR conditions (Fig. 54). The same phenotype was observed for the *sir2Δ* cells expressing the α-syn variants. Thus, CR intervention increased the CLS of cells expressing α-syn independently of Sir2 (Fig. 54). Furthermore, the determination of mean and maximum CLS demonstrated that *sir2Δ* cells expressing the toxic α-syn variants, WT or mutant A53T form, grown under CR conditions presented an increased mean and maximum CLS in comparison with the wild type cells expressing the same variants and submitted to the same conditions (Fig. 55). In contrast, *sir2Δ* cells harboring the vector control showed a similar CLS CR-dependent extension to the one displayed by wild type cells (Fig. 55). These results indicate that CR is still able to increase the CLS of the long-lived *sir2Δ* cells under proteotoxic stress (Fig. 55).

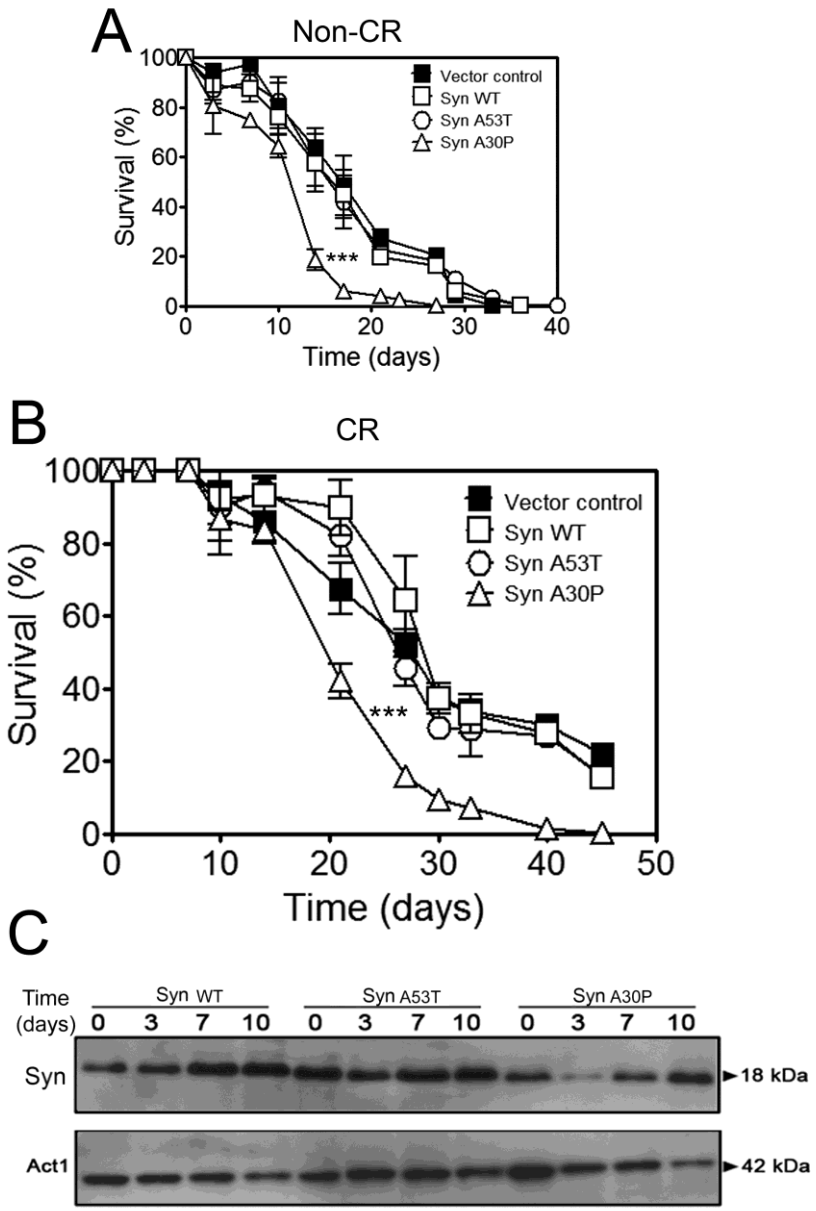


**Figure 54.** Caloric restriction (CR) increases CLS of *sir2Δ* cells expressing toxic α-syn variants. Chronological life span (A) under non-CR (Fig. 41A) and (B) CR conditions and (C) CR-α-syn levels of *sir2Δ* cells expressing the vector control, the α-syn WT, A53T or A30P mutant forms under the control of the constitutive *TPI1* promoter. Cell viability was measured at 2-3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (***p<0.001).

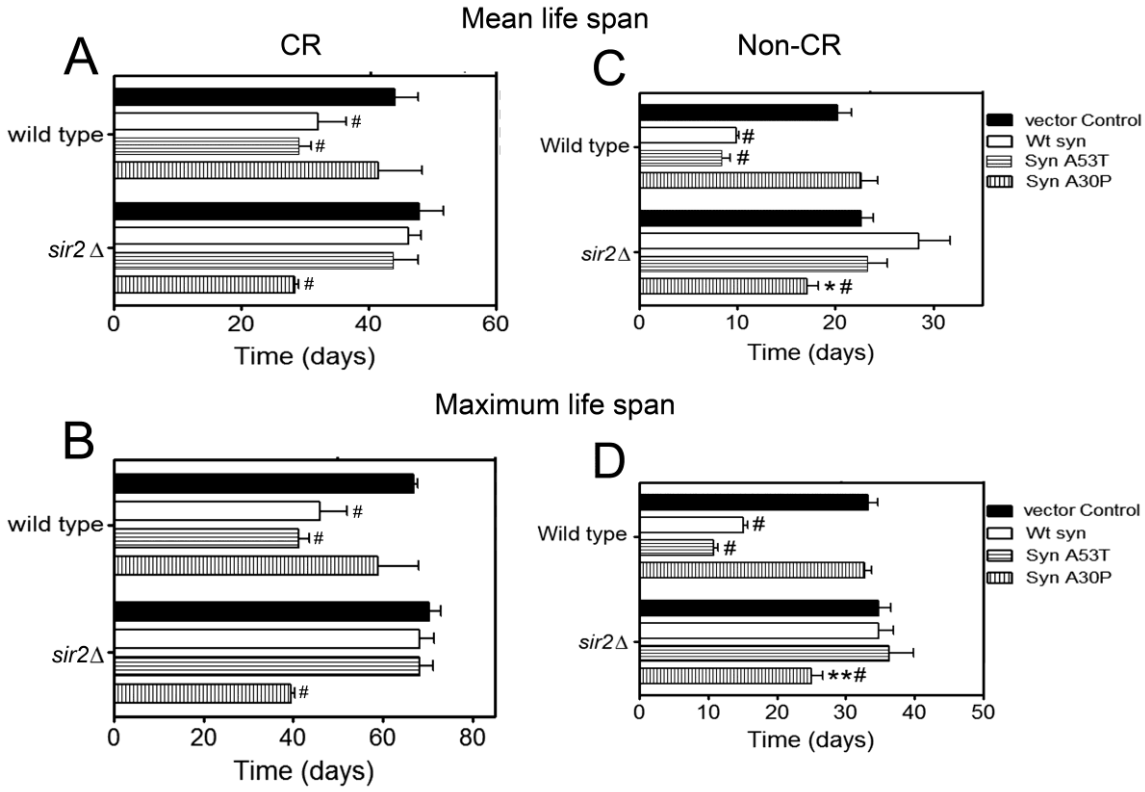


**Figure 55.** Caloric restriction (CR) increases the mean and maximum life span of *sir2Δ* cells expressing toxic α-syn variants. (A, C) Mean (50% survival) and (B, D) maximum (10% survival) chronological life spans of cells grown under CR or non-CR (Fig. 42) conditions, respectively, were determined from curve fitting of the survival data (Figure 46B and 54B) (from pair matched, pooled experiments) with the statistical software Prism (GraphPad Software). Significance was determined between wild type cells and *sir2Δ* cells expressing vector control or α-syn variants (*). The significance determined between cells expressing the vector control or α-syn variants within each strain (wild type or *sir2Δ*) was also determined (#) (#p<0.001; ** p<0.01).

As described in section 3.1, Sir2 is regulating autophagy and mitophagy by the transcriptional control of the *ATG8*, and particularly *ATG32* mRNA levels, in cells expressing the α-syn toxic variants. Thus, given the Sir2 increasing of autophagy/mitophagy activity in cells expressing the α-syn variants under non-CR conditions, we wondered if CR is also able to regulate the autophagy/mitophagy activity in cells where Sir2 is abolished. The data demonstrated that *SIR2* deletion statistically significantly reduces the autophagy and mitophagy activity in cells expressing α-syn toxic variants and also in the cells harboring the vector control (Fig. 56) in comparison with the wild type cells also grown under CR conditions (Fig. 48). Furthermore the autophagy and mitophagy levels were similar to the ones presented by the *SIR2Δ* cells grown

under non-CR conditions (Fig. 56). In these sense, the data suggests that CR is not promoting life span extension of *sir2Δ* cells by the modulation of autophagy/mitophagy.

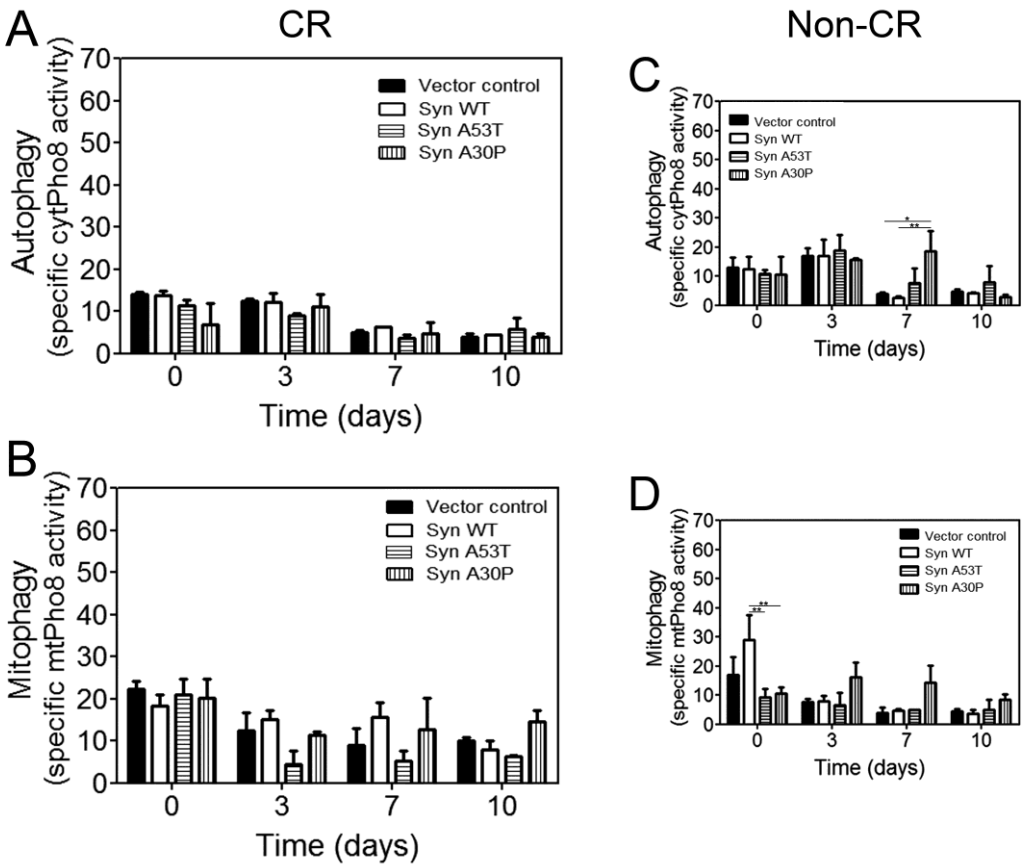


**Figure 56.** CR is not promoting life span extension of *sir2Δ* cells by the modulation of autophagy/mitophagy. (A, C) Autophagy and (B, D) mitophagy activity were measured through the alkaline phosphatase (ALP) assay in *sir2Δ* cells grown under CR or non-CR (Fig. 43) conditions, respectively. The data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA.

To complete this study further work needs to be performed such as the evaluation of the *ATG8* and *ATG32* mRNA levels to determine if CR is transcriptionally regulating autophagy/mitophagy and consequently alleviating α-syn toxicity. Additionally, to define the possible $O_2^{2-}$ involvement in the abolishment of α-syn toxicity under CR, the SODs activity must be also monitored. Furthermore, the evaluation of α-syn foci under CR intervention needs also to be assessed in an attempt to define the foci role in the α-syn toxicity.

Summing up, our data demonstrated that CR is associated with the maintenance of autophagy/mitophagy that consequently ameliorates the toxicity induced by α-syn toxic variants. Nevertheless the CR-mediated extension of CLS in cells expressing the α-syn toxic variants is independent of mitophagy and Sir2.

# CHAPTER 4

# CONCLUDING REMARKS AND FUTURE PROSPECTIVES

It has long been puzzling why protein aggregation and neurotoxicity develop late in life, even in familial forms of neurodegenerative disease where mutant proteins are present throughout life. In this context, aging is, by far, the strongest risk factor associated with the late-onset diseases. Indeed, aging is associated with a general and progressive decline of fundamental cellular housekeeping processes that are crucial for maintaining cellular homeostasis, such as autophagy [367, 368], which contributes to the accumulation of misfolded proteins [369], particularly in post-mitotic cells. In fact, the genetic and pharmacological inhibition or enhancement of autophagy is respectively correlated to decreased or increased longevity [345]. Moreover, the vast majority of interventions found to increase longevity are autophagy-dependent, supporting the crucial cytoprotective role of autophagy and the tight relation between autophagy and aging. To address the interplay between autophagy and aging under a proteotoxic context, the yeast *Saccharomyces cerevisiae* heterologous expressing human α-synuclein (α-syn) variants was used [290]. The yeast is a powerful model for investigating the cellular and molecular basis of α-syn toxicity and has several advantages such as the well characterized autophagy machinery and the fact of being an established system for aging research [370]. Herein, data is presented showing that the expression of α-syn WT or A53T mutant form in yeast cells results in shortening of chronological life span and increased α-syn toxicity. Furthermore, aging proved to be a major risk factor associated with α-syn toxicity, since the harmful phenotype was more pronounced when α-syn was expressed in stationary phase (aged) cells. These observations were made by using three distinct yeast models with different toxicity levels, from low to high toxicity, which highlighted that the modulation of the toxicity induced by α-syn depends on the physiological state of the cells.

The accumulation of misfolded proteins in the cells triggers the activation of the cellular protein quality control system. One of the major pathways of this system is the unfolded protein response (UPR). Our findings demonstrate that a sustained UPR activation is being elicited by the heterologous expression of the toxic α-syn variants. In yeast the UPR is mediated by the Ire1-Hac1 signaling pathway, which is ultimately responsible for the activation of several genes associated with the degradation of the misfolded proteins, particularly the *ATG* genes, like *ATG5, ATG7, ATG8*, *ATG14* and *ATG19* [194, 195]. Mounting evidence established that when UPR is unable to restore the endoplasmic reticulum homeostasis, the misfolded proteins are driven to proteolytic degradation, particularly autophagy [37]. Our findings confirmed that the UPR

activation was accompanied with the upregulation of at least two autophagic genes, *ATG6*, which participates in the formation of the autophagosome [54], and *ATG8*, which codifies for a protein that is predominantly associated with the autophagosome structure [118], suggesting that autophagy plays an important role in the α-syn-induced toxicity in yeast aged cells. Our data obtained by pharmacological manipulation of autophagy confirmed the association between autophagy induction and α-syn toxicity. The pharmacological inhibition of autophagy, with chloroquine or 3-methyladenine, rescues aged yeast cells from α-syn-induced toxicity. The pharmacological induction of autophagy with rapamycin also resulted in a slight reduction of the α-syn-induced toxicity. Nevertheless, this autophagy induction is mediated by the inhibition of TOR pathway that participates in a wide range of cellular response, namely proteins synthesis and cellular metabolism [259], and in yeast, its pharmacological or genetic abrogation is always associated with a strong chronological life span extension [244]. Thus, further conclusions cannot be drawn by the experiments with rapamycin.

Autophagy is a process that is present at basal levels, contributing to the turnover of cytoplasmic components and crucial to maintain cell homeostasis, particularly important in post-mitotic cells, such as neurons, since aged cells do not have the capacity to get rid of their waste through cell division [226, 227]. However, an imbalance on autophagy levels, either too much or too little, can be injurious to the cells [230]. Our findings confirmed that cells expressing PD-causing variants of α-syn displayed increased autophagic activity, which strongly supports our hypothesis and establishes an association between increased autophagic activity and α-syn-induced toxicity in aged cells. Notably, it was demonstrated that α-syn expression in mammalian cells impairs chaperone-mediated autophagy and induces the upregulation of autophagy (macroautophagy), which seems to contribute to neuronal cell death [299]. Although increased autophagic activity can mediate α-syn clearance in functionally competent cells [371] and thereby have anti-aging effects [372], it might also affect autophagy efficiency and selectivity in aged cells [373]. The controversial role of autophagy is also associated with oxidative stress and mitochondrial dysfunction, which play a central role in α-syn-induced toxicity [317, 319]. Although the relevance of mitophagy, induced by mitochondria-damaging agents, for the etiology of PD remains debatable [353], accumulating evidence on the key role of the PD-associated proteins PINK1 and PARK2, in targeting specific dysfunctional mitochondria for degradation by mitophagy [373], supports the interconnection of these cellular processes and their involvement

in the pathogenesis of PD. Based on those findings, we wondered if dysfunctional mitochondria were being targeted for degradation by the autophagic machinery. The performed gene analysis also demonstrated that genes specifically associated with mitophagy, *ATG32* and *ATG33*, were upregulated under α-syn-induced toxicity. Additionally, the *DNM1* gene, which codifies to a mitochondrial dynamic-related GTPase required for mitochondrial fission, that is through to be necessary for mitophagy [340], was also found overexpressed suggesting that probably before be engulfed by the autophagosome, mitochondria undergo fission to prepare them to fit into autophagosome [161]. Further findings showed that α-syn expression induces mitophagy and that deletion of *ATG11* or *ATG32*, both required for mitophagy, prevents α-syn-induced toxicity as revealed by the increased mean and maximum chronological life spans. A precondition associated with the α-syn-induced toxicity is the presence of functional mitochondria, as demonstrated by the resistance of *rho* 0 cells (lacking mitochondrial DNA) to α-syn-induced toxicity [319]. Our data showed that the decreased α-syn toxicity observed in cells with an impairment of mitophagy was not due to an inherent mitochondria dysfunction of *ATG11* or *ATG32* mutant cells, although these mutant cells display mitochondria dysfunction when subjected to severe nitrogen starvation [374].

To elucidate the pathways/molecules associated with α-syn-induced autophagy/mitophagy activation during aging, reactive oxygen species (ROS) were investigated. ROS, particularly superoxide anion, are crucial signaling molecules implicated in the control and regulation of autophagy and aging progression. Data herein presented suggests that the sustained superoxide dismutase (SOD) activities observed over CLS in *atg11Δ* or *atg32Δ* mutant cells could be the mechanism behind the decreased superoxide anion levels displayed by these cells. In fact, under certain scenarios, it is described that the $H_2O_2$ detoxifying enzymes, catalase, are degraded by autophagy, suggesting that the specific removal of catalases by autophagy creates a loop, in which the increase in the $H_2O_2$ production further stimulates an autophagy overactivation culminating in cell death [225]. In this sense, it is possible that the expression of α-syn induces autophagy that will degrade the superoxide anion detoxifying enzymes, SODs, since we found that wild type cells expressing the toxic α-syn variants display a decrease in the SODs activity. Thus, this event leads to an enhancement of the superoxide anion accumulation that consequently upregulates the autophagic activity, contributing to the cell death due to α-syn. Contrariwise, the mutant *ATG11* and *ATG32* cells expressing the toxic α-syn variants experience a

reduction in the autophagic/mitophagic activity that consequently will neither induce the SODs degradation nor the superoxide anion accumulation. Therefore, our results do not rule out superoxide anions as mediators of autophagy/mitophagy due to α-syn accumulation [360] and aging [375].

Mammalian SIRT1 was recently described as an inducer of autophagy under starvation conditions [356]. Here, we investigated such a role for the yeast homologue, Sir2. Our findings revealed that deletion of the *SIR2* gene not only alleviated α-syn toxicity, as previously described [320], and as evidenced by the increased CLS, but also that this phenomenon is linked to a drastic inhibition of autophagy and mitophagy as demonstrated by the ALP assay. Notably, Sir2 was shown to be essential for the regulation of *ATG8*, and particularly, *ATG32* mRNA levels in stationary phase cells expressing α-syn toxic variants. Our findings further reveal that autophagy and mitophagy are mainly regulated through Sir2, in agreement with the role that alterations on the acetylproteome have on the regulation of autophagy [203, 255].

It is well established that caloric restriction (CR) is able to increase life span in multiple organisms including yeast, worm, flies and mammalian, nevertheless, the molecular mechanisms by which CR slows aging remains still poorly understood. CR promotes several physiological changes that may underlie its effects on longevity, including decreased activity of nutrient and growth factor pathways, increased resistance to stress, altered translation and ribosome biogenesis, and increased autophagy. In this context, CR has the capacity to delay aging and age-related neurodegenerative disorders. Our data suggests that CR ameliorates the α-syn-induced toxicity in aged cells. This is confirmed by the results obtained under CR conditions where the α-syn toxicity is reduced, resulting in a CLS extension that was accompanied by physiological levels of autophagy/mitophagy activity, preventing the generation of autophagic stress. CR-mediates CLS extension was also observed in cells with impaired mitophagy expressing α-syn, even when compared with the wild type cells submitted to the same conditions, suggesting that CR-mediated life span extension of cells expressing α-syn is independent, or at least partially independent, of selective autophagy, particularly mitophagy, pointing to other protective pathways elicited by CR. However, CR is able to regulate autophagy/mitophagy, and consequently this regulation ameliorates the α-syn-induced toxicity in wild type cells.

In contrast to non-CR conditions, CR intervention is linked to a decrease in the accumulation of superoxide anion that consequently is associated with the regulation/decrease of

autophagy/mitophagy activity and lack of α-syn toxicity. Furthermore, the data showed that even under proteotoxic stress, CR is able to activate a pathway involving $H_2O_2$ accumulation but independent of Rim 15 [358, 366], revealed by the increased DHR fluorescence levels of CR cells.

In yeast, it is well recognized that CR induces the activity of Sir2 and consequently extends the replicative life span [257]. It is also described that either overexpression or deletions of Sir2 do not result in CLS extension under moderated CR [257]. Our results contradict this observation, since in our experimental conditions with moderate CR, the abrogation of *SIR2* harboring the vector control resulted in higher CLS compared to the same cells submitted to non-CR conditions. Furthermore, under CR interventions the absence of *SIR2* in cells expressing α-syn toxic variants led to a higher CLS extension in comparison to the wild type cells submitted to the same conditions, suggesting that probably, under CR, Sir2 transcriptionally regulates autophagy and mitophagy by controlling the *ATG8*, and particularly *ATG32* mRNA levels. However, the further CLS extension observed in the absence of *SIR2* indicates that possibly CR is alleviating α-syn toxicity due to mechanisms independently of Sir2. To further understand the phenomenon of CR-alleviating of α-syn toxicity, additional work must be performed, like the evaluation of the *ATG8* and *ATG32* mRNA levels to determine if CR is transcriptionally regulating autophagy/mitophagy, the study of the possible $O_2^{2-}$ involvement in the regulation of autophagy/mitophagy under CR, by the SODs activity monitorization, and the evaluation of α-syn foci under CR intervention.

In summary, our data shows that increased autophagy/mitophagy, promoted by α-syn WT and A53T mutant, has a deleterious effect in yeast aged cells. This appears counterintuitive as autophagy and mitophagy are considered to represent pro-survival processes. Nevertheless, several explanations could be envisioned to explain how impaired mitophagy due to the presence of toxic α-syn may extend CLS. One possible explanation is that aged cells have a reduced ability to simultaneously upregulate anabolic processes in order to compensate for the loss of cellular material by increased mitophagy. Another conceivable hypothesis is that the selectivity of various types of autophagy, such as mitophagy, is lost and one or more factors, commonly required for various types of autophagy, may become limiting when mitophagy is upregulated. The loss of selectivity could also result in the degradation of functional competent mitochondria, crucial to sustain cell survival of post-mitotic cells, contributing to the toxicity observed.

Future studies are necessary to be done in an attempt to dissect how different pathways of cellular quality control system influence each other and how they are regulated in a coordinated manner.

## 5.1 Future prospectives

Autophagy is a highly conserved proteolytic mechanism of protein quality control system essential for the maintenance of metabolic and cellular homeostasis and for an efficient cellular response to stress. It declines with aging and is believed to contribute to diverse aspects of aging phenotype. The results presented in this thesis yield new insights into the role of autophagy/mitophagy in the α-syn-induced toxicity, but raised several question on the regulation of autophagy/mitophagy during aging.

One of the primordial events associated with aging is the DNA replication stress that is sensed by different signaling pathways and has the capacity to regulate different cellular processes, modulating the expression of diverse genes and ultimately impairing the cells entry into mitosis. DNA replication stress is also responsible for the induction of autophagy, however the mechanism(s) underlying this event remain poorly understood. The yeast sucrose non-fermenting protein (Snf1) kinase, the homologue of the mammalian AMP activated protein kinase (AMPK), functions as an energy sensor that is able to reprogram the cellular metabolism in response to DNA replication stress, in order to restore the normal energy levels essential to sustain cell metabolism and to support a stress response. In fact, Snf1 participates in the cell cycle metabolic checkpoint activated by DNA replication stress regulating the G1/S transition and also controls S-phase entrance by regulating *CLB5* transcription [8]. Notably, Snf1 activity increases in aged cells even when glucose is abundant. In contrast to AMPK, Snf1 is negatively regulated by TORC1, but Snf1 can positively regulate autophagy. The molecular mechanisms underlying Snf1 regulation of autophagy are still elusive, but appear to be partially dependent on the inactivation of TOR kinases and on the direct Atg1 phosphorylation. Accumulating evidence suggests a strong interconnection between DNA replication stress, autophagy and nutrient signaling pathways. This axis has strong impact on aging regulation and progression. In fact, it was demonstrated that low α-syn levels protect yeast cells from growth inhibition and ROS

accumulation induced by the presence of hydroxyurea, a DNA damage inducer [19]. Apparently, α-syn modulates S-phase checkpoint, by up-regulating histone acetylation and ribonucleotide reductase levels [19]. Furthermore, the proteotoxic stress is able to modulate autophagy during aging [20]. Thus, in this context, the chronological life span, cell cycle, autophagy, DNA damage and phosphorylation state of Snf1 pathway regulatory proteins can be assessed. In this sense, the expression of α-syn, condition with implications both in cell cycle progression and autophagy can be explored to further understand the coordinated regulation of DNA replication stress and autophagy during aging. The identification of the mechanisms underlying the connection between DNA replication stress and autophagy in the aging context and even under proteotoxic conditions is of great importance for the continuous expansion on the knowledge of the growth signaling, DNA replication stress and autophagy impact in aging, providing a new paradigm for investigating how these pathways contribute to aging and to age-related diseases.

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# ATTACHMENT

**Attachment I**

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# Yeast chronological lifespan and proteotoxic stress: is autophagy good or bad?

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## Abstract

**Autophagy, a highly conserved proteolytic mechanism of quality control, is essential for the maintenance of metabolic and cellular homoeostasis and for an efficient cellular response to stress. Autophagy declines with aging and is believed to contribute to different aspects of the aging phenotype. The nutrient-sensing pathways PKA (protein kinase A), Sch9 and TOR (target of rapamycin), involved in the regulation of yeast lifespan, also converge on a common targeted process: autophagy. The molecular mechanisms underlying the regulation of autophagy and aging by these signalling pathways in yeast, with special attention to the TOR pathway, are discussed in the present paper. The question of whether or not autophagy could contribute to yeast cell death occurring during CLS (chronological lifespan) is discussed in the light of our findings obtained after autophagy activation promoted by proteotoxic stress. Autophagy progressively increases in cells expressing the aggregation-prone protein $\alpha$-synuclein and seems to participate in the early cell death and shortening of CLS under these conditions, highlighting that autophagic activity should be maintained below physiological levels to exert its promising anti-aging effects.**

## Introduction

Macroautophagy, hereafter autophagy, is the main proteolytic cellular system that guarantees the quality of proteins and organelles through their sequestration within double-membrane autophagosomes that are delivered to lysosomes for digestion [1]. As a result, the indispensable recycled components can be reprocessed not only for the synthesis of new macromolecules, but also as a cellular energy source, attributing an imperative role to autophagy in response to nutrient starvation or under specific physiological conditions requiring extensive cellular remodelling such as differentiation, development and homoeostasis (reviewed in [1–3]). However, an excessive activation of autophagy might result in the abolishment of its cytoprotective function and its enrolment in a self-destructive process [4].

Accumulating evidence suggests that different signalling pathways regulating aging converge on autophagy, and recent evidence obtained in multiple species ranging from invertebrates to mouse models reveals an interplay between autophagy activity and long-lived phenotypes (reviewed in [3,5]). However, several pieces of the interplay between autophagy and aging are poorly understood, and more detailed studies are needed in order to answer several key questions and to avoid generalization and oversimplifications made in the light of findings obtained in particular contexts. The yeast *Saccharomyces cerevisiae* CLS (chronological lifespan) has emerged as a powerful tool to study the connections between signalling pathways, autophagy and aging [5–7] in post-mitotic cells where failure of quality control systems such as autophagy is particularly detrimental [8,9]. The present review focuses on the regulation of autophagy by nutrient-sensing pathways in yeast, especially the most explored one, the TOR (target of rapamycin) pathway. The role of the signalling pathways in autophagy and aging will be discussed and the interventions known to promote longevity extension dependent on autophagy will be highlighted. The pro-survival and pro-death effects of autophagy will be addressed by data obtained during yeast CLS under proteotoxic stress, a condition known to induce activation of autophagy.

## Regulation of autophagy and aging

Aging is associated with a general and progressive decline of cellular processes that ultimately culminate in cell death. The age-dependent decrease in the activity of quality control mechanisms such as autophagy is believed to be the main cause of the accumulation of dysfunctional organelles and damaged molecules, particularly critical in post-mitotic cells [8,9]. The genetic and pharmacological inhibition or enhancement of autophagy is respectively correlated to decreased or increased longevity. Moreover, the vast majority of interventions found to increase longevity are autophagy dependent, supporting the crucial cytoprotective role of autophagy and the close relation between autophagy and aging.

In yeast, abrogation of the conserved TOR, Ras/cAMP-dependent protein kinase [or PKA (protein kinase A)] or Sch9 proteins, which integrate the network of

**Key words:** autophagy, chronological aging, proteotoxic stress, Ras/cAMP-dependent protein kinase (protein kinase A), Sch9, target of rapamycin (TOR).

**Abbreviations used:** ALP, alkaline phosphatase; Atg, autophagy-related gene; CLS, chronological lifespan; CR, caloric restriction; PKA, protein kinase A; TOR, target of rapamycin; TORC1, TOR complex 1.

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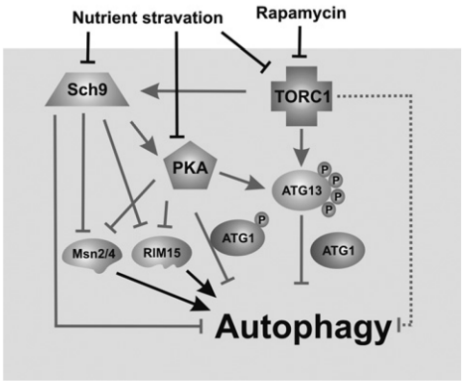
nutrient-sensing pathways, is known to promote longevity [6,10–12]. These signalling pathways are negative regulators of autophagy, reinforcing that autophagy and aging are co-ordinately regulated by a complex network of different signalling pathways, with partial overlapping branches and not yet disclosed hierarchical connections.

As in various species [13–17], in yeast, accumulating evidence has disclosed mechanisms that establish the direct regulation of autophagy by the TOR pathway, especially TORC1 (TOR complex 1). TORC1 negatively regulates autophagy by a direct targeting of ATG (autophagy-related gene) proteins or indirectly by still elusive mechanisms, such as transcriptional and translational control or through interactions with proteins that further regulate autophagy players (Figure 1; reviewed in [18]). In yeast, the transcriptional control of autophagy relies mostly on the regulation of the autophagy-related genes *ATG8* and *ATG14* [19–21]. The levels of *ATG14*, controlled by Gln3, could reach more than 20-fold in nitrogen starvation conditions [21]. The Atg8 protein increases 10–20-fold after induction of autophagy by starvation conditions [22]. Nevertheless, the transcription factor(s) that regulate the expression levels of the *ATG8* gene remain elusive. The transcriptional regulation of other *ATG* genes by starvation conditions was also described in [23], but to reveal the relevance of autophagy genetic control, further studies are still needed. On the other hand, the induction of autophagy in yeast is initiated by the activation of Atg1 kinase complex that includes Atg13 and the sub-complex formed by Atg17, Atg31 and Atg29 [24]. TORC1 affects the formation of the Atg1 complex by inducing hyperphosphorylation of Atg13, decreasing its affinity for Atg1 and consequently hampering the activation of the first step of the autophagy pathway. In contrast, TORC1 signalling inhibition results in partial dephosphorylation of Atg13, which allows its binding to Atg1 [25]. Atg13 phosphorylation is believed to be a crucial step in the activation of Atg1 complexes given the ability that a non-phosphorylable Atg13 form has to induce autophagy independent of TORC1 regulation [26]. In yeast, TORC1 also inhibits autophagy by regulating the phosphorylation of several proteins required for autophagy via Tap42 and PP2A (protein phosphatase 2A) [27].

Modulators of the TOR pathway have also been used as forefront evidence supporting the relation between signalling of autophagy and aging. Rapamycin and the so-called rapalogues are the most effective inducers of autophagy dependent on the TOR pathway and are emerging as potential enhancers of health and lifespan. TOR signalling inhibition by rapamycin was shown to up-regulate autophagy in multiple species (reviewed in [3,6]) and functional autophagy has proved to be necessary for the lifespan extension by rapamycin-mediated TOR inhibition in yeast [15]. TOR pathway inhibition mimics the physiological characteristics of starvation, including activation of autophagy (Figure 1) and yeast CLS extension dependent on autophagy [15]. On the other hand, the *S. cerevisiae TOR1* mutant cells present increased CLS associated with increased autophagy activity as detected by *ATG8–GFP* (green fluorescent protein; Figure 2).

**Figure 1 | Regulation of autophagy and its implications for yeast longevity**

TORC1 directly or indirectly regulates autophagy. Direct mechanism of negative autophagy regulation by TORC1 includes hyperphosphorylation of Atg13 that reduces the activation of the Atg1 complex. Starvation conditions or rapamycin results in autophagy induction through TORC1 inactivation. PKA and Sch9 pathways also negatively regulate autophagy through the involvement of Atg1 complex assembly or through the downstream targets Msn2/Msn4 and the protein kinase Rim15. Sch9 is a substrate of TORC1 and acts on PKA further, highlighting the complex network of these nutrient-sensing pathways. Inactivation of these pathways is also associated with long-lived phenotypes that require autophagy indication. See the text for details.

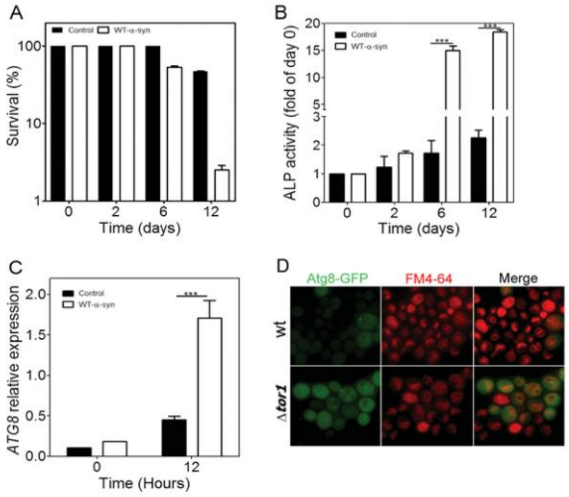



Although rapamycin is considered an inducer of yeast CLS extension and ameliorates neurodegenerative proteinopathies via activation of autophagy, opinion concerning its effective role is still controversial given the pleiotropic impact of the TOR pathway on various downstream targets besides autophagy and on the cross-talk of TOR with other signalling pathways.

In addition to TORC1, the nutrient sensory kinases PKA and Sch9 also play a role of negative regulators of autophagy, and the inactivation of both PKA and Sch9 release autophagy inhibition [28]. Hyperactivation of Sch9 or constitutive activation of PKA results in the suppression of autophagy even when TOR is inhibited by rapamycin or nutrient starvation [28] respectively. These data indicate that TORC1, PKA and Sch9 signalling pathways have a co-ordinated and parallel action on the regulation of autophagy. The common downstream targets of PKA and Sch9 pathways, the stress resistance transcription factors Msn2/Msn4 and the protein kinase Rim15, are required for autophagy induced by inactivation of these pathways, but not for autophagy induced by inactivation of TORC1 [28]. On the other hand, autophagy induction by Sch9 or PKA inhibition is also dependent on Atg1 kinase complex [28]. Atg1 [29] and Atg13 [30], as well as other Atg proteins, have also been assigned as PKA, Sch9 and TORC1 substrates [30,31] (Figure 1).

**Figure 2 | Evaluation of the autophagy activity in yeast control cells or cells expressing wild-type α-synuclein, during CLS**

(**A**) Survival, determined by colony-forming units. (**B**) Autophagy activity measured by the ALP assay. (**C**) *ATG8* mRNA levels, expressed as the ratio between the *ATG8* gene and the reference gene, *ACT1*. (**D**) Images of wild-type and Δ*tor1* cells expressing Atg8–GFP and stained with the fluorescent vacuole marked FM4–64. The images reflect the co-localization of Atg8-GFP in vacuolar membranes. Results represent means±S.E.M. for three independent experiments. Statistical significance (***$P < 0.001$) was determined by two-way ANOVA. WT α-syn, wild-type α-synuclein.




Besides the convergence of TORC1, PKA and Sch9 pathways on common downstream targets, they also cross-talk between each other. The originally proposed yeast Akt (also known as protein kinase B) homologue, Sch9, now believed to be an S6K1 (S6 kinase 1) homologue [32], is a substrate of TORC1 and an intermediate of the signalling from TORC1 to PKA [33] (Figure 1). Nevertheless, it is still not known whether or not PKA is also a substrate of TORC1 [33].

Another important aspect to consider when analysing the regulation of autophagy and aging by nutrient-sensing pathways is the dietary intervention, CR (caloric restriction). CR has been shown to promote longevity in various species, including yeast, and is known to inactivate TOR, PKA and Sch9 signalling pathways (reviewed in [11]). Although there is no direct evidence in yeast, it can be expected that increased autophagy underlies part of the effects of CR on promoting longevity as demonstrated in other species (reviewed in [3,34]).

Although TORC1 is the most studied regulator of autophagy, it is controlled by a complex network of different pathways also implicated in aging. Molecules such as the acetylase inhibitor spermidine and the deacetylase (Sirtuin1) activator resveratrol prolong the lifespan of multiple species in a TOR-independent autophagy-dependent fashion [35]. Interestingly, in yeast the longevity effects of CR are also associated with sirtuin activity (reviewed in [3,34,36,37]) pointing to a complex and elaborate network regulating autophagy and aging.

Regulation of autophagy is complex, but several pieces of the puzzle have already been firmly established; nevertheless, the picture is far from being complete, since numerous components have a yet unknown place. For example, an issue that merits concern is whether the change in Atg13 phosphorylation is a crucial step in the activation of the Atg1 complex given the ability a non-phosphorylable Atg13 form has to induce autophagy independent of TORC1 regulation [26]. Additionally, although the strategies to enhance longevity using autophagy as an anti-aging intervention are appealing, some crucial aspects have to be taken into consideration. The mechanisms to modulate and to keep autophagy activity at physiological levels after its induction and the effects that persistent activation of autophagy might have in the aging process remain unknown. The study of these aspects could be facilitated by using a condition of constant autophagy activation such as proteotoxic stress.

## Autophagy and aging under proteotoxic stress

During CLS yeast cells do not remain tight; in contrast, they are metabolically and biosynthetically active, responding to alterations in nutrient availability [10,17]. Accumulating evidence suggests that autophagy is required in order to regulate this phase, since during CLS the non-dividing cells are unable to dilute or to get rid of 'metabolic waste' by cell division [8,9]. The relationship between these two processes,

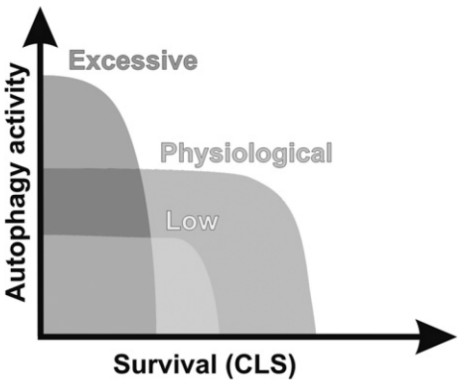
CLS and autophagy, is extremely complex and not fully understood and one of the most important questions relies on whether persistent autophagy induction would be an ideal anti-aging intervention [3]. Although autophagy is a well-established cytoprotective process that promotes survival of cells in metabolic distress, autophagy can also be a self-destructive process by its contribution and participation in cell death [4]. To further analyse the interplay between CLS and autophagy and to elucidate if the persistent autophagy induction indeed has a harmful effect, we assessed the autophagy activity during CLS of yeast cells under proteotoxic stress. The heterologous expression of proteins such as α-synuclein, an aggregation-prone protein involved in the pathogenesis of Parkinson's disease (reviewed in [38]) that is known to induce autophagy, could be helpful in clarifying some aspects of the role of autophagy in CLS. Our data show that during CLS of wild-type cells the autophagy activity [evaluated by the ALP (alkaline phosphatase) assay and *ATG8* mRNA expression levels] is maintained at low levels until day 12 of CLS (Figure 2). However, the heterologous expression of α-synuclein-induced toxicity and a reduction of yeast longevity associated with a pronounced increase in the autophagy activity and of the *ATG8* mRNA levels over time (Figure 2). This increased autophagy activity was coincident with the early appearance, at days 6 and 12, of cell death, described to be of an apoptotic as well as necrotic nature during CLS [39,40]. These results seem to indicate that the excessive and persistent activation of autophagy due to proteotoxic stress might lead to autophagy participation in the cell death occurring under these conditions in CLS, revealing the other face of the coin and the sinister nature of autophagy.

## Conclusions

Evidence gathered from different genetic and pharmacological manipulations reveals that different signalling pathways converge on autophagy to regulate longevity. Pharmacological manipulation of autophagy appears as an attractive strategy to delay aging and has been motivating the development and search for drugs that directly or indirectly, through nutrient signalling pathways, regulate autophagy activity. Nevertheless, the pleiotropic nature of nutrient signalling pathways regulating autophagy and the dual and opposite functions, cytoprotective or detrimental according to the context, of autophagy generate serious limitations. Our data on yeast CLS under proteotoxic stress claim that the cytoprotective role of autophagy could not be generalized and that, although autophagy activity could be initially beneficial, its maintained activation is associated with cell death and shortening of lifespan (Figure 3). Among the multiple unanswered questions regarding the interplay between autophagy and aging, one that is particularly important and that requires further studies is the level of autophagy activity and the phase of CLS that should be targeted by anti-aging strategies.

**Figure 3 | Role of autophagy in cell survival and death**

Impaired or low autophagy activity induces cellular damage, reducing the clearance of damaged proteins and organelles and the energy supply for essential cell functions, contributing to the aging phenotypes. Physiological levels of autophagy act as a cytoprotective mechanism and maintain cellular homoeostasis, allowing protein and organelle turnover and sustaining the energetic levels. Excessive levels of autophagy culminate in cell death.




## Funding

This work was supported by a grant from the Fundação para a Ciência e Tecnologia [grant number PTDC/BIA-MIC/114116/2009]. A.S. and B.S.-M. received fellowships from the Fundação para a Ciência e Tecnologia [grant numbers SFRH/BD/33125/2007 and SRFH/BD/41674/2007 respectively].

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Received 5 June 2011
doi:10.1042/BST0391466

**Attachment II**

BASIC RESEARCH PAPER

Autophagy 8:10, 1-16; October 2012; © 2012 Landes Bioscience

# SNCA (α-synuclein)-induced toxicity in yeast cells is dependent on sirtuin 2 (Sir2)-mediated mitophagy

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**Keywords:** alpha-synuclein, autophagy, mitophagy, sirtuins, chronological aging, yeast, synucleinopathies

**Abbreviations:** ALP, alkaline phosphatase; Atg, autophagy-related; BSA, bovine serum albumin; CQ, chloroquine; CLS, chronological life span; CMA, chaperone-mediated autophagy; CFU, colony-forming units; DHE, dihydroethidium; IRC, index of respiratory competence; PBS, phosphate-buffered saline; PD, Parkinson disease; PI, propidium iodide; ROS, reactive oxygen species; Sir2, sirtuin 2; SNCA, α-synuclein; SOD, superoxide dismutase; UPS, ubiquitin-proteasome system; UPR, unfolded protein response

SNCA (α-synuclein) misfolding and aggregation is strongly associated with both idiopathic and familial forms of Parkinson disease (PD). Evidence suggests that SNCA has an impact on cell clearance routes and protein quality control systems such as the ubiquitin-proteasome system (UPS) and autophagy. Recent advances in the key role of the autosomal recessive *PARK2/PARKIN* and *PINK1* genes in mitophagy, highlighted this process as a prominent new pathogenic mechanism. Nevertheless, the role of autophagy/mitophagy in the pathogenesis of sporadic and autosomal dominant familial forms of PD is still enigmatic. The yeast *Saccharomyces cerevisiae* is a powerful "empty room" model that has been exploited to clarify different molecular aspects associated with SNCA toxicity, which combines the advantage of being an established system for aging research. The contribution of autophagy/mitophagy for the toxicity induced by the heterologous expression of the human wild-type SNCA gene and the clinical A53T mutant during yeast chronological life span (CLS) was explored. A reduced CLS together with an increase of autophagy and mitophagy activities were observed in cells expressing both forms of SNCA. Impairment of mitophagy by deletion of *ATG11* or *ATG32* resulted in a CLS extension, further implicating mitophagy in the SNCA toxicity. Deletion of *SIR2*, essential for SNCA toxicity, abolished autophagy and mitophagy, thereby rescuing cells. These data show that Sir2 functions as a regulator of autophagy, like its mammalian homolog, SIRT1, but also of mitophagy. Our work highlights that increased mitophagy activity, mediated by the regulation of *ATG32* by Sir2, is an important phenomenon linked to SNCA-induced toxicity during aging.

## Introduction

SNCA is a ubiquitously expressed protein in the brain with an intrinsic ability to bind to lipids and membranes. The function of SNCA is still unclear, but it regulates synaptic plasticity, dopamine neurotransmission, endoplasmic reticulum-Golgi trafficking, and acts as a molecular chaperone.[1] Overexpression or mutation of SNCA results in its misfolding and the formation of oligomeric and aggregated species that are associated with autosomal dominant forms of Parkinson disease.[2,3] Impairment and deregulation of protein quality control systems has emerged as a central, and common, pathogenic event in different models of synucleinopathy.[4] Cell proteostasis is maintained by a precisely regulated protein quality control system, consisting of a balance between the folding and refolding of misfolded proteins, by molecular chaperones, and the proteolytic systems, by the UPS and autophagy.[5] Soluble SNCA is degraded by the UPS pathway[6,7] and by chaperone-mediated autophagy (CMA).[8] Nevertheless, studies in yeast and cell line models show that insoluble SNCA and SNCA aggregates interact with the proteasome leading to its inhibition,[7,9-11] an observation also corroborated by assays performed in the substantia nigra of PD brains.[12] Mutant and dopamine-oxidized forms of SNCA also impair the CMA pathway leading to an upregulation of macroautophagy

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Submitted: 02/27/12; Revised: 06/24/12; Accepted: 06/26/12
http://dx.doi.org/10.4161/auto.21275