Universidade do Minho
Escola de Medicina

Hélder Ricardo Cardoso Pereira

The role of cell signalling during yeast chronological lifespan

dezembro de 2021

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UMinho | 2021

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The role of cell signalling during yeast chronological lifespan

Tese de Doutoramento
Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Maria de Belém de Sousa Sampaio Marques
e da
Professora Doutora Paula Cristina da Costa Alves Monteiro Ludovico

dezembro de 2021

Foreword and Acknowledgments

I have no idea where this will lead us, but I have a definite feeling it will be a place both wonderful and strange.

Dale Cooper in "Twin Peaks" (1990)

Eles não sabem nem sonham que o sonho / é vinho, é espuma, é fermento / bichinho álacre e sedento (...)

António Gedeão in "Pedra Filosofal" (1956)

Gostaria de agradecer às minhas orientadoras, Belém Sampaio-Marques e Paula Ludovico; a todas as pessoas que conheci nas bancadas do ICVS, a linha da frente da Ciência; ao Instituto de Investigação em Ciências da Vida e da Saúde e à Escola de Medicina da Universidade do Minho, onde este trabalho de investigação foi desenvolvido; ao Presidente da Escola de Medicina, Professor Doutor Nuno Sousa, e ao Diretor do ICVS, Professor Doutor Jorge Correia-Pinto. Por fim, gostaria de agradecer à minha família, aos meus pais, sou aquilo que sou graças a eles. Os valores ensinados desde tenra idade são o canivete-suíço e a bússola moral que me acompanha. Este projeto foi apoiado por bolsas da Plataforma de Microscopia Científica do ICVS, membro da infraestrutura nacional PPBI - Plataforma Portuguesa de Bio-imagem (PPBI-POCI-01-0145 FEDER-022122; por fundos nacionais, através da Fundação para a Ciência e Tecnologia (FCT) - projeto UIDB/50026/2020 e UIDP/50026/2020; e pelos projetos NORTE-01-0145-FEDER-000013 e NORTE- 01-0145-FEDER-000023; pelo Programa Operacional Regional Norte Portugal (NORTE 2020).

The present work was carried out at the Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal. Financial support was provided by a PhD grant from the Foundation for Science and Technology (FCT) - SFRH/BD/133087/2017 developed under the scope of the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER), by FEDER, through the Competitiveness Factors Operational Programme (COMPETE).

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

O papel da sinalização celular durante o envelhecimento cronológico da levedura

O envelhecimento é um fenómeno biológico complexo que no sentido mais lato pode ser definido como uma perda estocástica de funcionalidade dos organismos vivos ao longo do tempo. O envelhecimento pode ser modulado por mecanismos celulares autónomos que englobam uma rede de sinalização celular diversa e interconectada, coordenada por reguladores de longevidade conservados ao longo da evolução e vias de sinalização de nutrientes, dentro de uma célula individual. A perda de homeostasia e sinalização de nutrientes desregulada são eventos-chave do processo de envelhecimento. Sistemas de controlo de qualidade das proteínas (UPS e autofagia) ficam sobrecarregados ao longo do envelhecimento. Intervenções para contrariar a progressão do envelhecimento como a restrição calórica (CR) foram empregues no modelo de expressão de aSyn em levedura, de forma a compreender o papel da comunicação celular autónoma nos sistemas de controlo de qualidade de proteínas durante o envelhecimento. Os dados evidenciam que CR contraria a progressão do envelhecimento e a toxicidade de aSyn promovendo o UPS e mantendo a autofagia em níveis homeostáticos. Adicionalmente, um conjunto de evidências sugere que o envelhecimento só pode ser explicado também com uma dimensão de mecanismos celulares não-autónomos, mediando comunicação intercelular de várias moléculas que atuam como fatores transmissíveis entre células. Estes fatores podem ser segregados dentro de EVs de origem em vias convencionais e não-convencionais de secreção. O secretoma de levedura envelhecida foi analisado e detetadas proteínas, ácidos nucleicos e lípidos, bem como a presença de EVs (partículas semelhantes a exossomas e microvesículas). O modelo aSyn foi empregue para evidenciar que a proteína é segregada para o meio condicionado e modula a longevidade de células recetoras. aSyn está presente em EVs no meio e é capaz de entrar noutras células. A inibição de uma via de biogénese de EVs (MVBs) anula os efeitos na longevidade e bloqueia a sua entrada nas células. A inibição da endocitose sugere também que esta via é essencial para a formação de exossomas e para a sua entrada nas células recetoras. Com esta tese, pretendemos evidenciar o papel da sinalização celular na coordenação dos sistemas autónomos de controlo de qualidade de proteínas a diversidade de factores transmissíveis e a conexão das vias de sinalização e tráfego membranar por mecanismos celulares não-autónomos no processo de envelhecimento.

Palavras-chave: envelhecimento, mecanismos celulares autónomos, mecanismos celulares não-autónomos, sinalização celular, levedura

Abstract

The role of cell signalling during yeast chronological lifespan

Ageing is a complex biological process that can be defined in the broadest sense as a stochastic, loss of function over the course of time in most living organisms. Ageing can be modulated by cell-autonomous mechanisms that comprise a diverse and interconnected network of cell signalling coordinated by evolutionary conserved master longevity regulators and nutrient sensing pathways, within the scope of a single cell. Loss of proteostasis and deregulated nutrient are hallmarks events of ageing progression. Protein quality control (UPS and autophagy) systems become overburdened with ageing. In this work, interventions to counter ageing progression such as CR were employed on the yeast aSyn expression model to understand the role of the cell-autonomous crosstalk of protein quality control systems during ageing. Our data supported that CR countered ageing progression and aSyn toxicity by upregulating the UPS and maintaining autophagy at homeostatic levels.

In addition, a body of evidence suggests that the ageing phenomenon cannot be explained by cell-autonomous mechanisms alone, and that another cell-nonautonomous dimension involving intercellular communication of a variety of molecular transmissible factors between different cells also has deep impacts in ageing modulation. Moreover, these transmissible factors could be released within EVs of different origins (conventional and unconventional pathways of secretion). We performed a screening study on the secretome of chronologically ageing yeast. We were able to detect proteins, nucleic acids and lipids in the secretome. We also characterised the EVs present as exosomes and microvesicles. Using aSyn as a proof-of-concept model for the cell-nonautonomous mechanisms of intercellular communication, we found that aSyn is secreted to the conditioned media and able to modulate ageing (decreasing CLS) on receptor cells. aSyn seems to be present in EVs in the secretome and to enter receptor cells. Abrogation of one EV biogenesis (MVBs) pathway annuls the CLS decreasing effects of aSyn conditioned media on receptor cells and block its uptake, suggesting the importance of exosome-like vesicles in the horizontal transmission of aSyn between cells. Abrogation of endocytosis suggested that this pathway is essential for both exosome biogenesis and release, and the uptake of aSyn into receptor cells. This thesis aimed at novel insights into yeast ageing: The role of cell signalling, and exquisite protein quality control and coordination is evidenced; The diversity of transmissible factors, and the interconnectedness of various signalling networks and vesicle trafficking pathways is supported with new findings.

Keywords: ageing, cell-autonomous, cell-nonautonomous, cell signalling, yeast

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List of Abbreviations and Acronyms

ALP Autophagy-lysosome pathway

aSyn α-Synuclein

ATG Autophagy-related gene

Bort Bortezomib

CLS Chronological lifespan

CM Conditioned media

CME Clathrin-mediated endocytosis

CP Catalytic core particle

CR Caloric restriction

CUPS Compartment for unconventional protein secretion

CV Control vector

Cvt Cytoplasm-to-vacuole targeting

CQ Chloroquine

DDR DNA damage response

DLS Dynamic light scattering

DUB Deubiquitinase enzyme

ER Endoplasmic reticulum

ERAD ER-associated degradation

EV Extracellular vesicle

GA Golgi apparatus

ILV Intraluminal vesicle

IPOD Insoluble protein deposit

ISG Immature secretory granule

MV Microvesicle

MVB Multivesicular bodies

NTA Nanoparticle tracking assay

PACE Proteasome associated control element

PAS Phagophore assembly site

PD Parkinson's disease

PGK1 3-phosphoglycerate kinase

PI Propidium iodide

PKA Protein kinase A

PSG Proteasome stress granule

UPR Unfolded protein response

UPS Ubiquitin-proteasome system

RLS Replicative lifespan

RP Regulatory particle

ROS Reactive oxygen species

SDS-PAGE Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SV Secretory vesicle

TGN Trans-Golgi network

TLC Thin-layer chromatography

TOR Target of rapamycin

TORC1 Target of rapamycin complex 1

List of Figures and Tables

CHAPTER 1

GENERAL INTRODUCTION

1.1. Ageing, a biological process

Ageing is a complex biological phenomenon that affects most living organisms and can be defined in the broadest sense as a stochastic, time-dependent functional decay. It is characterised by a progressive loss of physiological integrity, impaired cellular functions, and increased vulnerability to death. Ageing has peaked mankind curiosity and ingenuity throughout history, but only more recently scientific research on the field has experienced unprecedented breakthroughs. Ageing is recognized as the primary risk factor for many human pathologies (including cancer, diabetes, cardiovascular or neurodegenerative disorders) and its progression is regulated by several evolutionary conserved genetic and biochemical pathways [1-3]. The progressive accumulation of cellular damage over the course of time is considered the general cause of ageing [4, 5]. The following sections will summarize the main ageing hallmarks associated with damage accumulation.

1.1.1. Hallmarks of ageing

To better categorize the complex ageing phenomenon at the molecular and cellular levels, several hallmarks of ageing were proposed [1]. This initiative drew inspiration from a landmark paper in the cancer field first published in 2000 by Hanahan and Weinberg, which result in a significant momentum gain for that area of research. Afterwards, Lopez-Ótin and colleagues, hoping to achieve a similar outcome on the study of ageing, proposed nine candidate hallmarks, as can be seen in Figure 1. The nine hallmarks are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [1] According to the authors, an ideal hallmark candidate should adhere to the following criteria: it should occur during ageing progression, experimentally induced aggravation of the hallmark should result in acceleration of ageing, and experimentally induced enhancement should lead to a delay of the ageing, increasing the organism lifespan. The proposed hallmarks do not always meet the ideal requirement criteria. The last criteria, the delay or reversal of an ageing phenotype, is notoriously difficult to fulfil for all criteria, as known experimental interventions are not proven to ameliorate ageing on all hallmarks. This limitation needs to be viewed with the notion that ageing is a multi-factorial, interconnected

process, and therefore, interventions that target one hallmark may have desired or undesired consequences on others.

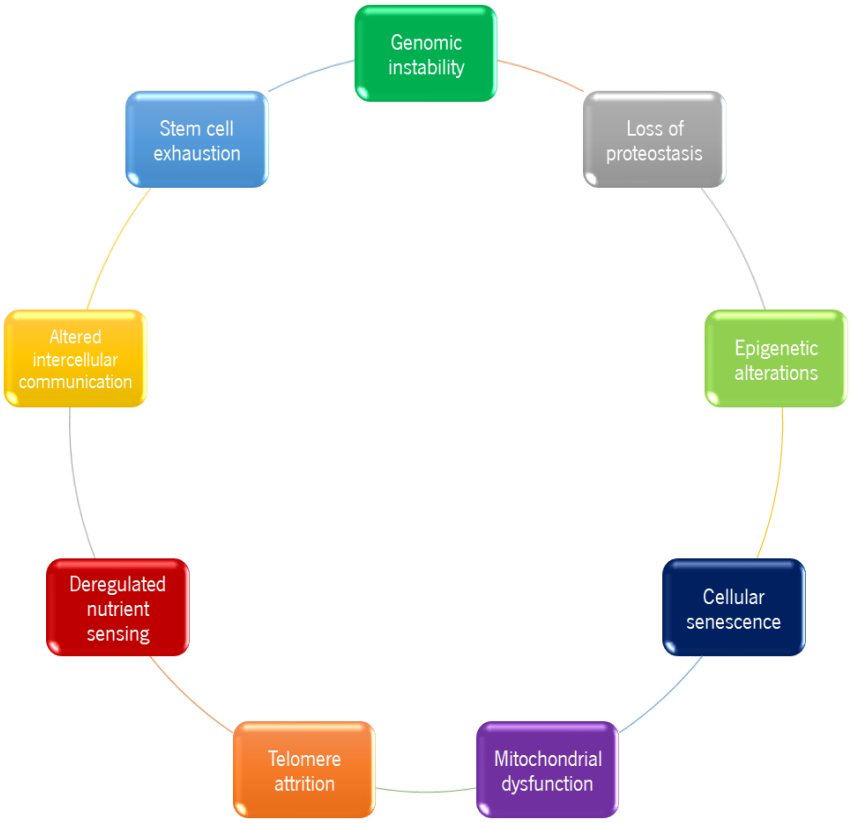

Figure 1. The hallmarks of ageing. Nine hallmarks were proposed by Lopez-Ôtin and colleagues to characterise the ageing phenomenon. Adapted from [1].

Genomic instability, the accumulation of genetic damage over the course of time, is associated with ageing across all eukaryotic organisms [6, 7]. The rate at which this instability occurs is increased with ageing progression. Premature ageing syndromes such as Werner or Bloom syndrome are described as the result of DNA damage [8]. DNA integrity and stability is constantly threatened by both exogenous (chemicals, radiation) and endogenous (ROS, replication errors, spontaneous hydrolytic reactions) agents [9-11]. Genomic stability is also achieved by maintaining telomere length and integrity of mitochondrial DNA [5, 7, 12], both shall be addressed in this section as separate hallmarks, highlighting once more the interconnectedness of the hallmarks.

Ageing-associated DNA damage affects the entire genome in a quasi-random manner, but some regions of the chromosomes are more susceptible than the rest. This is the case of telomeres, the terminal sections of the chromosomes. Telomere attrition is a common phenomenon during ageing because most DNA polymerases lack the ability to replicate the terminal end of linear DNA molecules in its entirety. Only a particular DNA polymerase, telomerase, can replicate completely the terminal ends of linear DNA molecules [5, 13]. Most somatic cells lack telomerase leading to progressive loss of telomeres during ageing on many organisms. In yeast, TERT (telomerase reverse transcriptase) was discovered to be

encoded by the *EST2* gene [5]. The limited proliferative capacity of some cells, also called the Hayflick limit [14], is due to this telomere attrition [5].

Another hallmark of ageing is the accumulation of epigenetic changes. Epigenetic changes are alterations on patterns of DNA methylation, post-translational modification of histones and chromatin remodelling that alter genetic expression without changing the DNA sequence [15]. The molecular mechanisms involve methylation of cytosine in C-G nucleotide pairs, histone modifications and non-coding RNAs to influence how accessible a certain genome locus is to be transcribed [15]. Sirtuins are a family of highly conserved histone deacetylases involved in transcription regulation. Several studies in ageing model organisms from yeast to mammals, suggest a role for these proteins in ageing progression [16, 17]. In yeast, the model organism where sirtuin 2 (Sir2) was first described [18, 19], *SIR2* overexpression extended lifespan in a mitotic (replicative) lifespan [1, 17, 20], while in post-mitotic (chronological) lifespan, Sir2 is implicated in aggravated protein toxicity [17].

Ageing and age-related diseases are associated with protein misfolding, aggregation and accumulation, which leads to impaired protein homeostasis (proteostasis), another ageing hallmark (Fig.1) [1]. Eukaryotic cells have quality control mechanisms that deal with misfolded proteins and keep protein folding at homeostatic levels (Fig. 2).

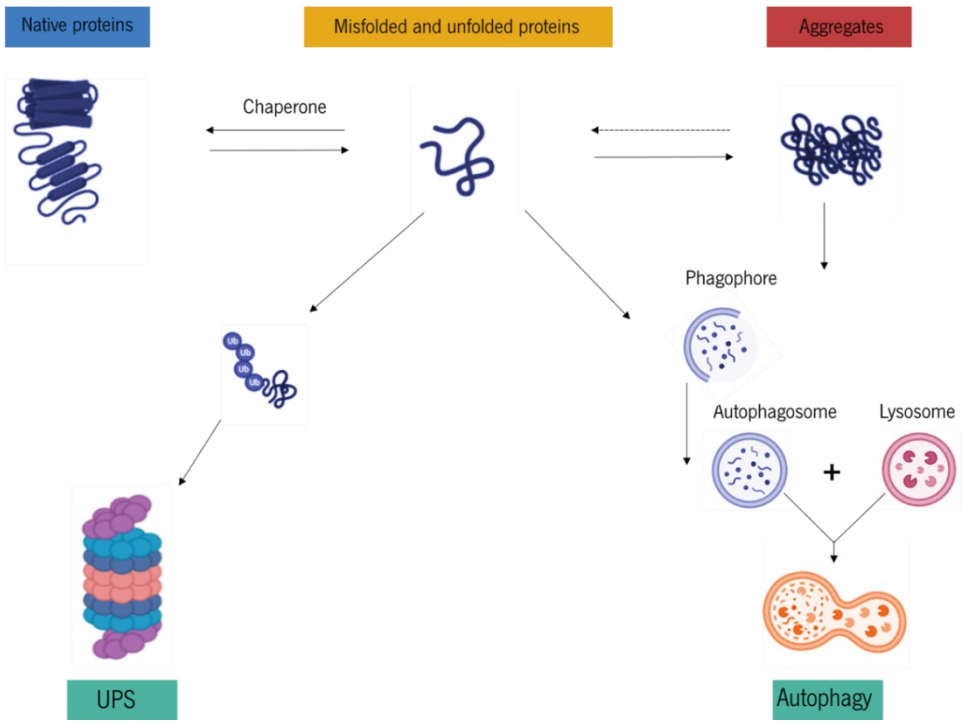

Figure 2. Proteostasis is maintained by an intricate network of proteolytic mechanisms that recognize and degrade misfolded proteins. Adapted from [2].

These include chaperone-mediated protein folding, mediated by several chaperones as those from the heat-shock family of proteins, the unfolded protein response (UPR) activated upon ER stress and the proteolytic systems, autophagy-lysosome (ALP) (vacuoles in yeast) and ubiquitin-proteasome systems (UPS). UPR itself can upregulate the ALP system, translocate misfolded proteins to be degraded by the UPS, or promote the synthesis of ER chaperones to cope with proteotoxic stress [21, 22]. Proteolytic systems degrade harmful protein aggregates but are themselves affected by ageing progression [20, 23]. These topics will be described in further detail on the **section 1.2.2** (The proteostasis network).

Overall, loss of proteostasis is associated with ageing. There is mounting evidence across several models that inducing proteotoxicity with the expression of proteins associated with age-related pathologies precipitates premature ageing, and several genetic and non-genetic interventions that promote proteostasis improve lifespan [1, 2, 24].

Nutrient sensing pathways are highly conserved across evolution (Fig. 3) and can modulate ageing progression and longevity [25, 26]. Current evidence suggest that anabolic signalling promotes ageing progression [24]. This longevity regulation senses changes in glucose and amino acids availability in yeast [25, 26], and growth hormones factors such as insulin and IGF in mammals [24]. There are two main nutrient-sensing pathways conserved from yeast to mammals: the Ras/Protein Kinase A (PKA) and the TOR (target of rapamycin)/S6 Kinase (S6K) pathways [24, 27]. Environmental or genetic interventions such as reduced caloric intake by caloric restriction (CR) or abrogation of key genes in the nutrient sensing pathways, respectively, can increase longevity [25-29]. Reduced caloric intake without malnourishment, also called dietary restriction, is a robust dietary, non-genetic intervention, with reported longevity increase effects on several model organisms (flies, worms, yeast or rodents) [24]. It is thought that the beneficial effects of CR are promoted by the inhibition of nutrient-sensing pathways [24, 27].

This will be addressed in more detail in the upcoming **section 1.2.1** (Master longevity regulators of ageing: nutrient and energy signalling pathways in yeast).

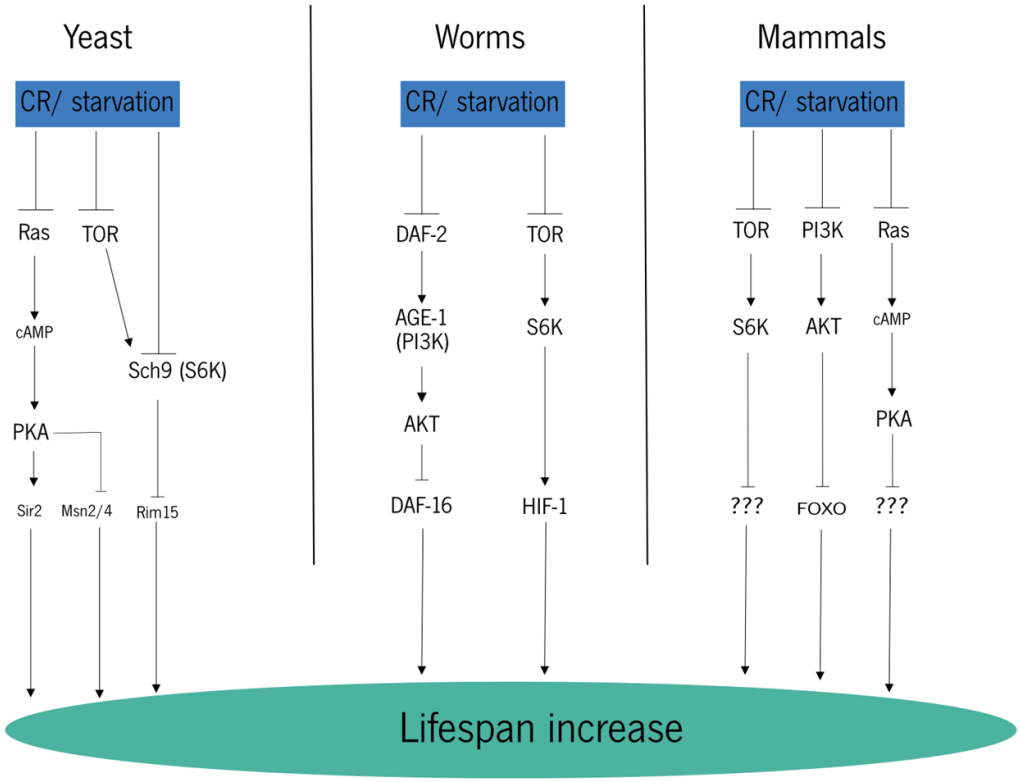

Figure 3. Nutrient-sensing longevity pathways are highly conserved between *S. cerevisiae*, *C. elegans* and *Homo sapiens*. Adapted from [26].

Reactive oxygen species (ROS) accumulation over the course of ageing is postulated as the main cause for mitochondrial loss of function, another hallmark of ageing (Fig. 1) [1]. This mitochondrial loss of function in turn can lead to the production and accumulation of even more ROS and cellular damage, according to the classical consensus on the subject, the free radical theory of ageing [30]. However, recent evidence has paved the way to a new conceptual framework for the role of ROS in ageing and mitochondrial dysfunction. Genetic manipulations that impair mitochondrial function, but do not increase ROS production, and studies that report increased ROS levels as lifespan extending in yeast [31-36] and worm models [37], at a first glance may seem wildly contradictory with the original free radical theory. Evidence suggests that ROS can have a beneficial effect activating homeostatic stress responses [1, 38, 39]. Nevertheless, after a certain threshold, ROS accumulation eventually ends up aggravating ageing and cellular damage [39].

Cellular senescence consists in cell cycle arrest. Cell cycle arrest can be triggered by DNA damage, telomere attrition or signalling pathways over the course of time, to counter the deleterious effect of ageing.

Stem cell quiescence is important for long-term maintenance of stem cell niches and tissue regeneration on complex, multicellular organisms. Its exhaustion is, therefore, another hallmark of ageing [1].

The last hallmark goes beyond cell-autonomous mechanisms, *i.e.*, the ensemble of all the mechanisms within a single cell that modulate ageing progression, into cell-nonautonomous mechanisms of ageing. Alterations of patterns of intercellular communication between cells, can modulate cellular ageing by low-molecular weight transmissible longevity factors [1, 40]. The relevance and mechanisms of this cell-nonautonomous mechanisms will be addressed in the upcoming **section 1.3** (Cell-nonautonomous mechanisms of ageing).

Lopez-Ótin and colleagues also proposed the subdivision of these nine hallmarks into three categories: primary, antagonistic, and integrative hallmarks [1]. Succinctly, primary hallmarks (genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis) represent the causes of ageing-associated damage and are all unequivocally detrimental to the cell. Antagonistic hallmarks (deregulated nutrient-sensing, mitochondrial dysfunction, and cellular senescence) can be a double-edged sword, and have opposite effects depending on their intensity. At low levels they contribute to maintaining homeostasis, at high levels become deleterious. These hallmarks can be thought of as designed for protecting the organism from damage or lack of nutrient availability. However, when exacerbated, they invert their function and cause further damage. Integrative hallmarks (stem cell exhaustion and altered intercellular communication) pertain to tissue homeostasis and function, and cell-nonautonomous mechanisms of cell-to-cell communication [1, 40]. Moreover, the authors suggested, in spite of the interconnectedness between hallmarks, a certain sequential order to these three categories. First the primary hallmarks trigger cellular damage, whose aggravation over the course of time then lead to imbalances on some antagonistic hallmarks. When, the cumulative damage caused by the primary and antagonistic hallmarks can no longer be compensated by molecular mechanisms, the integrative hallmarks start to manifest [1, 41]. With the characterisation of the hallmarks of ageing a consistent framework for research on the molecular mechanisms and interconnectedness of hallmarks was built. With this knowledge, research on the ageing field can gain focus and momentum to design novel interventions to improve lifespan from model organisms to humans [4, 41].

1.1.2. Ageing models

Ageing, as above described, is a complex, multifactorial biological process that for centuries has peaked mankind's curiosity. However, serious scientific research stumbled on the practical barriers: How can one test a theory about human ageing and longevity when it takes literally a lifetime to collect the data? To

study ageing progression, it is crucial to understand that many of the processes ruling ageing are conserved throughout evolution [1, 3, 38] and that model organisms such as mice (*Mus musculus*), flies (*Drosophila melanogaster*), worms (*Caenorhabditis elegans*) or yeast (*S. cerevisiae*) possess many advantages to facilitate ageing research. The short lifespan of these organisms is a key advantage, as well as reduced cost and simpler ethical concerns regarding humans [42].

1.1.2.1. Yeast as a model of ageing

The budding yeast *S. cerevisiae* is considered the prototype of a eukaryotic cell. Yeast is ideally tailored to study many phenomena transversal to all eukaryotes [7], from cell cycle to protein folding to name a few [42]. With a small genome size (12 Mbp), short doubling time (1.5 hours), and the existence of stable haploid and diploid phases allowing recombination analysis, yeast has been at forefront of ageing research. Yeast is one of the few model organisms where "knock in" gene replacement can be performed with relative ease and its genome was the first eukaryote genome fully sequenced since 1996 and annotated over the past decades. As above referred, many of the master longevity regulators of ageing and nutrient-sensing pathways are conserved from yeast to humans, and moreover yeast has proven itself instrumental in uncovering them first [3, 25-27]. Two of major longevity regulators studied in ageing are the sirtuins and the TOR signalling pathway, of which the yeast model was essential for their uncovering [25].

Yeast can be used to study ageing with two different ageing models: the Replicative Lifespan (RLS) and Chronological Lifespan (CLS). RLS pertains to the number of mitotic divisions each single mother cells generates, whilst in CLS, ageing is defined by the ability to survive from the moment cells enter post-mitotic stationary phase, when nutrients become limiting [7] (Fig. 4). Common laboratory strains of yeast can only divide a certain number of times, producing typically between 20 to 30 daughter cells from a single mother cell [7, 43]. This replicative limitation draws some parallels with the Hayflick limit [14]. A cell's lifespan is measured in generations, counted by the number of buds, daughter cells, produced. Yeast usually takes 2 to 3 days to reach quiescence and stop mitotic divisions. Over a hundred replicative ageing genes have been identified. Sir2, the NAD-dependent deacetylase is a key master longevity regulator of yeast during RLS [27]. Sir2 suppresses homologous recombination in ribosomal DNA. Ribosomal DNA instability is one of the main defects leading to premature ageing on RLS [25]. Other pathways such as TOR or Sch9 also have a role on longevity modulation during RLS. Under CR intervention, reduced Ras/PKA, TOR/Sch9 signalling leads to increased lifespan [24, 25].

Once yeast cells lose replicative capacity and enter stationary phase in a state of quiescence (a reversible state of growth arrest in G_0/G_1), the CLS paradigm can be employed to study ageing [44, 45]. CLS is defined by the amount of time a yeast cell can survive on the same nutrient-deprived medium. Even on CLS, yeast has two distinct populations: one is essentially homogenous and is constituted of quiescent, viable cells, while the other subpopulation is heterogeneous and comprised of dying nonquiescent cells under stress and cellular senescence processes. Senescence on yeast CLS is an irreversible state, and cells eventually die from regulated cell death processes[45]. Quiescent cells increase their survival on nutrient limiting conditions by decreasing transcription, translation, and metabolism [45, 46]. On CLS, the cell's clonogenicity is measured by plating aliquots on new nutrient-rich media, which will prompt the cells that remained viable to return to a non-quiescence state and re-enter the cell cycle in order to divide [25, 27]. As discussed before, the main nutrient sensing pathways, TOR, Sch9 and Ras/PKA, are the master longevity modulators during CLS. Sirtuins have less pronounced anti-ageing roles during CLS [18]. Sir2 promote exacerbate autophagy during yeast CLS, which has been tied to the proteotoxic stress induced by proteins associated with age-associated diseases [17, 20]. Reduced TOR signalling and PKA inhibition lead to the activation of many cellular stress responses such as Rim15 or the Msn2/4 or Gis1 transcription factors [25, 27, 47, 48] and the maintenance of cells in G_0/G_1 cell cycle arrest that promote lifespan [45].

Both yeast ageing models, replicative and chronological, have been useful tools in understanding the dynamics of evolutionary conserved longevity modulation, allowing for the uncovering of genes and signalling pathways. In the present thesis, we will focus on the post-mitotic, chronological ageing paradigm.

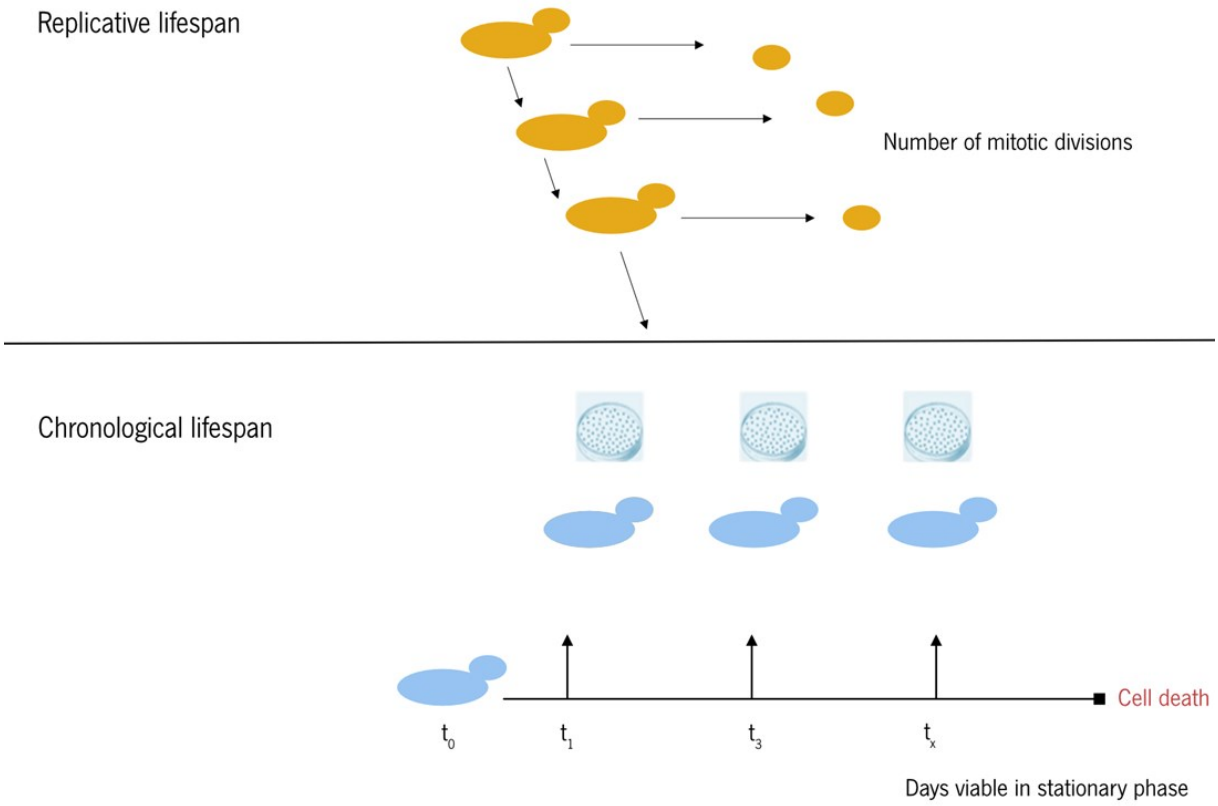

Figure 4. The two different ageing models provided by yeast *S. cerevisiae,* Replicative (RLS) and Chronological lifespan (CLS)

1.2. Cell-autonomous mechanisms in the regulation of ageing

Cell-autonomous mechanisms comprise the diversity of interconnected cell signalling networks within the scope of a single cell that can modulate the progression of ageing and longevity of organisms. Longevity can be defined by these cell-autonomous mechanisms that are essential for nearly all molecular and physiological cellular processes from metabolism, growth, proliferation, stress resistance, cellular homeostasis, survival, to death. These mechanisms are orchestrated by evolutionary conserved nutrient and energy sensing pathways, culminating in the regulation of the so-called master longevity regulators of ageing [49]. On the following lines, the main nutrient and energy sensing pathways conserved throughout evolution are to be detailed further. Their modulation by master longevity regulators is a prime example

of cell-autonomous processes coordinated regulation of ageing that can be observed even on the yeast model.

1.2.1. Master longevity regulators of ageing: nutrient and energy signalling pathways in yeast

Genetic studies in several model organisms, from yeast to worms and mammals, demonstrated that an organism's longevity can be modulated by single gene mutations [3, 49]. These master longevity regulator genes are part of evolutionary highly conserved nutrient and energy signalling pathways, many of which owe their discovery to the single-cell eukaryote, *Saccharomyces cerevisiae*. For the sake of simplicity, this section will focus on the signalling pathways present on the yeast model. The TOR (target of rapamycin)/S6 Kinase (S6K) (homolog of yeast Sch9) pathway and sirtuins are major ageing regulators firstly discovered in yeast [18]. Alongside them, is the Ras/Protein Kinase A (PKA) pathway. Sirtuins are mostly relevant in promoting longevity in yeast models with actively dividing, mitotic cells, whereas TOR and PKA affect mostly post-mitotic aged yeast cells [3]. TOR pathway is activated by the concentrations of amino acids and other nutrients, while PKA pathway is mainly activated by glucose (Fig. 5). These latter two are interconnected, as they both act on the stress-resistance regulon of the protein kinase Rim15 and transcription factors Msn2/Msn4 and Gis1. Sir2 is also connected to Msn2/4 activity [3, 25]. Also on yeast, Snf1 (homolog of mammalian AMP-activated protein kinase, AMPK), is an energy sensing protein capable of changing transcription patterns of glucose-repressed genes and support a stress response. Snf1 interacts with other nutrient sensing pathways and proteins such as TOR, Sch9 or PKA [50-52].

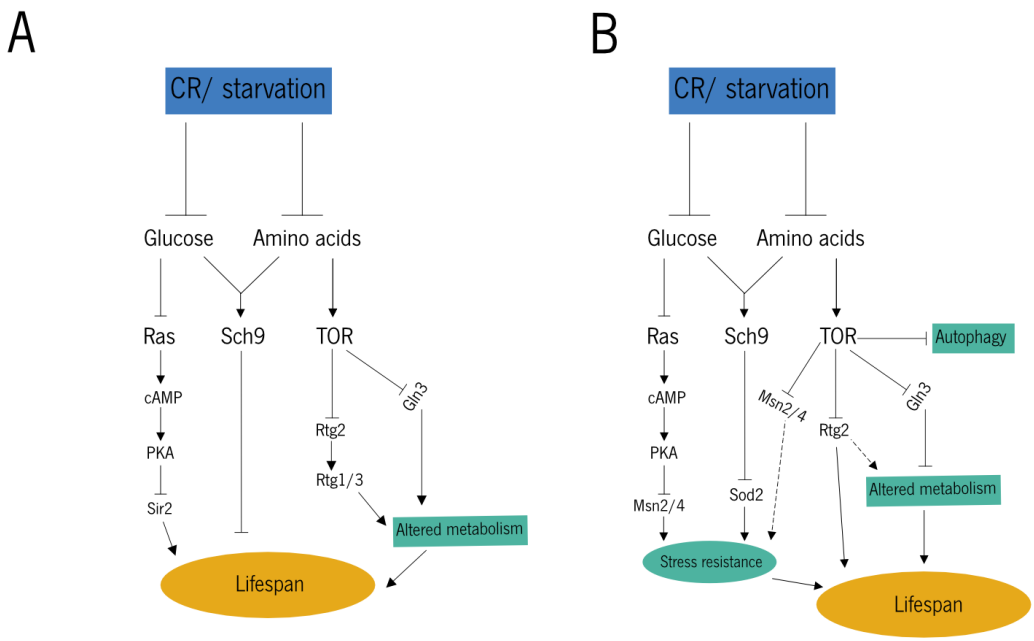

Figure 5. Nutrient and energy sensing pathways in yeast ageing and the role of caloric restriction, with replicative, mitotic cells, (**A**) and post-mitotic, non-dividing chronologically ageing cells (**B**). Adapted from [3].

The Ras/PKA pathway is activated through changes in levels of glucose availability. These changes are transduced by multiple sensors: the G-protein-coupled receptor Gpr1 and the Gpa2 proteins, several hexokinases and glucokinases, and adenylate cyclase Cyr1. Glucose phosphorylation by hexokinases (Hxk1/2) or glucokinases (Glk1) occurs when glucose availability is high within the cell. Cyr1 produces cyclic AMP (cAMP) when stimulated by the GTP/GDP binding Ras proteins (Ras1, Ras2), activating PKA [26, 53-55]. PKA consists of a heterotetramer containing a regulatory subunit, Bcy1, and three catalytic subunits (Tpk1/2/3). cAMP binds to the regulatory subunit of PKA, Bcy1, detaching it from the catalytic subunits and allowing for the activation of PKA. When activated, PKA inhibits the Msn2/Msn4 transcription factors, which would promote stress resistance and increased lifespan on post-mitotic cells (Fig. 4B). On mitotic (replicative) cells, an activated PKA inhibits Sir2 (Fig. 5). Upon lower levels of glucose, which could be induced by starvation or dietary intervention, this cascade of protein interactions does not occur, leading to the expression of the Msn2/Msn4 transcription factors, the ensuing stress resistance response and increased lifespan [3, 26, 27].

TOR is part of another evolutionary conserved nutrient sensing pathway, important to longevity regulation. TOR signalling network regulates growth of eukaryotic cells in response to nitrogen, amino acids and glucose or other carbon sources. TOR is a serine/threonine protein kinase of the phosphatidylinositol kinase-related kinase family (PIKK). TOR is targeted specifically by rapamycin, a drug with known antiproliferative effects [56]. When TOR is downregulated by prolonged rapamycin treatment or genetic abrogation, yeast cells switch transcription patterns leading to a downregulation of protein synthesis and

cell cycle arrest at a quiescence (G_0) state [57, 58] (Fig. 6). Autophagy in yeast is initiated by the activation of Atg1 kinase complex, which includes Atg13[56]. TORC1 affects the formation of the Atg1 complex hyperphosphorylating Atg13, impeding the complex assembly and therefore, autophagic flux.

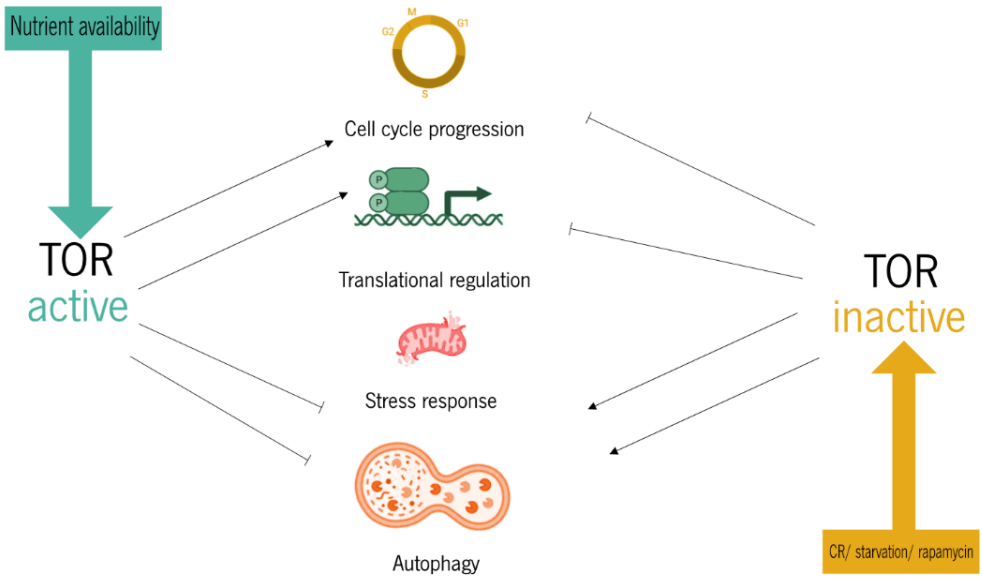

Figure 6. TOR signalling network is a prime example of a nutrient-sensing master longevity regulator implicated in a coordinated response to stress, damage, and ageing. Adapted from [27].

Autophagy is also regulated by Atg8 and Atg14 *ATG14* expression levels are regulated by Gln3, and TOR can inhibit Gln3. ATG14 and ATG8 transcription levels soar after autophagy induction by starvation [59]. TOR also interacts to inhibit Rtg2. Rtg2 is a sensor of mitochondrial dysfunction , and regulates the localization of Rtg1 and Rtg3 [60], transcription factors that activate the retrograde response, when not downregulated by TOR signalling. The retrograde response is a mitochondrion-to–nucleus signalling pathway, mediated by the transcription factors Rtg1 and Rtg3, which coordinates the anapleurotic production of α-ketoglurate [27, 61]. The retrograde response is implicated with longevity regulation in mitotically active yeast cells [27, 62].

The yeast protein Sch9 has been originally described as an homologue of mammalian protein kinase B (PKB/Akt) [54] and is now believed to be an homologue of S6 kinase [54, 63]. As such, Sch9 is considered the main downstream effector of TORC1 in yeast [33, 63]. Sch9 is phosphorylated by TORC1 in specific C-terminal residues. Sch9 can also be phosphorylated by different pathways independent of TOR signalling such as sphingolipids activated kinases Pkh1/2 or the energy sensing regulator Snf1 kinase. Nevertheless, once phosphorylated by TORC1, Sch9 can inhibit the longevity extending activities of cytoplasmic Rim15 by phosphorylation on specific residues. Rim15 induces cell cycle arrest at G_0 and initiates quiescence and longevity extension on yeast by activating Msn2/4 transcription factors. Sch9 is also essential for the activity of Gis1 transcription factor, independently of Rim15 and TORC1. In addition to oxidative stress resistance on nitrogen-starved yeast cells, Sch9 also connects with the nutrient sensing longevity regulation pathways like TOR and PKA (Fig. 7). In response to both glucose and amino acid availability, Sch9 inhibits Sod1/2, the mitochondrial superoxide dismutase enzyme, involved in the stress

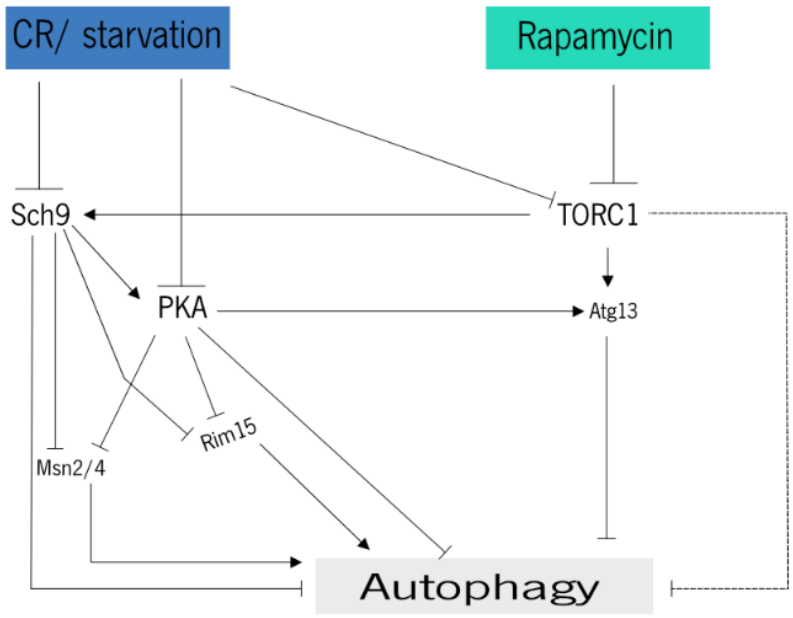

Figure 7. Regulation of autophagy by the master longevity regulators and nutrient sensing pathways in yeast. Adapted from [64].

response to ROS [3, 25, 26]. Oxidative stress poses a significant challenge to stationary phase cells. Deletion of either *TOR1* or *SCH9* increases yeast chronological lifespan [26, 33]. Mitochondrial ROS species such as superoxide anions reduce chronological lifespan by inducing DNA damage and mitochondrial dysfunction. Sch9 also inhibits the transcription factors *HCM1* and *HAP4* ensuring the transcription of respiratory genes and increased mitochondrial respiration [33]. Although toxic in high amounts with ageing progression, early stationary phase accumulation of ROS species such as hydrogen

peroxide can, however, result in an adaptive response known as hormesis. Hormesis can activate stress responses early on stationary phase resulting in extended CLS [31, 35, 65].

Considering the nutrient-sensing pathways above described, it seems clear that eukaryotic organisms, like yeast, have evolved to coordinate responses to fluctuations in nutrient availability in their environment. Many of these revolve around the sirtuins and TOR, Sch9 and PKA pathways impacting the organism's longevity. It is thought that cell signalling networks of nutrient-sensing pathways [24, 27] can be modulated by genetic, pharmacological or even dietary interventions (*e.g.* CR) to promote increased longevity [49, 66].

1.2.2. The proteostasis network

The proteome is the bulk of all proteins within a single cell, packed in diverse subcellular compartments. Proteins are essential players to almost all physiological processes within a cell. To maintain cellular homeostasis, the proteome must be kept intact - proteostasis - and protected from proteotoxic damage. To do so, cells evolved a complex network of molecular mechanisms (the proteostasis network, also called protein quality control), that accompany proteins from the moment they are synthetized in the ribosome to their eventual clearance by proteolytic degradation. This network relies on the activities of molecular chaperones, UPR, UPS and ALP systems [67, 68]. Loss of proteostasis is a known hallmark of ageing [1], and the activity of molecular mechanisms of the proteostasis network are prone to decline with ageing [20, 67, 68]. The proteostasis network is further regulated by several signalling pathways in response to genetic and epigenetic alterations or environmental stresses and processes like transcription and cell cycle progression are tightly coordinated with proteostasis and protein folding [69].

Protein homeostasis (proteostasis) can only be achieved when there is a balance between protein synthesis, folding, trafficking and degradation. To retain function, proteins must maintain a defined three-dimensional structure, called the native conformation. In their physiological environment, proteins are only slightly thermodynamically stable (also called metastable) and stress stimuli of several types (oxidative stress, heat shocks or alterations on the physiological pH of the cytosol), can compromise their stability [70] Proteins are very dynamic molecules that undergo frequent conformational changes and refolding/unfolding to traverse membranes, assemble into functional complexes or interact with other proteins. Some proteins are unable to achieve any ordered three-dimensional conformation by themselves and are dependent on protein-protein interactions to properly fold [71, 72]. When proteins misfold, they lose their intended functionality and start to aggregate within cells in toxic inclusions. These protein inclusions are oligomeric complexes of non-native conformations: they arise from non-native interactions

in protein folding potentiated by stress conditions. Aggregates are insoluble within the aqueous composition of the cytosol [73]. These toxic protein aggregates are associated with the aetiology of several ageing-associated diseases in humans, such as synucleinopathies [2, 20, 68, 74]. These oligomers have hydrophobic surfaces exposed that can interact with other proteins or cellular membranes in aberrant ways. Given the delicate thermodynamic balance of a protein under regular physiological conditions, some random protein-protein interactions might interfere with protein's native conformation. Some metastable proteins are targeted preferentially by these interactions and end co-aggregating with the oligomeric complex [68, 75]. Molecular chaperones assist protein surveillance and aid in proper *de novo* folding or refolding. In the cytosol, the heat shock response is responsible for the stress-inducible expression of chaperones like HSP70, HSP40, HSP90 and small HSPs of the heat-shock protein family [76]. The unfolded protein response (UPR) is a non-degradatory protein quality control mechanism to salvage misfolded proteins in the ER [22]. Proteotoxic stress can result in the accumulation of unfolded or misfolded proteins in the ER lumen and the UPR downregulates protein synthesis and activates a plethora of transcription factors, orchestrated by three different signalling pathways (ATF6α, IRE1α, and PERK) [21]. For instance, the non-conventional splicing of *HAC1* mRNA is triggered by the IRE1α pathway. The spliced Hac1 protein travels to the nucleus and mediates the transcription of ER chaperones, secretory proteins, and machinery for the ERAD (ER-associated degradation) response. ERAD recognizes misfolded ER proteins and transports them to the cytosol, where they can be degraded by the UPS [22, 77, 78]. Autophagy can also be directly activated by the PERK and ATF6 signalling pathways of UPR [79].

1.2.3. The ubiquitin-proteosome system (UPS)

The ubiquitin-proteasome system (UPS) is one of the major proteolytic systems of the proteostasis network, responsible for protein quality control and clearance. The proteasome consists of a proteolytic complex with a catalytic core (CP) particle and various regulatory particles (RP). The 20S proteolytic core is the complex responsible for the catalytic activities of the UPS. Regulatory subunits can also attach to this core, resulting in the assembly of several proteasomes species with the 19S regulatory particle being the most common. The 26S proteasome is a 2.5 MDalton complex comprised of the 20S proteolytic CP

and the 19S RP [80]. The 20S complex is a barrel-like structure made of 4 rings with two different types of catalytic subunits (α and β) (Fig. 8).

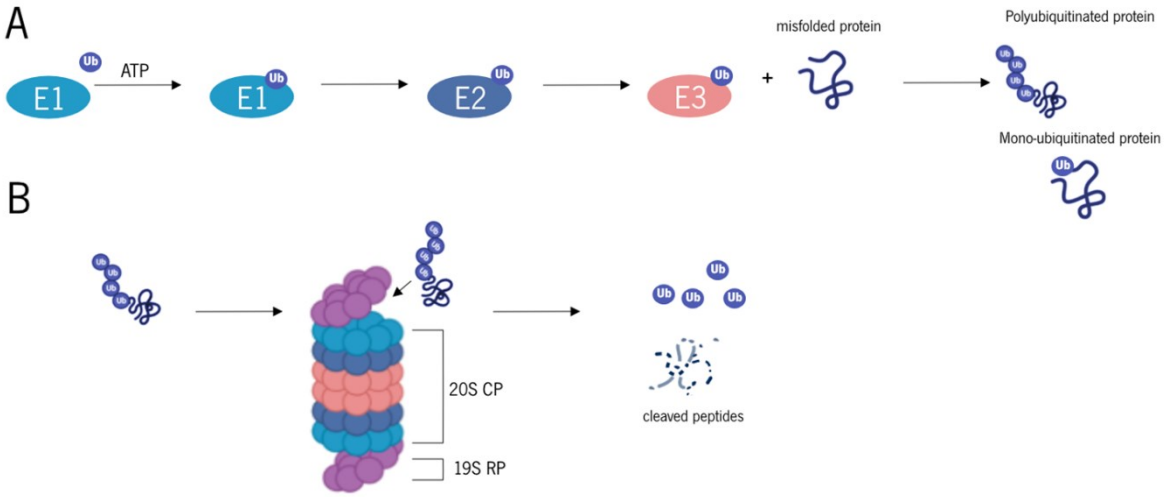

Figure 8. The ubiquitin-proteasome system (UPS) **(A)** Ubiquitin moieties bind to target proteins via three different ligases. **(B)** Target proteins tagged with ubiquitin are then recognized and degraded by the UPS. Adapted from [80].

The 19S RP attaches to the CP and recognizes the polyubiquitinated target proteins. The RP is further divided in two smaller regions, the base and the lid. The base of the RP complex is composed by ATPases (Rpt1-6), responsible for the unfolding of the target protein, and non-ATPase enzymes (Rpn1, Rpn2 and Rpn13). Rpn1 and Rpn2 are responsible for the translocation of the target protein to the proteolytic chamber along with the ATPases, which provide the necessary energy. Rpn13 is a ubiquitin receptor, recognizing the target protein. Inside the "barrel", the protein is cleaved into small peptides. The lid is composed of 9 other Rpn subunits (Rpn3, 5-9, 11,12, and 15). Lid subunits like Rpn11 stabilize the weak interactions between the CP and RP [81]. Three different types of catalytic activity were described for UPS: trypsin-like, that cleaves hydrophobic bonds, chymotrypsin-like, that cleaves at basic residues, and caspase-like activity, that cleaves after acidic amino acids [2, 81].

The regulatory subunits recognise proteins tagged with ubiquitin moieties as their targets. Ubiquitin is a small (8kDa) protein that covalently binds to proteins on specific amino acids residues in a post-translational modification called ubiquitination. To add a ubiquitin moiety to a target protein, there are three different steps characterised by the action of three different ligases, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-carrier enzyme) and E3 (ubiquitin-protein ligase) [67, 80-82]. This process can occur once, leading to mono-ubiquitination of the target, or several times on the same residue (usually lysine), leading to polyubiquitination. At least, 4 ubiquitin moieties are required for a protein be targeted by the UPS. The most common signal for recognition by the 26S proteasome is K48-linked polyubiquitinated chains [82].

Once the target protein is recognized by the proteasome for degradation, specific deubiquitinase enzymes (DUBs) remove the ubiquitin moieties, which can be latter reused [83].

1.2.3.1. Regulation of UPS

Studies on several model organisms revealed that the proteasomal activity is transcriptionally regulated by several genes. Rpn4 is a yeast transcription factor that controls levels of the UPS genes with the common sequence PACE (proteasome associated control element) in their promoters. Importantly, besides activating the UPS, Rpn4 is also a substrate target by proteasomal degradation upon its assembly. Rpn4 can, therefore, regulate the UPS assembly and function at two different levels, transcriptionally (by the PACE promoter sequence) and post-translationally (being targeted by the UPS itself) [81, 84, 85].
Evidence suggests that UPS can also interact with the other major proteolytic system, autophagy-lysosomal system (ALP), though the exact mechanisms of crosstalk remain elusive [86]. Recent findings suggest there is an overlap of target proteins between both proteolytic systems, as ubiquitin-tagged proteins can also be degraded in the cytosol by autophagy. Proteasomal inhibition with various compounds (bortezomib, MG132, lactacystin, etc.) or by genetic abrogation is reported to modulate autophagy [87-89]. For instance, bortezomib is reported to increase relative expression levels of *ATG5* and *ATG7*, known autophagy related genes [89, 90].

1.2.3.2. UPS and Ageing

Loss of proteostasis is a hallmark of ageing and thus, with the progression of ageing over time, the proteotoxic burden on the protein quality control systems is aggravated by the accumulation of damage and misfolded proteins [1, 67, 91]. In addition, many reports across several model organisms described a functional decline in UPS activities with ageing [92], associated with different molecular defects. For instance, diminished expression of proteasomal subunits [93], imbalances in α and β catalytic subunits, defective expression of regulatory subunits [94], and deleterious post-translational modifications in proteasomal subunits [95]. Other factors not directly related with the UPS might contribute to this aggravated UPS impairment during ageing, such as lower ATP levels, lower levels of free ubiquitin molecules, and major oxidative stress to UPS target substrates [96]. ATP molecules are necessary for proper 26S proteasome assembly with ATPases on the RP complex, and severe oxidation of proteins impairs their recognition by the UPS. In yeast, UPS activities are reported to decline during stationary phase [91, 97]. The decrease UPS activity during ageing is suggested to be linked to increased 26S

proteasomes disassembly and proteophagy, inactive proteasome degradation by autophagy [98, 99]. During starvation, proteasomal subunits can also be stored in cytoplasmic structures called proteasome stress granules (PSGs) that protect them from autophagic degradation [87]. PSGs have physiological relevance as they protect proteasome from stress and degradation and give a fitness advantage to cells during ageing [100]. PSGs can disband and release active proteasomes when nutrient availability is no longer limiting [99]. PSG formation requires fully assembled 26S proteasomes [101]. During stationary phase, impaired or inactive proteosomes can also be protected from degradation via storage in PSGs [98]. Just as functional decline of the UPS is associated with ageing, maintaining UPS activity seems to be required for lifespan extension on genetic and dietary interventions, highlighting the importance of this proteolytic system to cellular homeostasis [67, 94, 97].

1.2.4. The autophagy pathway

The autophagy-lysosome (vacuole in yeast) pathway is another major proteolytic system of the proteostasis network for the disposal of cargos such as long-lived proteins and whole organelles. Basal levels of autophagy promote the clearance of old proteins and the removal of damaged organelles. Autophagy is also induced in response to stress conditions, by nutrient and energy sensing pathways [102]. Deregulated or impaired autophagy is deleterious to cell's homeostasis [103-105].

Autophagy is also a membrane trafficking process that translocates its cargo to the lysosome/ vacuole for degradation. In higher eukaryotes, three different mechanisms exist: Microautophagy is the direct invagination of the lysosome/vacuole membrane to envelope small portions of cytoplasm [106]; Macroautophagy, herein called autophagy, involves the formation of a double membrane vesicle - the autophagosome - that later fuse with the lysosome (vacuole in yeast) where degradation occurs; Chaperone-mediated autophagy (CMA) degrades soluble intracellular proteins through direct engulfment by lysosomes.

Although autophagy was considered for a long time to be a bulk, non-selective process in the target of its degradation, selective autophagy processes also exist. CMA requires the molecular chaperone Heat shock cognate 70 (Hsc70) to recognise a specific amino acid motif (KFERQ) on the target protein promoting its binding to the LAMP-2A receptor on the surface of the lysosome [107]. This particular type of macroautophagy does not exist in yeast. Organelle-specific autophagy pathways exist for the degradation of damaged organelles such as mitochondria (mitophagy), peroxisome, the endoplasmic reticulum (ER), nucleus and lysosomes [108].

In the yeast *S. cerevisiae*, autophagy occurs mainly by two mechanisms: macroautophagy and microautophagy. Other pathways in yeast include cytoplasm-to-vacuole targeting (Cvt) [109] and secretory autophagy [110]. Cvt pathway transports cytosolic proteins to the yeast vacuole where they are degraded. This pathway depends on heat shock proteins of the Hsp70 family for protein vacuolar import and a functional vacuole ATPase for degradation [109]. Secretory autophagy has been recently described, as a non-degradative autophagic process that ties with unconventional protein secretion pathways. In contrast to degradative autophagy, and sharing parts of the canonical autophagic machinery, cytoplasmic constituents may be transported inside autophagosomes and secreted to the extracellular milieu [110]. For example, the secretion of Acyl coA binding protein 1 (Acb1) requires proteins from the autophagic machinery and proteins involved in the multivesicular bodies (MVBs) pathway like the ESCRT complex [111, 112]. These recent findings in unconventional secretory pathways in yeast are detailed further in the upcoming **section 1.3.3** (Endosomal/Multivesicular bodies (MVBs) pathway). Nevertheless, autophagy relies on an extensive and intricate molecular machinery, which is to be described in further detail on the section below for the yeast model.

1.2.4.1. Autophagic machinery

Autophagy has been observed in higher eukaryotes for a long time. The discovery of the lysosome compartment by De Duve coined the term in the 1960s, but only with the yeast model organism, some of the genes and molecules involved in this pathway started to be identified at the end of the 20th century by Ohsumi and colleagues [113, 114]. More than 40 different autophagy-related (ATG) genes were discovered in yeast so far [115-119].

Briefly, autophagy starts with a step called induction, followed by cargo recognition (only for specific types of autophagy), packaging where autophagic structures called phagophores are built to carry the cytoplasmic components [81, 120]. Vesicle nucleation, expansion and completion steps occur when phagophores elongate to close themselves into double membrane vesicles called autophagosomes. Later the autophagosomes fuse with lysosome or vacuoles. There, the inner membrane and cytosolic cargo is degraded, and the metabolic building blocks exported out of the vacuole/lysosome in a final recycling step [119, 120]. Atg proteins are involved with different steps of this pathway: Atg1, Atg13 and Atg17 form a complex involved in induction; Vps34, Vps15, Atg6 and Atg14 form the class III phosphatidylinositol 3-kinase (PtdIns3K) complex I, to coordinate the formation of the autophagosome; two ubiquitin-like protein conjugation systems, Atg12 and Atg8, participate in the expansion of the autophagosome membranes, Atg9 on the vesicle completion step, Atg15 on lysosomal/vacuolar breakdown and Atg22 on the recycling

final step [119, 121]. Amongst all the proteins that participate in the formation and degradation of autophagic machinery, Atg8 is the only one associated with autophagic membranes until the end of the process. This makes Atg8 an important marker for monitoring both the phagophore, autophagosome, and overall autophagic flux [105, 122]. Atg8 homologue in mammals, LC3 (microtubule-associated protein 1 light chain 3), is also the most widely used autophagy marker. All this autophagic machinery in yeast localizes at a perivacuolar site, the phagophore assembly site (PAS) [121] (Fig. 9).

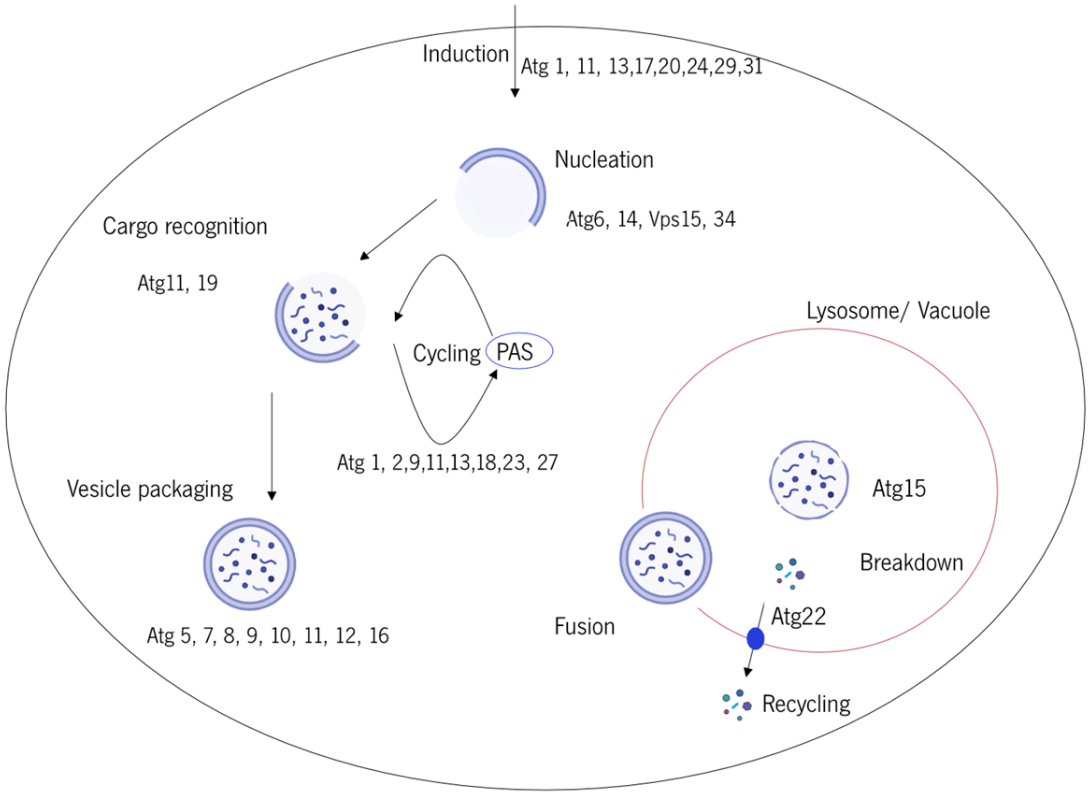

Figure 9. Different steps and proteins involved in the macroautophagy machinery assembly, from induction, cargo recognition, packaging, to cargo degradation and recycling steps in yeast. Adapted from [119].

1.2.4.2. Signalling pathways that regulate autophagy

Autophagy can be regulated by nutrient-sensing pathways coordinated by the master longevity regulators on yeast. TOR signalling network is involved with autophagy induction in yeast by the activation of the TOR-Atg1-Atg13-Atg17 kinase complex. TORC1 itself is a negative regulator of the complex, dependent on nutrient availability. Under nutrient rich conditions, TORC1 is activated promoting autophagy inhibition, while during starvation conditions, the complex is inactive, promoting the induction of autophagy. An activated TORC1 hyperphosphorylates Atg13, preventing it to interact with the PtdIns3K complex [121,

123]. *ATG1* expression is upregulated during autophagy induction [102, 123, 124]. Besides this direct interaction with the Atg proteins, the TOR pathway can also regulate autophagy through other downstream actors. TOR, a negative regulator of autophagy, can act upon the Tap42 protein, under nutrient availability conditions, inhibiting its association with PP2A. PP2A acts on several TOR associated proteins, such as by dephosphorylating Gln3. Under starvation, Gln3 is dephosphorylated and several genes, including *ATG8* and *ATG14* expression levels are upregulated, inducing autophagy [59, 120].

The Ras/PKA pathway and Sch9 can also both negatively regulate autophagy [54, 64, 125]. Atg1, Atg13, Atg18 and Atg21 were found to be PKA substrates [124, 125]. The stress resistance transcriptions factors Msn2/Msn4 and the Rim15 protein are the common downstream target of both PKA and Sch9 [59, 67, 102]. The involvement and overlapping of these master longevity regulators and nutrient-sensing pathways in the autophagy induction regulation, demonstrates the relevance of autophagy for ageing and organismal longevity.

As mentioned before, recent findings suggest regulatory or compensatory mechanisms of crosstalk between autophagy and UPS, though the exact mechanisms of crosstalk remain elusive [104]. Organelle-specific autophagy, proteophagy, is responsible for degradation of inactive proteasomes, which is especially relevant during ageing or stress and starvation conditions. Proteophagy in yeast requires the Cue5 ubiquitin receptor, the Hsp42 chaperone to promote the aggregation of proteasomes into insoluble protein deposit (IPOD) structures, and extensive ubiquitination of the UPS complexes [99]. Cue5 can simultaneously bind to Atg8 and ubiquitinated proteasomes [100].

Interestingly, as mentioned before, yeast proteasomes can localize in structures called proteasome stress granules (PSGs), to protect against degradation during stationary phase. Proteasomes are translocated from the nucleus to the cytoplasm within PSGs, in a swift and reversible process [99, 100]. Conversely, several autophagy-related proteins are ubiquitylated and degraded by UPS. Autophagy is reported to be upregulated when proteasomal activity is impaired and some reports associated failure in autophagic machinery with an increased UPS activity compensatory mechanism [86, 87, 126]. These putative crosstalk mechanisms could be affected in conditions of ageing and disease [86].

1.2.4.3. Autophagy and ageing

Like the other systems of proteotasis network, autophagy as a quality control system, plays a cytoprotective role on homeostatic levels [64, 127]. Autophagy is thought to be critically important in maintaining protein homeostasis, especially in post-mitotic cells, that do not benefit from the dilutions of

cellular damage that comes with cell division [128]. Autophagy was also described to be required for G_1/G_0 cell cycle arrest and quiescence in yeast cells [129]. However, decreased autophagic activity during ageing has been described [107, 130] in several model organisms such as flies, worms, yeast and mammals [115, 128]. Cargo packaging may still occur, but the fusion with the lysosomal compartment can be significantly impaired [67, 115]. Immature autophagosomes are reported to accumulate over time in the worm model organism [131]. Autophagy could also lose its selectivity for cargos and sequester inappropriate cargo, *i.e.*, properly folded proteins or functional organelles [20, 67]. A decrease in the *ATG* genes expression levels is reported also in most of these organisms [128]. This loss of proteostasis could be further aggravated by ageing-associated diseases that cause proteotoxic stress by protein misfolding and aggregation [2, 107] such as several neurodegenerative conditions (like Alzheimer's or Parkinson's) [20, 128]. Impairment of a major protein clearance pathways such as autophagy hampers the degradation of the protein aggregates formed by these diseases and thus facilitate ageing and disease progression. Importantly, mitochondrial function and mitophagy quality control are essential for cellular homeostasis during ageing. Mitophagy, organelle specific autophagy, is necessary for the clearance of damaged mitochondria, but also to promote the synthesis of new, functional ones, in a quality control balancing mechanism. In spite of this, decreased mitophagy is described on several organisms to be associated with ageing. Mitochondrial damage mediates the recruitment of Parkin protein by the PINK1 serine/threonine kinase in the inner mitochondria. Parkin protein then promotes mitophagy by the ubiquitination of mitochondrial surface proteins for autophagic degradation [17, 132, 133]. Genetic abrogation of *ATG* genes reduces lifespan of several model organisms [23, 25, 64, 134]. On the other hand, preservation of autophagic activities by overexpression of autophagy related genes in transgenic mice throughout their later life resulted in increased lifespan and protein homeostasis [135, 136]. Pharmacological or environmental interventions (such as CR) known to promote lifespan across several model organisms depend on autophagic activity for their beneficial effects [67, 97, 128].

All the above data from genetic and pharmacological manipulations seems to point towards autophagy as a key proteolytic system targeted by several signalling pathways to modulate lifespan. Despite all the gathered evidence showing that autophagy is needed for lifespan extension, late-life exacerbate levels of autophagy could also prove deleterious to cells and accelerate ageing itself [64, 97, 127]. Studies on yeast point towards shortening of lifespan and increased cell death associated with sustained activation of autophagy at later stages of CLS [64, 97, 137]. Autophagy targets key proteins in the DNA damage response (DDR) and DNA repair mechanisms, such as Rnr1, Mec1 and Rad53 [137]. Untimely or inappropriate cell cycle re-entry of post-mitotic yeast into the cell cycle, promoted by proteotoxic stress,

activates the DDR and autophagy, leads to cell death and premature ageing [137]. When prematurely aged yeast cells are subject to CR intervention, lifespan extension is accomplished through the maintenance of autophagy at homeostatic levels [23, 97]. This data hints at a threshold when the deleterious effects of autophagy start outweighing the benefits to homeostasis and that a tight balance is paramount to extend lifespan.

1.3. Cell-nonautonomous mechanisms of ageing

A growing body of evidence strongly hints that the ageing phenomenon cannot be explained only by the cell-autonomous mechanisms, and that a cell-nonautonomous dimension of coordinated, multidirectional signalling between different cells, tissues or even organisms also exists [40, 138-142]. A plethora of transmissible factors can modulate yeast longevity, either acting as lifespan-extending or -shortening, both on the cells that produce them and secrete them to the extracellular milieu, but also on other receptor cells on the same yeast population. These other cells exposed to the transmissible factors by cell-nonautonomous mechanisms of ageing, would then alter their lifespan and physiological or molecular stress responses through many of the already known master longevity regulators of cell-autonomous mechanisms [40, 143]. These metabolites can act as low molecular weight transmissible factors and those already identified include glycerol, hydrogen peroxide, amino acids, spermidine, hydrogen sulfide (H_2S), acetic acid and ethanol [31, 44, 47, 49, 66, 144-147]. Other metabolites that modulate lifespan through cell-autonomous mechanisms such as NADPH, trehalose, sphingolipids, fatty acids, or diacylglycerols are not yet described as transmissible factors of cell-nonautonomous mechanisms [143]. It is worth pointing out that even so, these metabolites within the “host” cell still trigger responses that

lead to the production of acetic acid and other metabolites that can act as transmissible factors themselves [143] (Fig. 10).

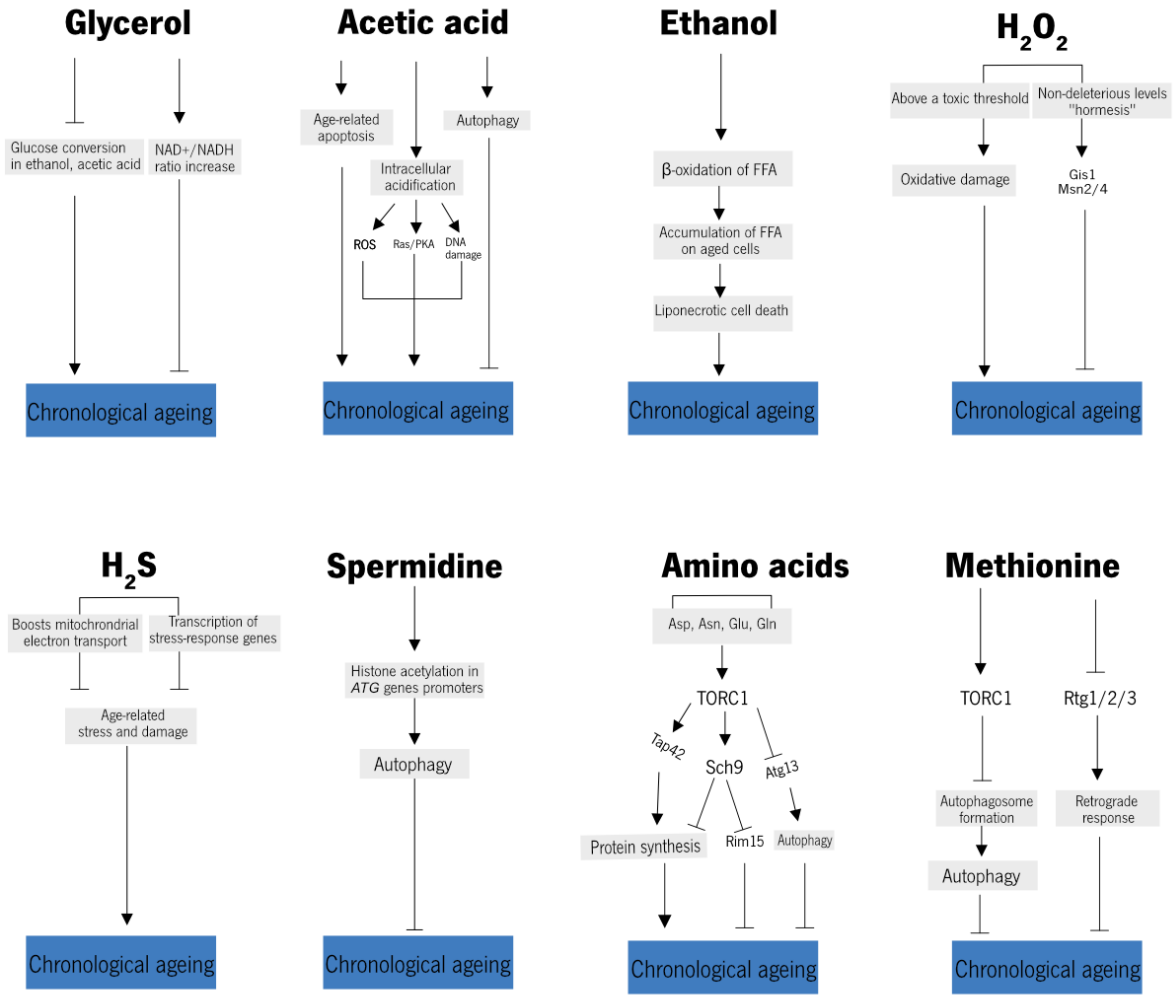

Figure 10. Several metabolites involved in cell-autonomous and cell-nonautonomous mechanisms of ageing in yeast can have either pro- or anti-ageing outcomes. Adapted from [143].

Ethanol and acetic acid are transmissible factors for yeast lifespan shortening. Both are products of glucose fermentation that are released to the liquid culture medium on stationary phase, post-mitotic yeast and interfere with yeast cell's ability to produce TCA cycle intermediates in the case of ethanol, and intracellular acidification that activates the Ras/PKA nutrient-sensing pathway in the case of acetic acid [40, 143].

Quorum sensing is a microbial mechanism to regulate physiological or morphological responses to differences in cell density [148]. Diffusible chemical signals called bacterial pheromones are essential for the formation of biofilms, bioluminescence, production of pathogenic factors, antibiotic, or other metabolites [149]. Signalling mechanisms akin to quorum sensing exist in several fungi species. [40, 49,

150, 151]. The pathogenic fungi *Cryptococcus neoformans* secretes proteins that function as virulence factors to the extracellular medium [152, 153].

Pheromone signalling is the most well-established pathway of quorum sensing and intercellular communication in yeast [149, 154]. Depending on environmental cues, this mechanism may contribute to switch the fungi from unicellular yeast form to a filamentous form. Farnesol and tyrosol were discovered to control filamentous growth in *Candida albicans* [148]. The parasitic fungus *Histoplasma capsulatum* exists on the soil as a saprophytic filamentous fungus. However, if aerosolized filaments are inhaled by animals, *H. capsulatum* switches to yeast form growth, invading macrophages. Some cell wall polysaccharides are described as the quorum sensing messenger molecules [155]. Quorum sensing molecules were also described in other fungi species like *Neurospora crassa* or *Ceratocystis ulmi* [148] Cell-autonomous and cell-nonautonomous mechanisms have been implicated with regulating yeast longevity[154]. In *S.cerevisiae*, aromatic alcohols, phenylethanol and tryptophol, are also described as quorum sensing molecules [148, 154]. These two aromatic alcohols are derived from the amino acids phenylalanine and tryptophan and induce filamentous growth on stationary phase, ageing yeast cells, via the Flo8 transcription factor which is a target of the Ras/PKA nutrient sensing pathway [149, 155]. Nitrogen availability also regulates yeast quorum sensing. High amounts of ammonium ion repress filamentous growth and two genes, *ARO9* and *ARO10*, required for aromatic alcohol synthesis [155]. Herker and co-workers had also reported that unidentified secretions from older yeast cell could stimulate other cell's survival in stationary phase. The authors proposed that older yeast cells commit altruistic suicide by apoptosis to provide nutrients to younger yeast cells [156, 157].

Though it was known for many years that secretory vesicles produced by yeast mother cells are required for the formation of a new daughter cell [158], only more recently, it was reported the formation and release of extracellular vesicles (EVs) through the cell wall on yeast and other fungi species [159-166]. EVs from fungi were found to contain several classes of molecules, from proteins, neutral lipids, polysaccharides, and RNA [160, 162, 164-168]. Nearly all species from the three domains of life produce EVs, which comprise larger microvesicles and smaller (<150 nm) exosomes of endosomal origin [169]. EVs are thought to be implicated in the pathogenesis of many diseases, and to play relevant roles not only on intercellular communication and cell-nonautonomous mechanisms of ageing as a cargo delivery vector for the transmissible factors, but also as pathways for disposal of unwanted or deleterious cell components [170, 171]. The latter could relate to the proteolytic systems (such as autophagy) [170, 172]. Recently, EVs have been associated with horizontal transmission of prions, self-replicating infectious protein particles [173, 174], in humans. An interesting example is that of the human protein α-synuclein, involved

in the pathogenesis of Parkinson's disease (PD), an age-associated neurodegenerative illness. Prions are mechanisms of epigenetic inheritance in yeast, like the Sup35 translation termination factor, recently described to be enveloped into EVs in yeast secretome [175].

EVs appear to be produced by several different secretory pathways including the conventional ER-Golgi secretory pathway and the endosomal/Multivesicular bodies (MVB) pathway [163, 170, 176]. Further details about EV biogenesis can be found on the upcoming **sections 1.3.1** to **1.3.3**. The relevance of EVs in the modulation of ageing and of the cell-nonautonomous mechanisms is still to be uncovered.

1.3.1. Extracellular vesicles, microvesicles and apoptotic bodies

Changes in intercellular communication are a known hallmark of ageing and can be regulated by cell-nonautonomous mechanisms [1, 40, 143, 176]. As referred to above, these mechanisms involve the transmission of low-molecular weight longevity factors, representing diverse classes of molecules, that elicit alterations in longevity and different cellular responses on the cells that receive them [40, 143, 160, 170]. Rather than the secretion of these molecules in free form into the extracellular milieu, recent research has focused on their delivery encapsulated within extracellular vesicles (EVs) [169, 170, 172]. EVs can carry and deliver cargos to receptor cells containing among others, metabolites, proteins, or RNA species in a horizontal transmission mechanism of biological information [172, 177]. EVs have been shown to play different roles on many physiological processes and pathogenesis in mammals [178-180]. Cancer cells secrete EVs that stimulate tumour progression [176]. Neurons and microglia secrete EVs containing pathogenic proteins such as prions [181], mutated superoxide dismutase [182], β-amyloid peptide [183] and α-synuclein (aSyn) [173, 174], most of them implicated in neurodegenerative diseases associated with ageing progression. The term EVs engulfs a wide array of different types of vesicles, with different origins and functions, but all are composed by a phospholipid lipidic bilayer and transmembrane proteins identical to those found in the plasma membrane and other biological membranes. Some types of membrane lipids were found to be enriched in EVs regarding other types of membranes, like sphingomyelin, phosphatidylserine, cholesterol, saturated fatty acids, and ceramides [184]. Some of these features (sphingomyelin and cholesterol) are similar to subdomains of the plasma membrane called lipid rafts. Lipid rafts act as scaffold for signal transduction and membrane trafficking pathways. In yeast, lipid rafts are enriched in sphingolipids and ergosterol, the fungal counterpart of mammalian cholesterol [169, 185]. Lipid rafts are part of the conventional secretory pathway, being synthetized in the yeast ER and help sorting immature secretory granules (ISGs) at the trans-Golgi network (TGN) [185, 186]. Given this

level of shared features between EVs and biological membranes, characterizing EVs on their molecular composition is not straightforward. On mammalians, membrane surface proteins have been described as EVs biomarkers, such as several tetraspanins (CD63, CD8 and CD9), ER chaperone calnexin and ESCRT-associated proteins (ALIX and TSG101) [169]. However, none of these markers is truly universal across EVs subtypes, and across different species. EVs can be characterised by their size and presumed biogenesis pathway into apoptotic bodies (>800 nm), microvesicles (100 to 1000 nm), and exosomes (ranging from 40 to 150 nm) [169, 176, 187, 188]. Apoptotic bodies have been well-known for decades, as the result of a regulated cell death process. During the final stages of apoptosis, plasma membrane blebbing occurs and the cell contents are divided into several membrane vesicles, named apoptotic bodies [189]. Only more recently, the notion that other types of vesicles also shed from the plasma membrane of viable cells has been uncovered. Exosomes are released through the endosomal/MVBs pathways, from endocytosis-derived clathrin-coated vesicles (CCV) to endosomal maturation, where late endosomes containing intraluminal vesicles (ILVs) fuse with the plasma membrane. Microvesicles bud directly from the plasma membrane, through a different process from endosomal/MVBs formation of exosomes, which is still not fully elucidated (Fig. 11).

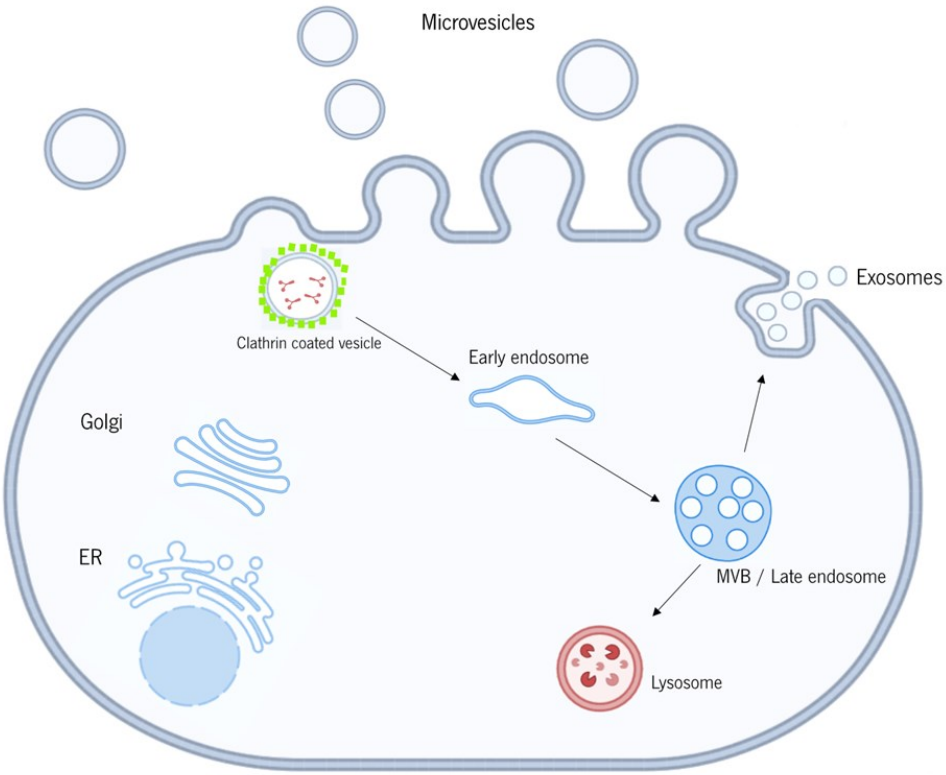

Figure 11. Biogenesis and release of microvesicles and exosomes in eukaryotes. Adapted from [179].

Several authors have attributed this process to ESCRT-mediated trafficking [190], GRASP/Grh1-mediated unconventional secretion [163] or elements of ER-Golgi conventional secretion including the Sec4 GTPase [162].

In fungi, though the conventional secretory pathway was already known, only more recently, the release of EVs was reported in pathogenic and non-pathogenic fungi species like *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Candida albicans*, *Sporothrix schenckii* and *S. cerevisiae* [153, 159, 160, 162, 164, 168, 191]. These vesicles were found to carry proteins, lipids, and polysaccharides [162, 191]. Oliveira and co-workers reported that both conventional and unconventional secretory pathways are required for EVs biogenesis in *S. cerevisiae*. Using *SEC4* and a *SNF7* mutants to genetically abrogate the conventional ER-Golgi and the endosomal/MVB pathways respectively, they reported that neither mutant impeded completely EVs' release. However, changes in vesicle diameter, kinetics of EVs release and membranes sterol contents were observed [162]. The authors also suggest the participation of other players between conventional ER-Golgi and unconventional endosomal/MVB pathways like the ESCRT machinery or GRASP (Grh1) -mediated unconventional secretion [163]. EVs' biogenesis in the yeast model is still not fully understood and underlines the interconnectedness of the secretory pathways and trafficking machinery.

1.3.2. Conventional protein secretory pathway

Secretion or vesicle trafficking is a fundamental physiological process on all living organisms, that delivers proteins and other cargo to the extracellular medium. Most proteins secreted to the extracellular milieu in eukaryotes follow a well-defined route, called the conventional pathway [186, 192]. These proteins have a common feature, a N-terminal signal peptide, which directs the protein to enter the endoplasmic reticulum (ER). The signal sequence is recognized by the signal recognition particle (SRP), which then interacts with the receptor SR. Inside the ER lumen, the signal peptide is cleaved, and the protein may be glycosylated. Then, the protein is folded by molecular chaperones [193]. If the proteins end up misfolding, the UPR or ERAD pathways are activated. If, by contrast proteins reach their proper conformation and are not ER-resident proteins, then they are trafficked into the Golgi apparatus (GA) inside coat protein II (COPII) vesicles. These COPII vesicles bud from specific sites on the ER membrane called ER-exit sites and are directed towards *cis*-facing Golgi apparatus. From the Golgi apparatus, proteins can go on two opposite directions, either shuttled back towards the ER via COPI vesicles (retrograde trafficking) or continue through the GA into the trans-Golgi network, where they mature into membrane bound secretory vesicles or ISGs (anterograde trafficking) [111].

1.3.2.1. The trans-Golgi network (TGN)

The Golgi apparatus (GA) is composed of a series of cisternae arranged in stacks, with a cis to trans polar orientation, with three distinct regions being recognized in each Golgi: *cis*, medial, and *trans*. In yeast, the GA comprises individual cisternae that occasionally associate, but form no stacks, although the cis-trans polarity feature is still present [194]. The trans-Golgi network (TGN) is adjacent to the last *trans* cisternae of the GA, and consists of a tubular-vesicular compartment, where COPII coated vesicles mature. The GA cisternae constantly mature from cis-to-trans and secretory proteins within are transported along, in an anterograde direction, up to the TGN. COPII vesicles fuse into newly formed *cis*-cisternae, *cis*-cisternae mature into *trans*-cisternae and the *trans*-most cisternae of the GA eventually mature into a TGN compartment. COPII coated vesicles in the TGN mature and are sorted to their destinations: into secretory vesicles (SV) and immature secretory granules (ISG) by different cargo adaptor protein complexes, or to lysosomal/vacuolar transport (Fig. 12). In yeast, the "exomer", is the single adaptor mediating protein transport from the TGN to the plasma membrane. Immature secretory granules containing large amounts of cargos accumulated in the cytoplasm, until they mature into mature secretory granules (MSGs) with a specific stimulus (*e.g.* lipid rafts) and are bound to the plasma membrane. Secretory vesicles in yeast are transported to the plasma membrane by the Sec4 GTPase, through actin filaments or microtubules [195]. Tethering between the plasma membrane and SVs or MSGs is mediated by the "exocyst" complex. The exocyst complex consists of eight subunits, including 6 proteins of the Sec family (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15), Exo70, and Exo84 [196, 197]. Vesicles then fuse with the plasma membrane, releasing their contents to the extracellular medium.

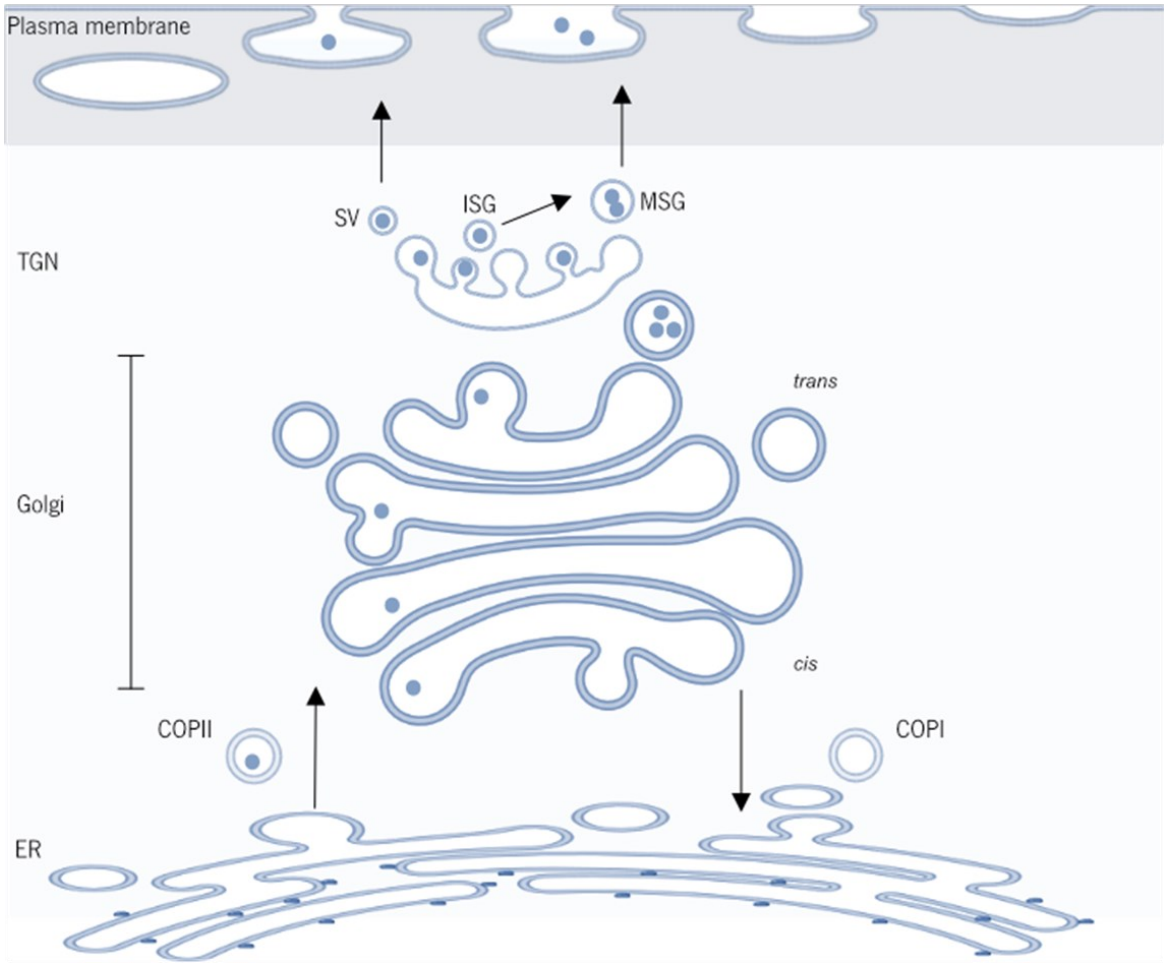

Figure 12. Schematic representation of conventional ER-Golgi protein secretion in eukaryotes. Adapted from [186].

1.3.3. Endosomal/Multivesicular bodies (MVB) pathways

A substantial number of proteins in eukaryotes are secreted without a signal peptide and bypassing the conventional ER-to-Golgi secretory pathway. These alternative trafficking pathways are known as unconventional protein secretion and encompass several diverse mechanisms. The most common are the endosomal/multivesicular bodies (MVB) pathways, which are interconnected [111, 192, 198].

The endosomal pathway starts with the retrograde trafficking pathway of endocytosis, by which plasma membrane invaginations form vesicles to carry extracellular contents to the cytosol. Various mechanisms of endocytosis exist in eukaryotes; however, the major pathway is clathrin-mediated endocytosis (CME), herein referred as endocytosis. The components of the endocytic machinery are largely conserved between yeast and mammals. Endocytosis starts when endocytic coat (Pan1, End3, Sla1/2, clathrin among others) proteins cluster around the inner leaflet of the plasma membrane. Cargo recruitment proteins deliver the cargo molecules to the coated area of the plasma membrane. Actin filaments, Las17

and Arp2/3 (actin-related protein 2/3) complex start to bend the plasma membrane around the coated area, forming an invagination. Actin filaments assemble to generate forces to overcome turgor pressure on yeast, allowing membrane invagination. Arp2/3 complex promotes actin polymerization around the invagination after being activated by Myo3/5 (Type I myosins) and Las17, promoting the elongation of the invagination. Scission proteins (Rvs161, Rvs167 in yeast, amphiphysin in mammals) cut the membrane invagination, separating the clathrin-coated vesicle from the plasma membrane. The vesicle coat is then removed by Hsc70 chaperones, releasing the endocytic proteins into the cytosol and the vesicle can then fuse with an early endosome (Fig. 13) [199].

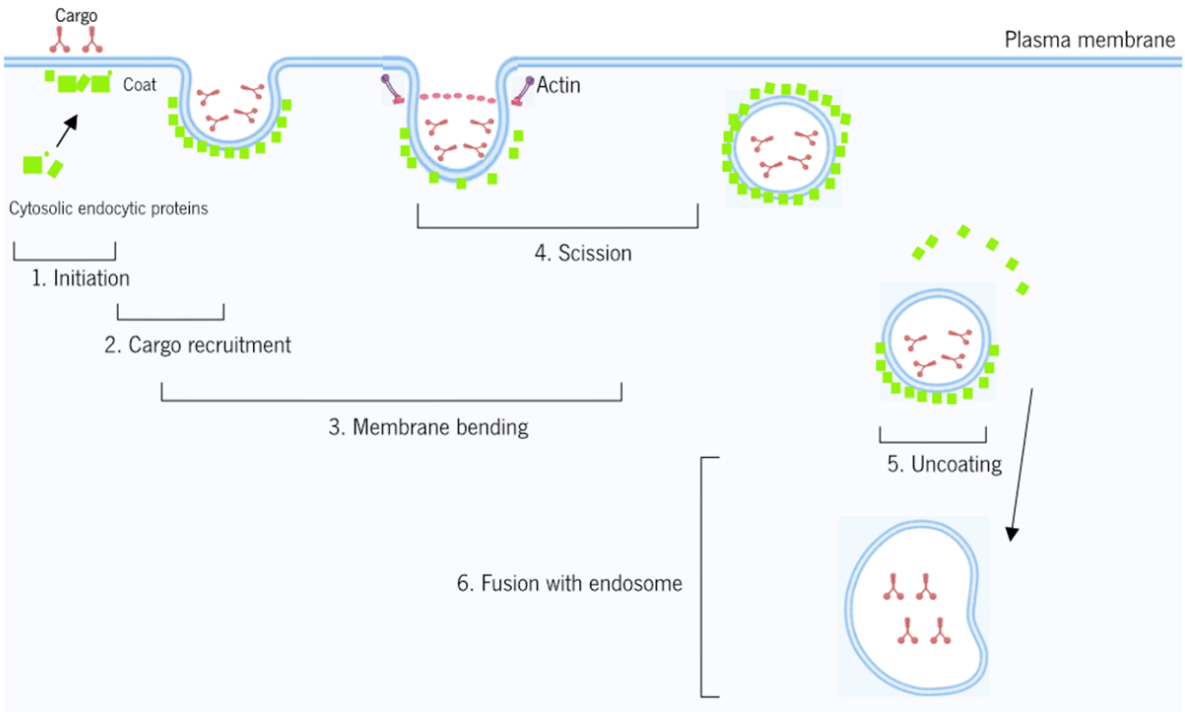

Figure 13. Stages of clathrin-mediated endocytosis and the associated molecular machinery, starting with the formation of clathrin-coated vesicles (CCV) that later on fuse with the endosome. Adapted from [199].

Early endosomes mature into late endosomes, also known as multivesicular bodies (MVBs) or multivesicular endosomes (MVEs), in the cytosol. MVBs can be very heterogeneous in size and composition, however they all contain intralumenal vesicles (ILVs). MVBs can fuse with the lysosome/vacuole compartment, secretory vesicles from the TGN, autophagosomes to form amphisomes for secretory autophagy, or be released to the extracellular space as exosomes upon fusion with the plasma membrane (Fig. 14) [176, 200, 201].

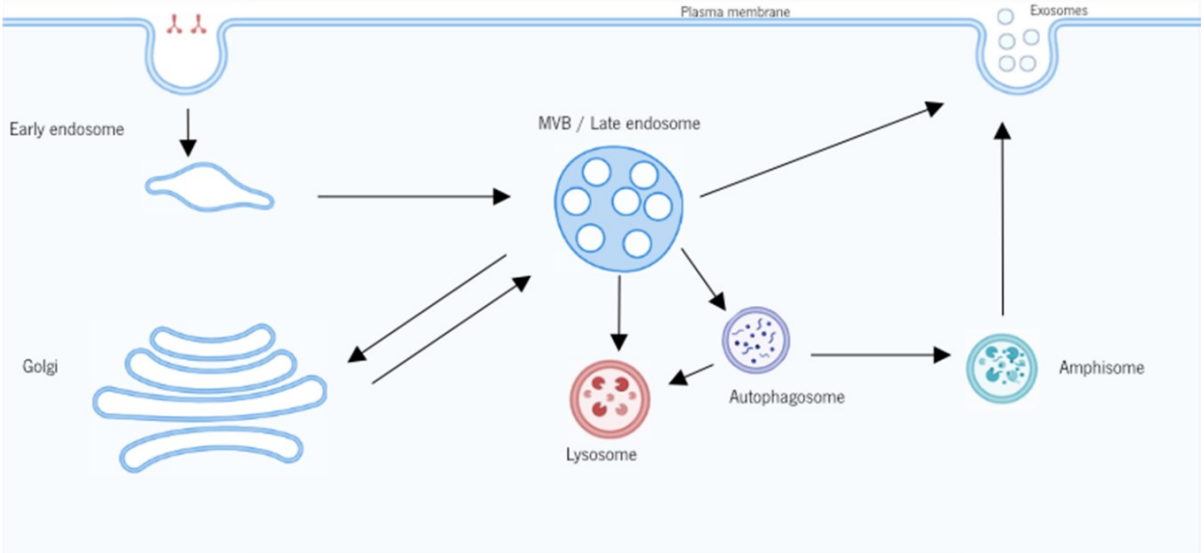

Figure 14. Vesicle trafficking pathways in eukaryotes: Early endosomes can be formed from plasma membrane invaginations and develop into MVBs and exosomes or fuse with other vesicles such as autophagosomes, secretory vesicles from TGN, or lysosomes. Adapted from [176].

The ESCRT (endosomal sorting complex required for transport) complexes are involved in many biological processes from endocytosis and endosomal trafficking to exosome secretion and autophagy [202]. ESCRT complexes are well-known to mediate processes that involve membrane bending and scission. ESCRT machinery is composed of four ESCRT complexes (0, I, II, III) and the AAA ATPase Vps4 complex [198]. ESCRT complexes sort out the cargo in early endosomes with ubiquitin tagged moieties and facilitate inward budding membrane vesicles, *i.e.,* ILVs. One accepted model for ESCRT-mediated ILVs formation is through oligomerization of Snf7, a subunit of the ESCRT-III complex that engulfs the cargo and recruits Vps24, Vps4 and Vps2 proteins to form a dome-like structure. The ESCRT machinery is essential for the formation of intralumenal vesicles in MVBs [201, 203].

Other forms of unconventional protein secretion in yeast include Acb1 proteins secretion by a special compartment for unconventional protein secretion (CUPS) and secretory autophagy. Acb1 secretion inside CUPS is reportedly tied to elements of both MVBs formation and secretory autophagy. Acb1 is only found in mature CUPS and not on MVBs. Acb1 secretion is dependent on the Grh1 protein, which upon starvation, is translocated from the ER-Golgi to larger membrane bound compartments, called CUPS. CUPS formation is independent of COPII and COPI mediated transport. Snf7, the ESCRT-III subunit, attaches to this compartment and is essential for its stability. It is suggested that CUPS might merge with autophagosomes to form amphisomes and Acb1 to be secreted via secretory autophagy [111, 204].

Secretory autophagy was first described in mammalian cells, where a variety of cytosolic proteins are exported to the extracellular milieu. These include the secretion of aggregation prone proteins and damaged organelles, also targeted by the canonical degradative autophagy. It is still not clear, what mechanisms regulate which autophagosomes and which cargo are directed to fuse with the lysosome/vacuole, and which are directed to fuse with the plasma membrane. In yeast, the protein Sso1 was identified as essential for the fusion of the amphisome with the plasma membrane. Secretory autophagy is still a novel concept, and many questions remains open-ended. However, it is safe to say that it depends on the autophagic machinery controlling autophagosome formation, the Grh1 protein in yeast (and its homologue GRASP in mammals), or the ESCRT complexes involved in MVBs biogenesis [110].

1.4. Synucleinopathies related to protein misfolding and ageing

Ageing is recognized as the primary risk factor for many major human pathologies, including neurodegenerative disorders [1, 2]. Loss of proteostasis, promoted by the age associated decay in the protein quality control systems that degrade misfolded proteins, is a common motif in most neurodegenerative diseases in humans. The prevalence of neurodegenerative disorders is much greater in the older population, which prompted many authors to refer to them as age-associated diseases [1, 205-207].

Parkinson's Disease (PD) is the second most common neurodegenerative disorder worldwide, affecting millions of elderly people and about 1% of the world population over 60 years [207-209]. The worldwide increase in life expectancy, with estimates pointing towards 2 billion people over the age of 60 in the year 2050 [208-210], stresses the urgency of understanding PD and other synucleinopathies pathophysiology to better improve patients' quality of life.

PD is characterised at the clinical level by a set of distinct symptoms on motor dysfunctions, including resting tremors, muscle rigidity, bradykinesia, mask face and postural reflex impairment [209, 211]. The main neuropathologic events behind these PD motor dysfunction symptoms are the loss of dopaminergic neurons in the *substantia nigra pars compacta* brain region and the formation of Lewy bodies or Lewy neurites composed of aSyn aggregates in the remaining dopaminergic neurons [211].

The pathogenesis of PD seems to revolve around the loss of proteostasis, oxidative stress, mitochondrial dysfunction, and ageing progression [20, 77, 209, 210, 212-214]. The majority of the cases of PD are sporadic, however familial forms also exist associated with mutations in several genes that encode aSyn, the synaptic protein Synphilin-1, the mitochondrial serine protease (Omi/HtrA2) and other proteins

involved in the proteolytic systems and mitochondrial clearance (Parkin, PINK1) [20, 211, 215]. Parkin and PINK1 were demonstrated to regulate mitochondrial fission, fusion, and the clearance of damaged mitochondria through mitophagy [133]. aSyn is a pre-synaptic protein present in neurons [216]. This 140 amino acid-long protein was originally identified in association with synaptic vesicles in the presynaptic nerve terminal [212]. It has a role on synapsis, plasticity, and neurotransmission. It is also suggested that this protein can interact with vesicle trafficking, lipid metabolism, mitochondrial clearance, and the proteolytic systems [205, 217-220]. Overexpression of wild type aSyn is reported to impair vesicles fusion with Golgi apparatus. aSyn is also described to hamper endocytosis and the proteasome, to induce accumulation of ubiquitin, lipid droplets and ROS, and mitochondrial fission [221]. Under healthy conditions, aSyn is expressed across the entire brain. This protein is also a major constituent of Lewy bodies, proteinaceous insoluble aggregates. Lewy bodies are implicated in the pathogenesis of a group of age-associated, neurodegenerative illnesses called synucleinopathies. Synucleinopathies include Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [20, 206, 211].

aSyn is composed of an amphipatic N-terminal region, a central hydrophobic region and an acidic C-terminal region (Fig. 15). The amphipatic and central regions contains several sequence repeats (KTKEGV) [222]. Some point mutations on the aSyn gene, like A53T, A30P, and E46K among others, are associated with familial forms of PD [211, 223, 224]. Genomic multiplication of the wild-type aSyn locus also leads to disease progression [223].

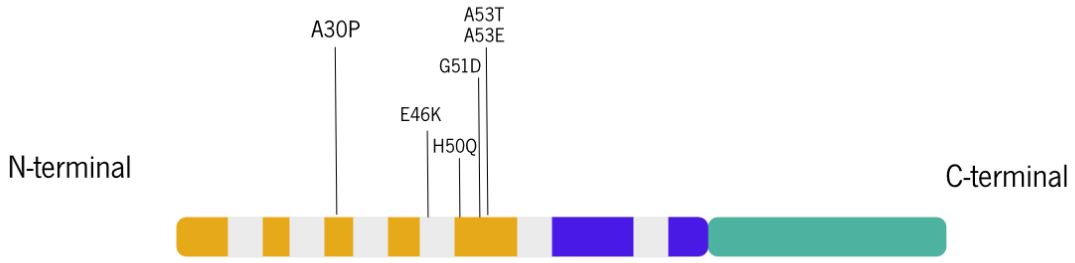

Figure 15. Schematic drawing depicting the three main regions of aSyn: N-terminal (yellow), a hydrophobic central region (blue), and C-terminal (teal). Several mutations associated with PD are listed on the amphipathic N-terminal. In light grey, several sequence repeats can be found in the N-terminal and central regions. Adapted from [210].

Some proteins like aSyn are unable to achieve any ordered three-dimensional conformation by themselves and are dependent on protein-protein interactions to properly fold [71, 72]. Moreover, aSyn is prone to

aggregation within cells in toxic inclusions composed of aSyn in fibrillar form. These aSyn protein inclusions are oligomeric complexes constituents of Lewy bodies: they arise from non-native interactions in protein folding potentiated by stress conditions. The precise principle behind toxicity on these inclusions is still unclear. The consensus so far is that the oligomeric complexes *en route* to become insoluble fibrils are the primary agents of cytotoxicity [74, 225]. Post-translational modifications have been implicated in aSyn misfolding and toxicity. aSyn in normal conditions is non-phosphorylated, while aSyn in Lewy bodies is ubiquitinated [212] and phosphorylated [226]. aSyn was also found to be glycated in PD patients, and that SUMOylation increases aSyn solubility and decreases aggregation [211].

aSyn is also implicated in PD progression between different neuronal cells through a horizontal prion-like transmission mechanism. Initially, it was thought that aSyn contribution to PD pathogenesis was exclusively of a cell-autonomous nature and that aSyn could only be located inside neuronal cells. Then, aSyn was first detected in human plasma [227] and cerebrospinal fluid [228], and that secreted aSyn could lower the viability of different types of neuronal cells [229]. The extracellular levels of aSyn are dependent on both the extent of its secretion and the effectiveness of its clearance by intracellular proteolytic systems. Various impairments of the proteostasis network within a cell such as those described during ageing and synucleinopathies pathogenesis [103, 230], could lead to an increased extracellular release of the protein [231]. Extracellular aSyn is cleared by extracellular proteolytic enzymes or taken into other cells in the vicinity to be degraded by intracellular protein quality control systems such as ALP [229]. Vesicle trafficking pathways such as endocytosis or phagocytosis have been reported to clear aSyn from the extracellular milieu of several types of neuronal cells. aSyn was found to be uptaken by clathrin-mediated endocytosis [229, 232]. CCVs are directed to the lysosome for degradation by the ALP machinery [232]. aSyn was also described to be secreted to the extracellular space by exosomes, through a calcium-dependent mechanism of exocytosis [173, 174]. By using calcium ion chelators, the aSyn-containing exosomes release can be greatly impaired [174]. aSyn enveloped in exosomes is more likely to enter receptor cells and induce proteotoxic stress than free aSyn molecules. Exosomes are a common shuttle vector for intercellular communication between neurons in the brain, activating signalling pathways and protein expression in the receptor cells. In addition, exosomes also play the role of disposal of unwanted molecules [229]. aSyn carrying exosomes have the potential to both facilitate the progression of PD and aberrant aSyn transmission between neurons, or to allow the proteolytic clearance of aSyn by other neighbouring cells, such as astrocytes and microglia, achieving homeostasis [229].

1.4.1. Ties between synucleinopathies and the proteolytic systems

aSyn, an aggregation-prone prone protein implicated in the pathogenesis of synucleinopathies, can be degraded by the ubiquitin-proteasome (UPS) and autophagy-lysosomal (ALP) systems of the proteostasis network. aSyn is a target protein for the main proteolytic systems, but concurrently, aSyn can also affect both systems' activity, aggravating disease progression [233-235]. Ageing further imbalances the tips of the scale, as both systems activities decline in a time-dependent manner[67].

aSyn aggregates sequester ubiquitin, heat shock proteins and components of the UPS [236], which could result in proteasomal dysfunction [213, 237, 238]. aSyn overexpression was also shown to inhibit UPS activity [239, 240], and genetic abrogation of 26S proteasome activity resulted in accelerated neurodegeneration and the formation of inclusion bodies in a murine model [233].

Autophagy is also reported to be impaired by aSyn in a PD context. Chaperone-mediated autophagy (CMA) is the preferred ALP pathway on mammalian brains. Mutated forms of aSyn, such as A53T, impair CMA by binding to the lysosomal membrane and CMA receptors [241, 242]. The inhibition of CMA by aSyn upregulates UPS activity in another example of coordinated crosstalk between the two proteolytic systems [86, 87, 126, 233]. However, when both CMA and UPS are impaired by aSyn, neuronal cells resort to autophagy. Autophagy in turn is also suggested to be inhibited by aSyn [242]. aSyn is reported to inhibit the Rab1a GTPase responsible for ER-Golgi transport of Atg9, essential for the formation of the autophagosome [243].

Oxidative stress and loss of mitochondrial membrane potential also play an important role in the progression of neurodegeneration in PD. Mitochondrial damage stabilizes PINK1 protein in the inner mitochondria and the recruitment of Parkin. Parkin ubiquitinates proteins for autophagic degradation by mitophagy. Overexpression of aSyn mutants increases Parkin-dependent mitophagy in neurons [244] and mitophagy in the yeast model [17]. It is hypothesized that aSyn promotes exacerbated, deleterious levels of mitophagy, removing not only damaged mitochondria, but also fully functional ones [20]. Wild-type Parkin protein was also found to activate the 26S proteasome. Parkin interacts with several subunits involved in proteasomal assembly. PD associated Parkin mutants impair the proteasome assembly [81, 245].

The cumulative failure of several protein degradative pathways (CMA, UPS, autophagy or mitophagy) provide insights into the cascade of events that leads to neurodegeneration, also potentiated by ageing. Overall, these findings support the notion of the proteolytic systems impairment and their crosstalk as a relevant hallmark in PD pathogenesis.

1.4.2. Yeast models on the study of synucleinopathies

Model organisms can be tailored to decipher the molecular basis of disease pathogenesis and progression. The establishment of good cellular or organismal models in PD, as with other neurodegenerative diseases, is a challenge and depends on the momentum in the research field. Different model systems have been employed for PD research, from higher eukaryotes immortalized cell lines and murine models, to simple unicellular organisms like the budding yeast.

The budding yeast model organism has been employed with great success as a model for ageing, as previous described. It has proven itself as a reliable, versatile system to study complex biological processes. The yeast genome is fully sequenced and annotated, yeast is very pliable to genetic manipulation and many of the key cellular pathways are evolutionary conserved from yeast to higher eukaryotes [27, 29, 68, 144]. Many of these conserved pathways (secretory pathways, master longevity regulators, proteolytic systems) bear relevance not only for ageing, but also to understand ageing-associated diseases like PD. With this notion, two different yeast systems were designed to study PD and neurodegeneration [17, 246, 247]. On the first approach, the human disease related gene has a yeast homologue, and it is possible to study the effects of genetic abrogation of the target gene or its overexpression. However, if the human gene does not have a yeast homologue, the human gene can be heterologously expressed in yeast and the relevance phenotypic changes that arise from this expression can be monitored [247, 248]. In the case of synucleinopathies, the latter, also referred to as the "humanized" yeast system, has been proven a useful approach, with different forms of aSyn expression in yeast. aSyn can be expressed in its wild-type (WT) form under a constitutive promoter, or with several mutations associated with familial forms of the disease (such as A30P, A53T or E46K) [29, 97, 137, 246]. The WT and A53T aSyn variants localize initially in the plasma membrane and are transported there via the conventional secretory pathway. The A30P variant does not enter the secretory pathway and is spread all over the cytosol. WT and A53T are toxic to yeast when overexpressed, while A30P is not [224, 246]. aSyn toxicity and aggregation in yeast cells provide a system to study both the pathogenesis of PD at a molecular level, as well as the consequences of loss of proteostasis and premature ageing [29].

With this introductory **Chapter 1**, the advances in the literature about the role of cell signalling networks during yeast ageing were summarized, whether involved in cell-autonomous or novel cell-nonautonomous

mechanisms, such as protein quality controls systems or horizontal transmission of longevity factors by conventional or unconventional secretion. Ageing is a complex biological process that can only be fully understood by an integrated view of both cell-autonomous and cell-nonautonomous mechanisms. The use of the yeast model organism and the human aSyn yeast expression model, allows for the unveiling of both dimensions, from exquisite protein quality control to metabolic and genetic modulation of ageing progression, within a single yeast cell or through horizontal transmission of molecular factors that impact longevity. The work on this thesis aimed fundamentally to understand the role of cell signalling networks during yeast chronological lifespan, at both dimensions and their impact on ageing modulation. More particularly, we aimed: 1) to elucidate the role and mechanisms of the crosstalk between the two major proteolytic systems during yeast chronological ageing, under a known non-genetic lifespan extending intervention; 2) to ascertain the presence of EVs as a cell-nonautonomous mechanisms related to cell-to-cell communication during aging; 3) to detect and identify the classes of molecules released that could conceivably act as transmissible molecular factors; 4) to investigate how the manipulation of ageing could modulate the profile of molecules released; and 5) to understand by which mechanisms did the secretion of aSyn to CM, potentially enveloped in EVs, occurred, and by which mechanisms it entered naïve receptor cells.

Over the next chapters, results will be presented about the role of cell-autonomous control of proteostasis, screening for signalling molecules involved in cell-nonautonomous mechanisms during yeast ageing, and a proteotoxic model (aSyn heterologous expression) for cell-nonautonomous mechanisms of ageing. All the protocols, materials and methods can be found on **Chapter 2**. In **Chapter 3,** we described the results obtained during the thesis. **Section 3.1**, entitled "Yeast chronological lifespan and cell-autonomous regulation of homeostasis", data will be presented about the role of cell-autonomous signalling in the crosstalk between proteolytic systems under a lifespan-extending intervention, CR, on prematurely ageing yeast cells expressing human aSyn. The data supports the notion that under CR intervention, proteasomal activities are upregulated, whilst autophagy is kept at non-deleterious, homeostatic levels. A novel body of evidence suggests the importance of cell-nonautonomous mechanisms in the modulation of ageing, involving the transmission of biological information between different cells through transmissible molecular factors. On **Section 3.2**, entitled "Cell-nonautonomous mechanisms during yeast chronological lifespan: Screening for messenger molecules", the results of a screening for different classes of molecules on the ageing yeast secretome, from nucleic acids to protein and lipids will be discussed. On **Section 3.3**, entitled "Cell-nonautonomous mechanisms and the modulation of yeast chronological lifespan: A proteotoxic stress model (aSyn)", we set out to understand

the role of cell-nonautonomous mechanisms in yeast ageing with a particular yeast expression model: the heterologous expression of human aSyn. This proof-of-concept model provided invaluable insights: aSyn containing CM induced premature ageing of naïve receptor cells; aSyn expressing cells produced particles compatible with both exosomes and microvesicles, likely carrying the aSyn protein inside. On **Section 3.4**, entitled "Cell-nonautonomous mechanisms and vesicle trafficking: Mechanistic links between retrograde and anterograde trafficking in a proteotoxic stress model (aSyn). Genetic abrogation of the endosomal/MVBs and endocytic pathways suggested the involvement of both anterograde and retrograde trafficking pathways in the horizontal transmission of aSyn to modulate yeast chronological ageing, mirroring some knowledge about aSyn prion-like behaviour on human cells.

Finally, **Chapter 4**, will feature an integrated and comprehensive discussion of the main findings and contributions of the present thesis, as well as a proposal for future advancements. With this thesis, we hope to contribute to a better understanding of the fundamental mechanisms modulating ageing.

CHAPTER 2

MATERIAL AND METHODS

2.1 Strains and plasmids

The yeast *Saccharomyces cerevisiae* strains and plasmids that were used in this study are listed on Table 1.

Table 1. Yeast strains and plasmids used in this study.

Yeast strain	**Genotype**	**Source**
BY4741	*MATa his3Δ1 lys2Δ0 met15Δ0 ura3Δ0*	Sampaio-Marques *et al.*, 2018.
BY4741 *Δpdr5*	*MATa his3D1 leu2D0 met15D0 ura3D0 pdr5::kanMX4*	*Euroscarf*
BY4741 *Δump1*	*MATa his3D1 leu2D0 met15D0 ura3D0 pdr5::kanMX*	*Euroscarf*
BY4741 *Δrpn4*	*MATa his3D1 leu2D0 met15D0 ura3D0 pdr5::kanMX*	*Euroscarf*
BY4741 *Δcue5*	*MATa his3D1 leu2D0 met15D0 ura3D0 pdr5::kanMX*	*Euroscarf*
BY4741 *Δsnf7*	*MATa his3D1 leu2D0 met15D0 ura3D0 pdr5::kanMX*	*Euroscarf*
BY4741 *Δend3*	*MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0 npi1*	Paiva *et al.*, 2009
Plasmids	**Type of Plasmid**	**Source**
pYX242-SynWT	2μ	Winderickx, J
pYX242-SynA30P		Winderickx, J
pRS416-GFPAtg8	2μ	Sampaio-Marques *et al.*, 2018.
pYES2-Ub-G76V-GFP	2μ	Addgene

2.2 Media and culture conditions

Cell stocks were maintained in YEPD agar medium containing 0.5% yeast extract, 1% peptone, 2% agar, and 2% glucose. All experiments were performed in synthetic complete (SC) medium containing glucose as a carbon source and 0.67% yeast nitrogen base without amino acids (Difco Laboratories) supplemented with excess amino acids and bases for which the strains were auxotrophic: 50 μg/mL histidine,100 μg/mL uracil and 100 μg/mL methionine.

2.2.1. Conditioned media harvest and media replacement on yeast cultures

To obtain the conditioned medium, during the cells' CLS, yeast cells were grown in SC medium at 26 °C for different time points. The cultures were then centrifuged, and the supernatants were collected. These supernatants are designed as conditioned media and were used to inoculate new cultures.

2.3 Chronological lifespan

For the chronological lifespan (CLS) assays, cells were grown on synthetic liquid media until reaching stationary phase, and this time point was considered day 0 of CLS. Survival was assessed by colony-forming units (CFUs) beginning at day 0 of CLS (when viability was 100%), and then again, every 2-3 days until less than 0.1% of the cells in the culture were viable. Cellular samples were serially diluted and platted on YEPD agar plates.

Cell's viability during CLS was also determined by flow cytometry with the fluorescence propidium iodide (PI) stain. Briefly, PI was added to about 106 cell aliquots at several timepoints of CLS. Cells were harvested, washed twice, resuspended in PBS, and stained in the dark with 5 μg/mL PI for 30 min at 30°C. The fluorescent signal was detected using a LSR II (BD Biosciences) flow cytometer with a 488 nm excitation laser. Signals from 30,000 cells/sample were captured at a flow rate of 1,000 cells/s. Data was analysed with FlowJo software (BD Biosciences).

2.3.1. Maximum and minimum lifespan

Mean and maximum chronological lifespan, which represent 50% and 10% of yeast cell's survival were determined by curve fitting of the survival data from pair-matched, pooled experiments with the Prism 8 statistical analysis software (GraphPad Software).

2.4 Pharmacological inhibition of autophagy and UPS

To block autophagy, 50 μg/μL chloroquine (CQ), or to inhibit UPS activity, 30 μM bortezomib or 50 μM MG132, were added to the culture medium at day 0 of CLS.

2.4.1. Autophagy monitoring by the GFP-Atg8 processing assay

For this assay, cells were transformed with the plasmid pRS416-GFPAtg8 with fusion gene under the control of the ATG8 endogenous promoter [249, 250]. GFP N-terminally tagged to Atg8 is delivered to the vacuole and given that GFP is more resistant to degradation than Atg8, bulk autophagy leads to accumulation of free GFP in the vacuole. The ratio between free GFP and total GFP is used as a readout for the autophagic flux. GFP-Atg8 and free GFP are detectable by immunoblotting using a GFP-specific antibody (see section Protein quantification and immunoblotting). Immunoblot bands were quantified by densitometric analysis using the Quantity One software (Bio-Rad).

2.5 Nucleic acids extraction

2.5.1. DNA and RNA precipitation from yeast culture media

DNA and RNA isolation was performed using the TRIzol reagent (Invitrogen) [251]. Briefly, 0.8 ml of TRizol reagent is added to 1 ml of culture media. The mixture is incubated on ice for 10 minutes, prior to snap-freezing at -80 C. 0.2 ml of chloroform is added to each sample. Samples are defrosted at room temperature and shaken vigorously. The mixture is centrifuged 12,000 x g, 30 minutes, 4 C. The upper aqueous phase is collected into new RNAse-free microtubes. 0.5 ml of 2-propanol is added, and tubes are left to precipitate overnight at -20 C. RNA is pelleted at 12,000 x g for 30 minutes, 4 C. The pellet was then washed once in ethanol 70% and dissolved in DEPC-treated water.
DNA and RNA were quantified by a NanoDrop (ND-100, NanoDrop Tecnhnologies) spectrophotometer and aliquots ran on 0.75 to 1% agarose gels electrophoresis with SYBR Safe (ThermoScientific) nucleic acid stain.

2.5.2. RNA extraction from yeast cells

Yeast samples were centrifuged (3500 rpm, 5 minutes, 4° C) and the pellets stored at -80 C, pending RNA extraction. RNA was extracted with 0.8 ml of cold TRIzol reagent (Invitrogen) added to the yeast pellet and the homogenate was transferred to microcentrifuge tubes containing 0.5 g of sterile glass beads, which had been soaked in sodium hypochlorite, washed with sterile water, and dried overnight in

an oven at 200 C. The suspension was shaken for 5 minutes in a vortex and incubated at room temperature for another 5 minutes. 0,2 ml of chloroform were added, and the tubes were shaken. The suspension was incubated 5 minutes on ice, prior to being centrifuged at 7,000 x g, for 15 minutes at 4 C. The upper aqueous phase was collected into new RNAse-free microtubes. 0.5 ml of 2-propanol is added, and tubes are left to precipitate 10 minutes on ice and centrifuged 11,000 x g for 10 min at 4 C. After discarded the supernatant, the pellet is washed in 1ml of cold ethanol 70%. The RNA pellet was dissolved in 50 µl of RNAse-free DEPC-treated water. The samples' purity was assessed by the A260/280 ratio on a NanoDrop (ND-100, NanoDrop Tecnhnologies) spectrophotometer and the RNA integrity by visualization on an electrophoresis 1% agarose gel with SYBR Safe (ThermoScientific) nucleic acid stain.

2.6 Quantitative mRNA expression analysis

The quantitative mRNA expression analysis was performed according to the MIQE guidelines (Minimum information for publication of quantitative real-time PCR Experiments [252]). Quantitative real-time PCR (qPCR) was used to measure the mRNA transcripts of the *RPN4* gene (FW: AAGGAAACCAGCAAAATCATC; RV: TTTCTAATGTGCCGTTTTCAT). Three reference genes (*ACT1*-actin (FW: GATCATTGCTCCTCCAGAA; RV: ACTTGTGGTGAACGATAGAT), *PDA1*-alpha subunit of pyruvate dehydrogenase (FW: TGACGAACAAGTTGAATTAGC; RV: TCTTAGGGTTGGAGTTTCTG) and *TDH2*-isoform 2 of glyceraldehyde-3-phosphate dehydrogenase (FW: CCGCTGAAGGTAAGTTGA; RV: CGAAGATGGAAGAGTTAGAGT)) were selected due to their stable expression and were tested in 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. Then, 22.5 ng of cDNA of each sample was 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. The PCR efficiency of each primer pair 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 $10^{(-1/slope)}$. 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).

2.7 Protein isolation and immunoblotting

2.7.1. Protein extraction from yeast whole-cell samples

For the detection of protein levels by immunoblot, yeast whole-cell extracts were collected at specific timepoints of CLS and extracted as follows: Cells were washed once with PBS, then pre-treated with lithium acetate 2M for 5 minutes at room temperature. Cells are pelleted by a quick centrifugation, and lithium acetate is removed. 0.4 M of NaOH were added and cells incubated 5 minutes on ice. Afterwards, cells were pelleted and resuspended in SDS-PAGE sample loading buffer and boiled for 5 minutes.

2.7.2. Protein precipitation from yeast culture media

Culture media was collected at specific time points of CLS and protein was precipitated from them, with either absolute acetone or trichloroacetic acid methods. For the first method, protein was precipitated from a volume of conditioned media using 6 volumes of cold acetone and kept overnight at -20°C. This was followed by two washing steps, with cold acetone and protein pellets were collected by centrifugation at 10,000 g, 4° C for 30 minutes as described by Niu and co-workers [253]. Pellets were then dissolved in SDS-PAGE sample buffer.

For the second method, 5 ml of pure trichloroacetic acid (TCA) were added to at least 40 ml of conditioned media. Then, the mixture is vortexed vigorously and incubated on ice for at least one hour. Samples are then centrifuged 4500 rpm for 15 minutes at 4 °C. The supernatant is discarded, and 0.5 ml of TCA 10% is added. Vortexed once more and centrifugation was carried out at 13,000 rpm, 15 minutes, 4°C. 0.5 ml of acetone were added to the resulting pellet. Centrifuged for 10 minutes at 13,000 rpm, 4°C. The supernatant was discarded, and the protein pellet was dried with a Speed Vacuum centrifuge. Finally, the pellet was resuspended in a small volume of SDS-PAGE sample buffer.

2.7.3. Protein quantification and immunoblotting

Total protein quantification was performed with the RC/DC protein assay (Bio-Rad) and processed in accordance with manufacturer's instructions. 30 μg of protein were resolved on a 12% SDS gel and transferred to a nitrocellulose membrane during 12 min at 25V in Trans-Blot Turbo transfer system. Membranes were blocked with tris buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% bovine

serum albumin (BSA). by incubation with primary antibodies against anti-α-syn (1:1000, Cell Signaling); anti-GFP (1:5000; SICGEN), and anti-PGK (1:5000; Invitrogen) in TBST containing 1% BSA and primary antibody. After washing with TBS, the membranes were incubated with the respective secondary antibody, HRP-conjugated anti-rabbit IgG, anti-mouse IgG or anti-goat IgG at a dilution of 1:5000 in 1% BSA. Membranes were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) or Clarity Western ECL Substrate (Bio-Rad) and images of the immunoblotting were obtained in a ChemiDoc XRS System (Bio-Rad) with Quantity One (Bio-Rad) software.

2.8 Determination of proteasomal activity

"Proteasome-Glo TM Cell-Based Assays" (Promega) was used to measure the proteolytic activity of the UPS. It consists in a luminescent assay to measure the chymotrypsin- and trypsin-like activities associated with the proteasome. Proteasome-Glo Cell-Based Reagents were both prepared and equilibrated at 22°C for 30 min before use. The luminescence was measured in a luminometer "Fluoroskan Ascent FL" by ThermoScientific. About $1x10^6$ cells were plated in a 96-well opaque plate at a volume of 100 μL together with "Proteasom-Glo TM Cell-Based Assays" (100 μL). The assay plates were equilibrated at 22 °C, and after incubation during 5 to 30 min, a luminescent signal was obtained. Protein concentration was used to normalize proteasome activity.

2.9 Neutral lipids extraction and characterisation

Neutral lipids samples for this study were extracted by the Bligh and Dyer [254] method, with some adaptations [255]. Briefly, 1 volume of culture media sample (from 1 up to 6 ml) is added to 3.75 volumes of chloroform/methanol (2:1) and the mixture is vortex thoroughly. Another 1.25 volumes of chloroform and water were added. The mixture was centrifuged 4,000 x g, for 15 minutes. The lower lipid enriched phase is collected to new centrifuge tubes, while the upper phase is centrifuged again with another 1.25 volumes of chloroform. The resulting lower phase is mixed with the first one. The solvent is left to dry at room temperature and the lipidic pellet is dissolved in a small volume of chloroform/methanol (2:1) (20 to 50 μl) and stored at -20 C.

Culture media was collected and stored immediately at -80C or dried in a lyophilizer for 2/3 days until all water was evaporated and a fine powder was obtained.

After extraction, lipid-enriched samples were run on TLC silica plates for lipid separation and visualization.

2.9.1. Neutral lipids extraction from vesicular fractions (EVs)

Vesicular fractions were obtained by ultracentrifugation as described (EV isolation by differential ultracentrifugation), and an adapted Bligh and Dyer method was employed to extract lipids. Briefly, about 100 µL of vesicular fractions in sterile PBS were mixed with 0.8 ml of chloroform and 0,4 ml of methanol. Extraction then proceeded as described in the above section (Neutral lipids extraction and characterisation).

2.9.2. Thin-layer chromatography (TLC)

Lipid samples were carefully loaded in silica TLC plates with glass capillaries. The plates were previously dried for 30 minutes at 100 C in an oven. After loading the samples, the plate is placed in a closed vertical glass chamber with a mixture of either chloroform/ methanol (96:4) or petroleum ether/ diethyl ether / acetic acid (70:30:1) as the mobile phase, with enough volume to touch the bottom of the plate, but not the site where the samples were loaded. After a few minutes, the mobile phase to the top of the plate and then the plate is removed and left to dry. The plate can then be incubated for a few minutes in a closed glass Petri dish with iodine crystals or dipped in $Mncl_2$ charring solution (3,15 g of $MnCl_2$, 60 ml of water, 120 ml of methanol, 8 ml of concentrated H_2SO_4) for 20 minutes at room temperature and then, incubated in an oven at 100 C for 30 minutes [256, 257].

2.10 Characterisation of yeast extracellular vesicles (EVs)

2.10.1. EV isolation by differential ultracentrifugation

To obtain EV-enriched vesicular fractions, 40 ml of culture media collected from yeast cells was centrifuged at 2,000 x g for 20 minutes, at 4 C and supernatant is carefully collected by pipetting. The supernatant is transferred to 40 ml polycarbonates bottles and centrifuged in an Avanti J-25I (Beckman Coulter) ultracentrifuge with a JA–21 rotor at 10,000 x g, for 30 minutes, 4 C. The supernatant is transferred carefully to smaller 10.4 ml polycarbonate bottles (leaving about half a centimetre of liquid on the tube to avoid contaminating the supernatant with the pellet) and centrifuged at 100,000 x g, for 70 minutes, 4 C in an Optima XE-100 (Beckman Coulter) ultracentrifuge with a Type 90Ti rotor (Beckman Coulter). The

resulting supernatant is discarded, and the pellet is vigorously resuspended, with repeated pipetting, in about 0.7 ml of sterile PBS. The suspension was centrifuged to wash the pellet at 100,000 x g, for 1 h. The pellet was then resuspended with vigorous pipetting in 100 µL of PBS, transferred to a clean microcentrifuge tube and stored at -80 C.

2.10.2. EV characterisation by Dynamic Light Scattering and Nanoparticle Tracking Assay

Vesicular fractions obtained by differential ultracentrifugation were analysed by dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Panalytical). A blank measurement was made with PBS and vesicular fractions were diluted 1:10 in a disposable plastic cuvette to be read and the effective diameter of the particles in suspension was determined by the dynamic light scattering principle [258].
Vesicular fractions were also analysed by nanoparticle tracking assay (NTA) with a NanoSight NS300 (Malvern Panalytical) to determine the particles diameter distribution and concentration.

2.11 Immunofluorescence microscopy

Immunofluorescence microscopy allows for the observation of the aSyn protein inside cells.
To do so, yeast cells were cultured and collected at several timepoints of CLS and fixed in 10% formalin (Sigma) for 15 minutes. After washing with cacodylate (100 mM), cells are incubated 10 minutes at room temperature with TDES buffer in order to soften the cell wall [100 mM Tris, pH 7.5, 25 mM DTT (Fermentas, R0862), 5 mM EDTA (Merck, 108418) and 1.2 M sorbitol (Sigma,51876)]. Another washing was performed with phosphocitrate buffer (100 mM) [(100 mM K2HPO4 (Merck,105104), 100 mM citric acid (Sigma, C1909)), mixed 1:1 with 2M sorbitol for the working solution]. Cells were then incubated 30 minutes with 100 mM phoscitrate buffer:1 M sorbitol containing 50 µl of ß-glucuronidase (Perkin Elmer, NEE154001EA) and 25 µl of 10 mg/ml zymolyase (Seikagaku Biobusiness, 12049) to produce spheroplasts. After another wash with 100 mM cacodylate: 5 mM CaCl2: 1 M sorbitol, cells were permeabilized with 0.1%Triton-X100 contained in 1x phosphate-buffered saline (PBS), during 10 minutes. 7. After 5 min rinsing with 0.1% BSA in PBS [137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4), 1.8 mM KH2PO4], cells were incubated overnight with the antibody anti-aSyn (Cell signaling, 2642) (1:400), followed by rinsing with 0.1% BSA in PBS for 15 minutes. Next, cells were incubated for at least 4h with the AlexaFluor 488 conjugated goat anti-rabbit secondary antibody (Invitrogen, A32766) (1:100) for 4 h.

Then, cells were rinsed with 0.1% BSA in PBS for 15 minutes, resuspended in PBS with DAPI nucleic acid staining (Sigma) and mounting media (Ibidi,50001) and visualized in an Olympus BX61 fluorescence microscope on an Olympus 100x/oil objective. Background reduction was performed with appropriate saturation levels using software FV1000 (Olympus) and Affinity Photo 1.10 (Serif).

2.12 Statistical analysis

Statistical analyses were determined using either Student's t-test with Welch's correction or two-way ANOVA with Tukey's multiple comparisons with the Prism 8 statistical analysis software (GraphPad). A p-value of less than 0.05 was considered as a significant difference.

CHAPTER 3

RESULTS AND DISCUSSION

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Section 3.1

Yeast chronological lifespan and cell-autonomous regulation of homeostasis

The results described on this chapter were published as follows:

Pereira H*, Sampaio-Marques B*, Santos AR, Teixeira A, Ludovico P. Caloric restriction rescues yeast cells from alpha-synuclein toxicity through autophagic control of proteostasis. Ageing (Albany NY). 2018;10(12):3821. [* equal contribution]

3.1. Cell-autonomous regulation of homeostasis by the proteolytic systems rescues ageing yeast from aSyn toxicity under caloric restriction

Ageing is a complex and multi-factorial physiological process, translated by the stochastic accumulation of damage and loss of cellular, tissue and organ function. Loss of proteostasis and deregulated nutrient sensing are amongst some of the proposed hallmarks of ageing [260]. Yeast cells have quality control mechanisms that deal with protein folding/misfolding. Two of them are the proteolytic systems ubiquitin-proteasome (UPS) and autophagy. aSyn is an aggregation-prone protein targeted for degradation by both systems [261]. aSyn's role in synucleinopathies and related proteotoxic stress is evident, though its roles in ageing and disease are not completely understood [206, 210, 212]. What is certain is that the proteolytic systems become overburdened by aSyn overexpression, which is aggravated by an ageing-led decline on their activity [20, 68, 97], therefore compromising the delicate protein homeostasis balance. Thus, interventions that delay ageing could have promising outcomes as demonstrated in several cellular and animal ageing models. We aimed to understand the role of the proteolytic systems activities on yeast during ageing and their contribution to longevity modulation through cell-autonomous mechanisms, with an established non-genetic lifespan extending intervention. To do so, we employed caloric restriction (CR) with a known yeast expression model [17, 224, 247] during chronological lifespan (CLS). The expression model consists of the heterologous expression of the human wild-type aggregation-prone aSyn under a constitutive promoter. CR, a reduced calorie intake without malnutrition, is one of the most effective non-genetic interventions reported to promote lifespan and ameliorate the age-related decline of proteostasis by enhancing the activities of the proteolytic systems [23, 24, 262], UPS [262] and autophagy. CR beneficial effects on proteostasis are associated with the modulation of nutrient and energy sensing pathways such as TOR or Snf1 (yeast homolog of AMPK) [51, 68, 97]. CR is also beneficial for several processes that occur during diauxic shift on yeast such as the accumulation of trehalose [66], ATP synthesis and cell cycle arrest at G_0/G_1 [45, 139]. Moreover, CR was already reported to reduce aSyn toxicity in several ageing models, like yeast, worms, mice and primates [23, 263], and in association with proteolytic systems' activities [23, 262]. Wild-type aSyn expression is reported to reduce yeast lifespan and increase autophagy to exacerbated, deleterious levels [17, 23, 97, 137]. CR intervention greatly extends control vector lifespan and rescues yeast expressing wild-type aSyn lifespan to near control vector levels. CR-promoted longevity is dependent on the two main proteolytic systems, ubiquitin-proteasome system (UPS) and autophagy [23]. The activity of both systems declines with ageing progression and is also affected by aSyn expression and aggregation [20, 23].

The proteolytic systems themselves are reported to have a degree of crosstalk between them. However, the precise dynamics of their molecular interactions and its impact in countering aSyn toxicity during ageing remains unclear, particularly under CR conditions [86, 87, 126, 134]. To shed light on the topic, we pharmacologically inhibited either UPS or autophagy activities under CR conditions and monitored lifespan and the proteolytic systems' activity to understand the contribution of each to the beneficial effects observed under CR conditions during premature ageing induce by aSyn. We also performed genetic abrogation of key genes in the proteasome assembly. On the present chapter, we shall detail the main findings and the insight gathered on the crosstalk between proteolytic systems under CR during CLS on this yeast model.

3.1.1. Caloric restriction enhances ubiquitin-proteasome activity of aged aSyn-expressing cells

Corroborating previous results from the laboratory by Guedes and colleagues [23], we monitored CLS of yeast cells expressing the human wild-type aSyn under caloric restriction (CR) and verified the reported increased lifespan promoted by CR intervention (Fig. 16A). We monitored the chymotrypsin and trypsin-like catalytic activities of UPS during CLS (Fig. 16B). Our results showed that, under normal growth conditions, aSyn expression dramatically reduces chymotrypsin-like activity in ageing yeast. Trypsin-like activity was affected to lesser extent, only after 7 days of CLS (Fig. 16B). This decrease in UPS activity was confirmed by the increased accumulation of a short-lived protein, UB^{G76V}-GFP, which with normal proteasomal activity would be quickly degraded (Fig. 16C and D). Further corroborating this result, an increase in protein ubiquitination profile (Fig. 16E and F), in *RPN4* (a transcriptional regulator of the 26S proteasome) mRNA transcripts relative expression and a decrease in *RPN5* (a non-catalytic subunit of the lid of 26S proteasome complex [264, 265], upregulated by *RPN4*) mRNA transcripts relative expression were observed (Fig. 16G and H). These results are in agreement with a previous study that reported an aSyn-driven proteasomal impairment affecting cell's survival during stationary phase [235]. By contrast, under CR intervention, we saw an increase in proteasome activity (Fig. 16B, C and D). This is corroborated by the decreased protein ubiquitination profile and decreased *RPN4* and increased *RPN5* relative expression levels in aSyn-expressing cells (Fig. 16E, F, G and H). UPS activity is increased since day 0, whilst shifts in *RPN4* and *RPN5* relative expression levels can only be observed at day 3 of CLS. This can likely be attributed to negative feedback regulation of proteasome homeostasis. Overall, this set of data

suggests that CR rescues ageing yeast cells from aSyn-induced toxicity partially due to an increase of UPS activity.

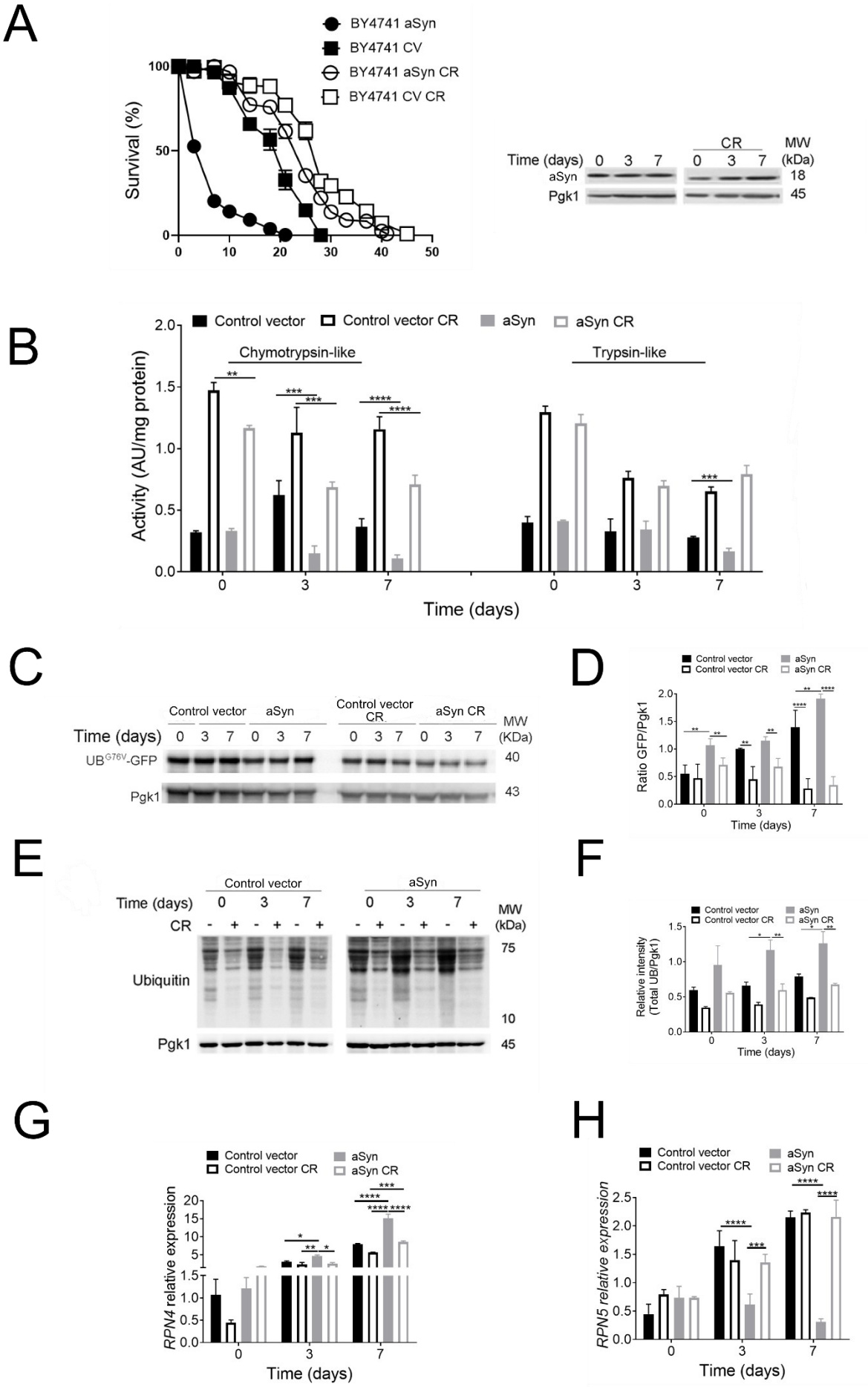
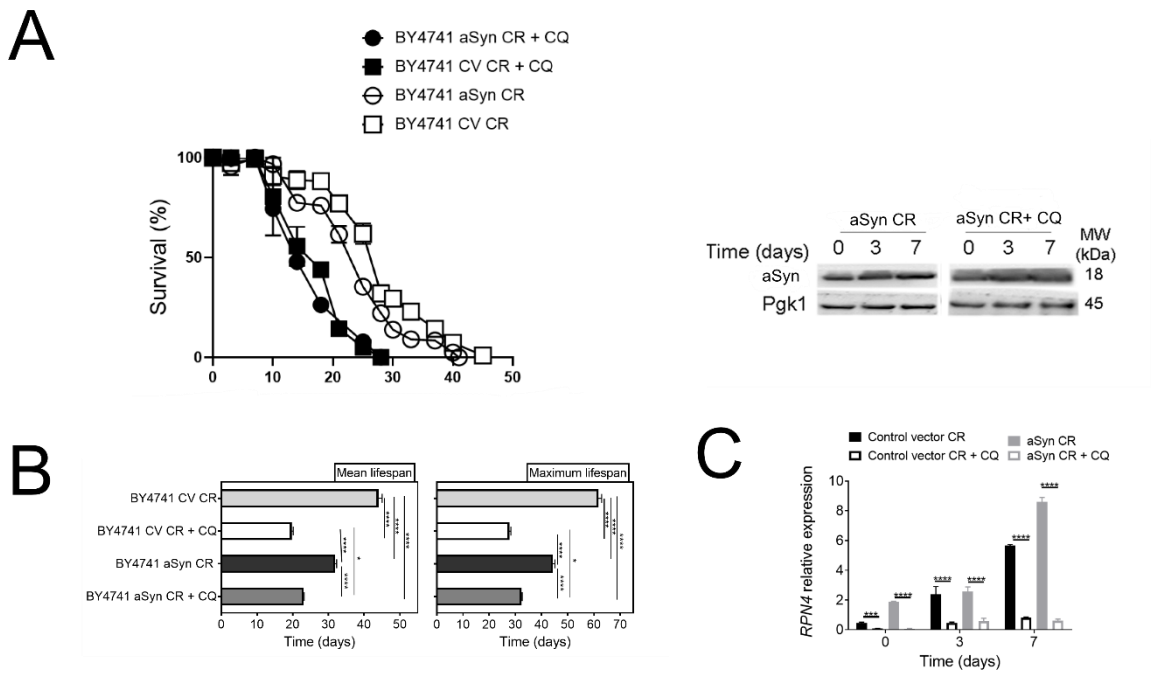

A
BY4741 aSyn
BY4741 CV
BY4741 aSyn CR
BY4741 CV CR
Survival (%)
Time (days) 0 3 7 CR 0 3 7 MW (kDa)
aSyn 18
Pgk1 45
B
Control vector
Control vector CR
aSyn
aSyn CR
Chymotrypsin-like
Trypsin-like
Activity (AU/mg protein)
Time (days)
C
Control vector
aSyn
Control vector CR
aSyn CR
Time (days) 0 3 7 0 3 7 0 3 7 0 3 7 MW (KDa)
UB^G76V-GFP 40
Pgk1 43
D
Ratio GFP/Pgk1
Time (days)
E
Control vector
aSyn
Time (days) 0 3 7 0 3 7 MW (kDa)
CR - + - + - + - + - + - +
Ubiquitin 75 10
Pgk1 45
F
Relative intensity (Total UB/Pgk1)
Time (days)
G
RPN4 relative expression
Time (days)
H
RPN5 relative expression
Time (days)

Figure 16. Caloric restriction abrogates α-synuclein (aSyn) induced toxicity by upregulating ubiquitin-proteasome system activity.
(**A**) Chronological lifespan (CLS) and aSyn levels of stationary wild type cells harbouring the control vector or expressing the human aSyn grown under regular (2% glucose) or CR (0.5% glucose) conditions. (**B**) Chymotrypsin- and trypsin like activities. The assay was normalized to the total protein amount. (**C**) UPS activity measured by monitoring the ubiquitin/proteasome dependent proteolysis of the short-lived protein UBG76V-GFP. GFP was detected by Western blotting using a GFP-specific antibody. (**D**) Graphical representation of GFP/Pgk1 obtained by densitometric analysis. (**E**) Ubiquitination profile determined by Western blotting using an anti-mono and polyubiquitination antibody. (**F**) Graphical representation of the intensity of total UB/Pgk1 obtained by densitometric analysis. (**G**) *RPN4* and **(H)** *RPN5* mRNA relative expression levels. 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. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) between cells grown under regular or CR conditions expressing control vector or wild-type aSyn. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

3.1.2. Autophagy inhibition in aSyn-expressing cells submitted to caloric restriction is associated with increased ubiquitin-proteasome activity

It is widely reported in the literature, that the crosstalk between UPS and autophagy is paramount to prevent protein misfolding and proteotoxicity [68, 266, 267]. To explore the hypothesis of a coordinated crosstalk between UPS and autophagy, we pharmacologically inhibited (with chloroquine) autophagy under CR and monitored aSyn-expressing cells during CLS. Autophagy inhibition resulted in a shorter lifespan, regarding yeast cells expressing control vector under the same CR conditions (Fig. 17A). Mean and maximum lifespans were also determined for yeast cells expressing wild-type aSyn or control vector, under CR or normal growth conditions (2% glucose), with or without chloroquine. Even though inhibition of autophagy decreased CLS of aSyn-expressing cells under CR, the lifespan of these cells is still extended when compared with that of aSyn-expressing cells on normal growth conditions (mean lifespan of 23.1 ± 0.2 days versus 7.7 ± 0.6 days) (Fig. 17B). To understand the contribution of UPS for the beneficial effects promoted by CR intervention on the lifespan of aSyn-expressing cells when autophagy is inhibited, UPS activity was measured. An upregulation of UPS was detected, as evidenced by the increased activities of chymotrypsin and trypsin-like (Fig. 17D), the decreased UB^{G76V}-GFP accumulation (Fig. 17E and F), the decreased protein ubiquitination profile (Fig. 17G and H) and decreased *RPN4* relative expression levels (Fig. 17C). This data strongly supports the notion that both proteolytic systems crosstalk under CR intervention, leading to an increase of UPS activity in response to autophagy inhibition.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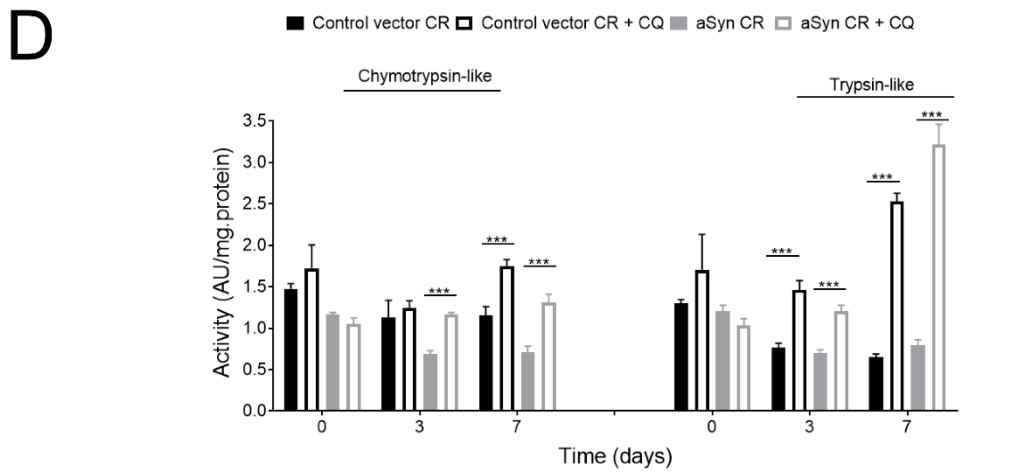
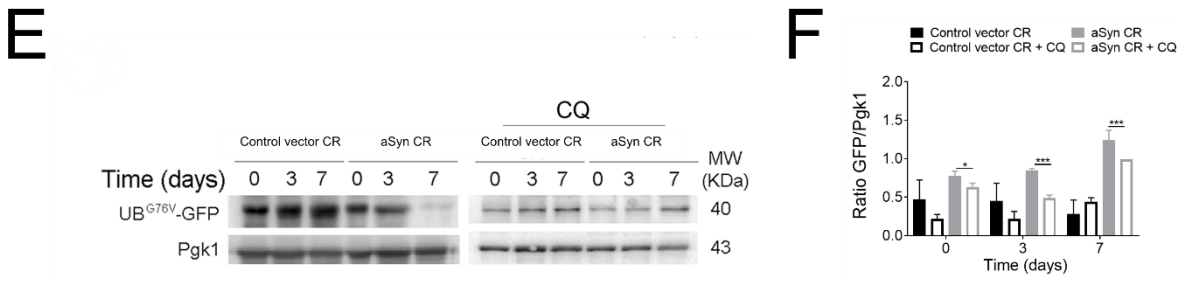
BY4741 aSyn CR + CQ
BY4741 CV CR + CQ
BY4741 aSyn CR
BY4741 CV CR
Survival (%)
Time (days)
aSyn CR
aSyn CR+ CQ
MW (kDa)
aSyn
Pgk1
18
45

B
Mean lifespan
Maximum lifespan
BY4741 CV CR
BY4741 CV CR + CQ
BY4741 aSyn CR
BY4741 aSyn CR + CQ
Time (days)
C
Control vector CR
Control vector CR + CQ
aSyn CR
aSyn CR + CQ
RPN4 relative expression
Time (days)


D
Control vector CR
Control vector CR + CQ
aSyn CR
aSyn CR + CQ
Chymotrypsin-like
Trypsin-like
Activity (AU/mg.protein)
Time (days)


E
CQ
Control vector CR
aSyn CR
Time (days)
MW (KDa)
UB^G76V-GFP
Pgk1
40
43
F
Ratio GFP/Pgk1
Time (days)

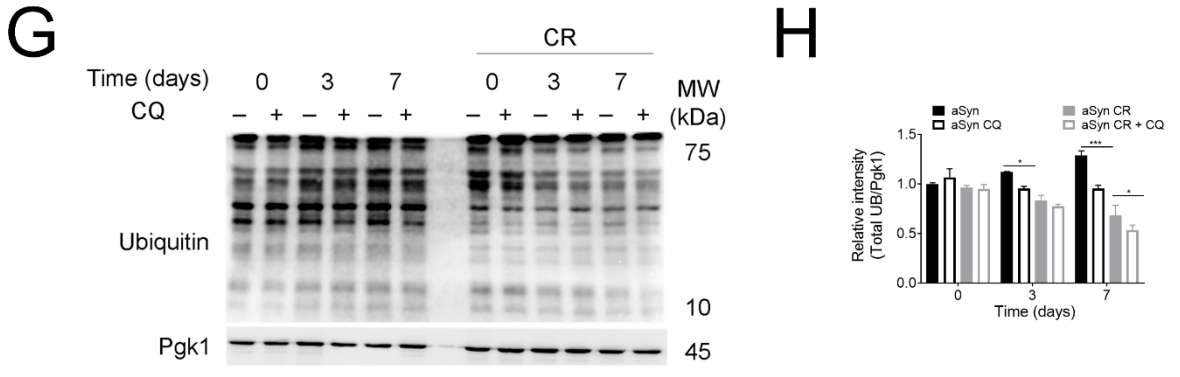

G
CR
Time (days)
CQ
MW (kDa)
Ubiquitin
Pgk1
75
10
45
H
aSyn
aSyn CQ
aSyn CR
aSyn CR + CQ
Relative intensity (Total UB/Pgk1)
Time (days)

Figure 17. Autophagy inhibition leads to upregulation of the ubiquitin-proteasome system activity in α-synuclein (aSyn) -expressing cells under caloric restriction. (**A**) Chronological lifespan (CLS) and aSyn levels of aSyn-expressing stationary wild type cells, under caloric restriction (CR, 0.5% glucose) conditions, in the presence or absence of chloroquine (CQ), an inhibitor of autophagy. (**B**) Mean (50% survival) and maximum (10% survival) lifespans determined from curve fitting of the survival data from CLS. Significance was determined by two-way ANOVA (*p≤0.05, ****p≤0.0001) between cells grown under CR conditions expressing control vector or aSyn in the presence or absence of CQ. (**C**) *RPN4* mRNA relative expression levels. (**D**) Chymotrypsin- and trypsin like activities. The assay was normalized to the total protein amount. (**E**) UPS activity measured by monitoring the ubiquitin/proteasome-dependent proteolysis of the short-lived protein UBG76V-GFP. GFP was detected by Western blotting using a GFP-specific antibody. (**F**) Graphical representation of GFP/Pgk1 obtained by densitometric analysis. (**G**) Ubiquitination profile determined by Western blotting using an anti-mono and polyubiquitination antibody. (**H**) Graphical representation of the intensity of total UB/Pgk1 obtained by densitometric analysis. Statistical significance represented in (**C**), (**D**), (**F**) and (**H**) was determined by Student's t-test (*p≤0.05, ***p≤0.001, ****p≤0.0001) comparing caloric restricted control vector or aSyn-expressing cells in the presence or absence of CQ. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

3.1.3. Ubiquitin-proteasome activity inhibition does not impact on autophagy of aSyn-expressing cells under caloric restriction

Next, to continue exploring the crosstalk between autophagy and UPS in aSyn-expressing yeast cells under CR intervention, we pharmacologically inhibited UPS activity with bortezomib. Given that proteasome inhibitors *on S. cerevisiae* require limited drug efflux to be effective, a *Δpdr5* null mutant was used. BY4741 *Δpdr5* cells expressing aSyn and control vector under CR conditions and treated with bortezomib (data not shown here for MG123) were monitored throughout CLS (Fig. 18E and F). UPS inhibition was confirmed by a sharp decrease in chymotrypsin- and trypsin-like activities (Fig. 18A), the increased relative levels of *RPN4* expression (Fig. 18B) and the accumulation of UB^{G76V}-GFP protein (Fig. 18C and D). UPS inhibition resulted in a shorter lifespan and decreased mean and maximum lifespans of aSyn-expressing cells under CR, regarding non-inhibited cells. This result points out that UPS, in addition to autophagy, is also an important mechanism for CR-led longevity extension in aSyn-expressing cells. However, when we monitored the autophagic flux on these conditions, we saw no major effects of proteasome inhibition on autophagy of aSyn-expressing yeast cells under CR (Fig. 18G, H, and I).

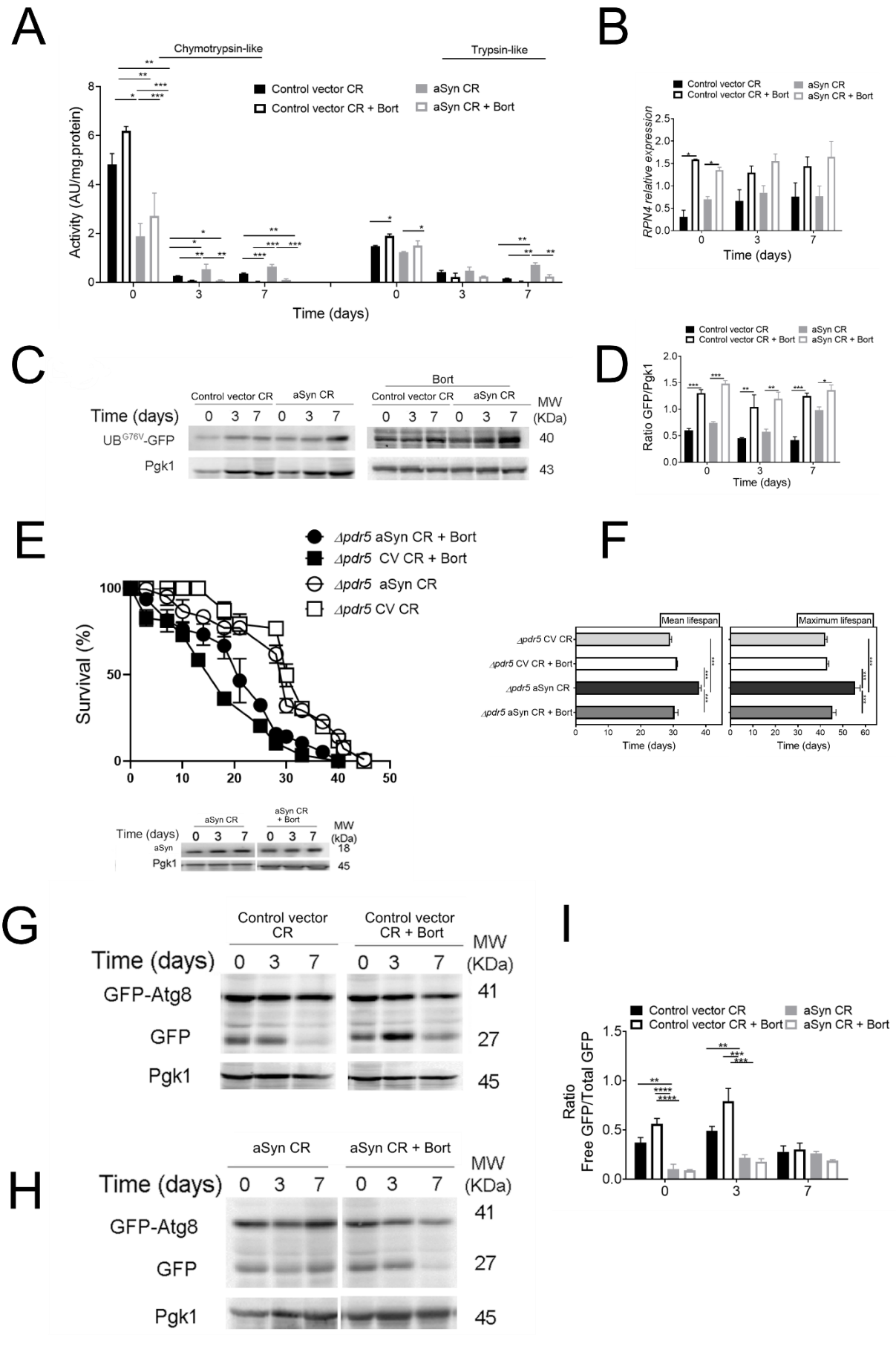

A
Chymotrypsin-like
Trypsin-like
Control vector CR
Control vector CR + Bort
aSyn CR
aSyn CR + Bort
Activity (AU/mg.protein)
Time (days)
B
RPN4 relative expression
Time (days)
C
Bort
Time (days)
MW (KDa)
UB^G76V-GFP
Pgk1
40
43
D
Ratio GFP/Pgk1
Time (days)
E
Δpdr5 aSyn CR + Bort
Δpdr5 CV CR + Bort
Δpdr5 aSyn CR
Δpdr5 CV CR
Survival (%)
aSyn
Pgk1
18
45
F
Mean lifespan
Maximum lifespan
Δpdr5 CV CR
Δpdr5 CV CR + Bort
Δpdr5 aSyn CR
Δpdr5 aSyn CR + Bort
Time (days)
G
GFP-Atg8
GFP
Pgk1
41
27
45
H
I
Ratio Free GFP/Total GFP

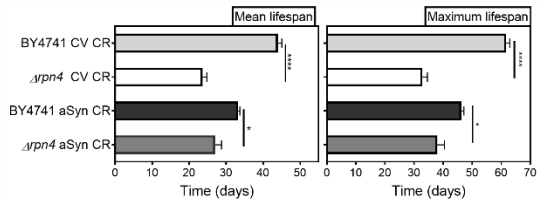
Figure 18. Pharmacological inhibition of the ubiquitin-proteasome system activity decreases lifespan of α-synuclein (aSyn)-expressing cells grown under caloric restriction but has no major impact on autophagy. (**A**) Chymotrypsin- and trypsin like activities. The assay was normalized to the total protein amount. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01, ***p≤0.001) between cells grown under CR (0.5% glucose) conditions expressing control vector or aSyn in the presence or absence of bortezomib (Bort). (**B**) *RPN4* mRNA relative expression levels as described in the legend of Figure 1. (**C**) UPS activity measured by monitoring the ubiquitin/proteasome-dependent proteolysis of the short-lived protein UBG76V-GFP. GFP was detected by immunoblotting using a GFP-specific antibody. (**D**) Graphical representation of GFP/Pgk1 obtained by densitometric analysis. Statistical significance represented in (**B**) and (**D**) was determined by Student's t-test (*p≤0.05, **p≤0.01, ***p≤0.001) comparing caloric restricted control vector or aSyn-expressing cells in the presence or absence of Bort. (**E**) Chronological lifespan (CLS) and aSyn levels of Δ*pdr5* cells expressing aSyn grown under CR conditions, in the presence or absence of Bort. (**F**) Mean (50% survival) and maximum (10% survival) lifespans determined from curve fitting of the survival data from CLS. Significance was determined by two-way ANOVA (***p≤0.001) between cells grown under CR conditions expressing control vector or aSyn in the presence or absence of Bort. Autophagy flux assessed by the GFP-Atg8 processing assay (immunoblotting analysis with antibody against GFP) of caloric restricted cells expressing control vector (**G**) or aSyn (**H**) in the absence or presence of Bort. Blots represented in (**G**) are from the same gel, as in (**H**). **(I)** Densitometric analysis of the ratio between the free GFP versus the total GFP. Bands were quantified by Quantity One software. Significance of the data was determined by two-way ANOVA (**p≤0.01, ***p≤0.001, ****p≤0.0001) between cells grown under CR conditions expressing control vector or aSyn in the presence or absence of Bort. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

To further corroborate these findings, we also attempted to inhibit UPS activity by a genetic abrogation. UPS activity is inhibited on Δ*ump1* and Δ*rpn4* strains. Both mutants were confirmed to have impaired UPS activity (data not shown). While *RPN4* is a transcriptional regulator for the 26S proteasome [264], as mentioned before, *UMP1* deletion triggers an incorrect assembly of the 20S proteasome [268]. Determining mean and maximum lifespans of both mutant cells (Fig. 19A and B) expressing wild-type aSyn or control vector showed that the genetic abrogation of UPS activity significantly impairs the CR-led beneficial effects on yeast CLS, which agrees with the results obtained from pharmacological inhibition of UPS (Fig. 18). Once again, UPS seems to be an important player in the CR-mediated longevity extension. Concerning autophagy, genetic inhibition of UPS, like what was observed on pharmacological inhibition (Fig. 18), does not cause major impacts on autophagic flux of aSyn-expressing ageing yeast cells, under CR (Fig. 19C, D, and E).

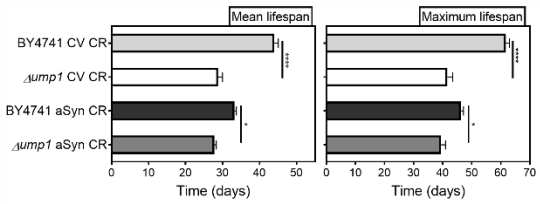
Finally, we were interested to understand the role of autophagy in an organelle-specific process, proteophagy, in the context of proteasomal impairment. The yeast 26S proteasome is degraded by Atg8-mediated autophagy. Proteophagy in yeast is a process activated by either nitrogen starvation or proteasomal impairment/inactivation. Proteophagy is dependent on Cue5, an ubiquitin receptor that targets inactivated proteasome for autophagic degradation. Cue5 can simultaneously bind ubiquitinated proteasomes and the Atg8 autophagy related protein. Inactive UPS complexes aggregate with the assistance of the Hsp42 chaperone, form insoluble protein (IPOD) structures and are extensively ubiquitinated to be recognized by the Cue5 receptor [98, 100, 269]. Therefore, we monitored the CLS of aSyn-expressing and control vector in these proteophagy impaired Δ*cue5* cells. Mean and maximum lifespans of Δ*cue5* cells expressing aSyn increased significantly (Fig. 19F) under normal growth conditions. Under CR conditions, however, no differences were observed between Δ*cue5* and wild-type aSyn expressing cells. When autophagy was pharmacologically inhibited, under normal growth conditions, both wild-type aSyn expressing and control vector cells, displayed significant lifespan extension (Fig. 19G).

These results along with the *Δcue5* cells data suggest the importance of maintaining autophagy at homeostatic levels, which even on normal growth conditions is beneficial for UPS activity rescuing cells from aSyn-induced toxicity. The reversible formation of proteasome stress granules (PSGs) during stationary phase to avoid autophagic degradation is described as an advantageous fitness gain for yeast cells [98, 99]. It is conceivable and compatible with our data that the beneficial effects of CR on aSyn-expressing cells are partly associated with the inhibition of proteophagy and formation of PSGs during early stationary phase.

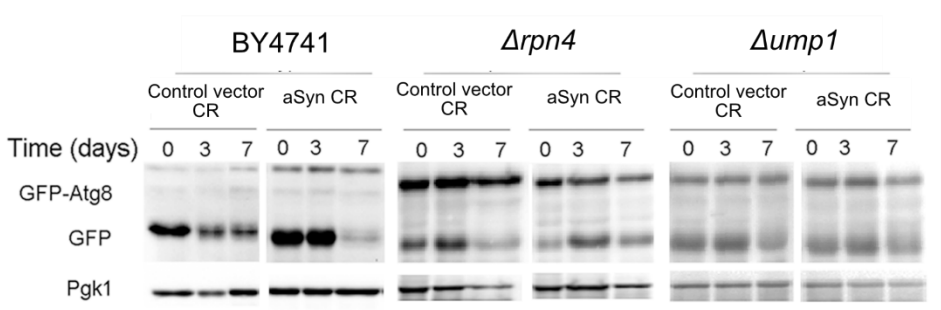
A


Mean lifespan
Maximum lifespan
BY4741 CV CR
Δrpn4 CV CR
BY4741 aSyn CR
Δrpn4 aSyn CR
Time (days)
Time (days)

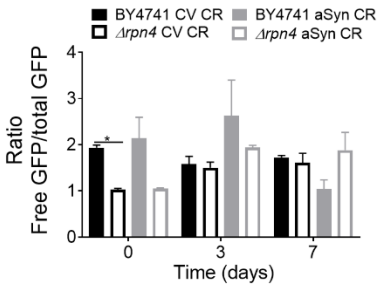
B


Mean lifespan
Maximum lifespan
BY4741 CV CR
Δump1 CV CR
BY4741 aSyn CR
Δump1 aSyn CR
Time (days)
Time (days)

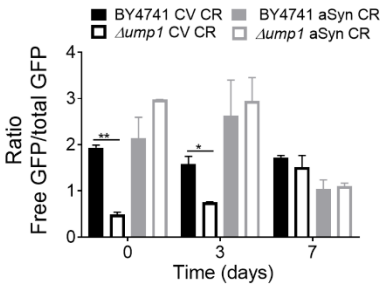
C


BY4741
Δrpn4
Δump1
Control vector CR
aSyn CR
Time (days)
0 3 7
GFP-Atg8
GFP
Pgk1

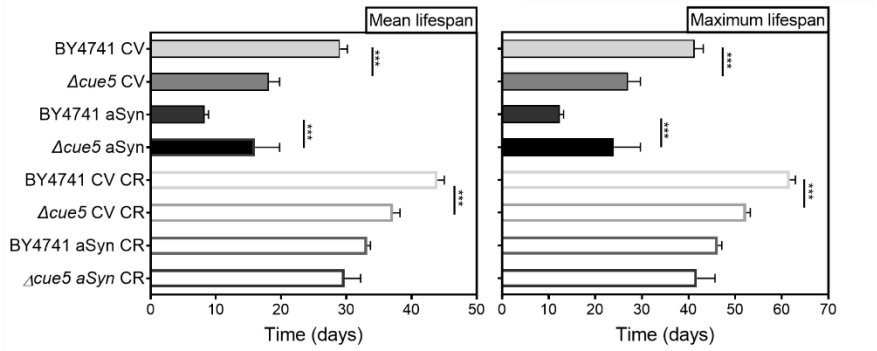
D


BY4741 CV CR
BY4741 aSyn CR
Δrpn4 CV CR
Δrpn4 aSyn CR
Ratio Free GFP/total GFP
Time (days)

E


BY4741 CV CR
BY4741 aSyn CR
Δump1 CV CR
Δump1 aSyn CR
Ratio Free GFP/total GFP
Time (days)

F


Mean lifespan
Maximum lifespan
BY4741 CV
Δcue5 CV
BY4741 aSyn
Δcue5 aSyn
BY4741 CV CR
Δcue5 CV CR
BY4741 aSyn CR
Δcue5 aSyn CR
Time (days)
Time (days)

G

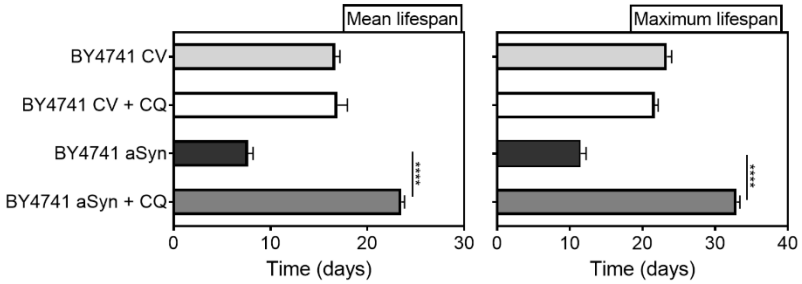

Mean lifespan
Maximum lifespan
BY4741 CV
BY4741 CV + CQ
BY4741 aSyn
BY4741 aSyn + CQ
Time (days)
Time (days)

Figure 19. Genetic inhibition of the ubiquitin-proteasome system activity decreases lifespan of α-synuclein (aSyn)-expressing cells grown under caloric restriction conditions but has no major impact on autophagy. Mean (50% survival) and maximum (10% survival) lifespans determined from curve fitting of the survival data from CLS of caloric restricted control vector and aSyn expressing wild-type cells compared with *RPN4* (**A**) and *UMP1* (**B**) deleted cells. (**C**) Autophagy flux assessed by the GFP-Atg8 processing assay (immunoblotting analysis with antibody against GFP) of wild-type, *Δrpn4* and *Δump1* caloric restricted cells expressing control vector or aSyn. Blots from same strain were run in the same gel. Densitometric analysis of the ratio between the free GFP versus the total GFP of *Δrpn4* (**D**) and *Δump1* (**E**) cells. Data from wild-type cells is repeated in (**D**) and (**E**) for easier interpretation. Bands were quantified by Quantity One software. (**F**) Mean and maximum lifespans determined from curve fitting of the survival data from CLS of wild-type and *Δcue5* cells expressing the control vector or aSyn in normal (2% glucose) or CR (0.5% glucose) growth conditions. (**G**) Mean and maximum lifespans determined from curve fitting of the survival data from CLS of wild-type cells expressing the control vector or aSyn in the absence or presence of chloroquine (CQ). Significance was determined by Student'st-test (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) comparing wild-type with each mutant strain in the same conditions. For (**G**) the comparison was done between control vector or aSyn-expressing cells in the presence or absence of CQ. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

The crosstalk interactions by the major proteolytic systems, UPS and autophagy during ageing are reported in the literature as essential for longevity extension across several model organisms [86, 87, 104, 126, 270, 271]. Overall, the bulk of our data points that in proteotoxic stress conditions, the reported beneficial effects attributed to CR are dependent on increased UPS activity and maintenance of autophagy at homeostatic, non-deleterious levels. We observed an increase in UPS activities when autophagy was pharmacologically inhibited, but no significant boost in the autophagic activities when the UPS was inhibited. This appears to contradict the ruling consensus on the literature where autophagic activities were increased in a compensatory mechanism when UPS was inhibited [86, 267, 270, 272, 273]. During stationary phase, proteasomes are reversibly translocated from the nucleus to the cytosol into PSGs, where then can bypass autophagic degradation (proteophagy) [98-100]. Our data points to a beneficial effect of the proteophagy genetic inhibition on aSyn expressing cells lifespan during normal growth conditions. No significant differences were found on CR conditions between *Δcue5* cells and wild-type cells expressing aSyn, which could tempt us to speculate that the CR promoted beneficial effects are also related with either the inhibition of proteophagy or the formation of PSGs.

In brief, the data herein presented suggests that while the inhibition of either UPS or autophagy under CR conditions reduces longevity, maintaining autophagy at homeostatic levels appears to be the major cell-autonomous mechanism behind rescuing ageing yeast cells from aSyn-induced toxicity.

Section 3.2

Cell-nonautonomous mechanisms during yeast chronological lifespan: Screening for messenger molecules

3.2. Screening for different classes of transmissible factors in the ageing yeast' extracellular milieu

Ageing is a complex biological phenomenon that affects most living organisms and can be defined in the broadest sense as a stochastic, time-dependent functional decay [1]. The budding yeast *S. cerevisiae* is a well-established model organism for ageing studies [17, 23, 27, 29, 97]. Ageing can be regulated by cell-autonomous mechanisms [49], encompassing all the mechanisms within a single cell that modulate ageing progression, but also by cell-nonautonomous mechanisms [40]. A growing body of evidence suggests that ageing progression, is not only regulated by the interactions between nutrient and energy sensing signalling pathways and concentration of specific metabolites within a cell, but also by the release of low molecular weight transmissible factors into the extracellular milieu [40, 143]. It is postulated that spatial and temporal shifts on the concentration of these molecules might regulate the cellular pathways that are widely recognized as modulators of ageing, via cell-nonautonomous mechanisms. However, the relevance and role of these mechanisms on ageing is still poorly understood [274]. Yeast cells have also been described to have cell-nonautonomous mechanisms regulating longevity [138, 151, 154, 275]. On the following chapter, we will detail our attempts to isolate and characterise the classes of molecules present in the extracellular medium of aged yeast cells, namely, nucleic acids, proteins, and lipids. First, we wanted to understand if the release of molecules into the extracellular media was associated with cell-nonautonomous mechanisms of ageing, and not a mere consequence of cell death and loss of plasma membrane integrity during yeast CLS. We characterised the survival by counting colony-forming units (CFUs) at various time points and assessed the membrane integrity by propidium iodide (PI) exclusion assay of BY4741 cells during CLS. BY4741 cells remained viable, with survival near 100% for the first 10 days of CLS. Only after 10 days survival starts to plummet significantly, reaching about 0.1% of the initial values at 30 days of CLS (Fig. 20A). The percentage of cells with plasma membrane integrity remained high during the first seven days of CLS (Fig. 20B). We established from this data that there was no substantial decrease in survival and membrane integrity on the first 7 days that could justify the leaking of cellular contents into the extracellular milieu. This would be an important piece of data for the experimental design of the following screening experiments.

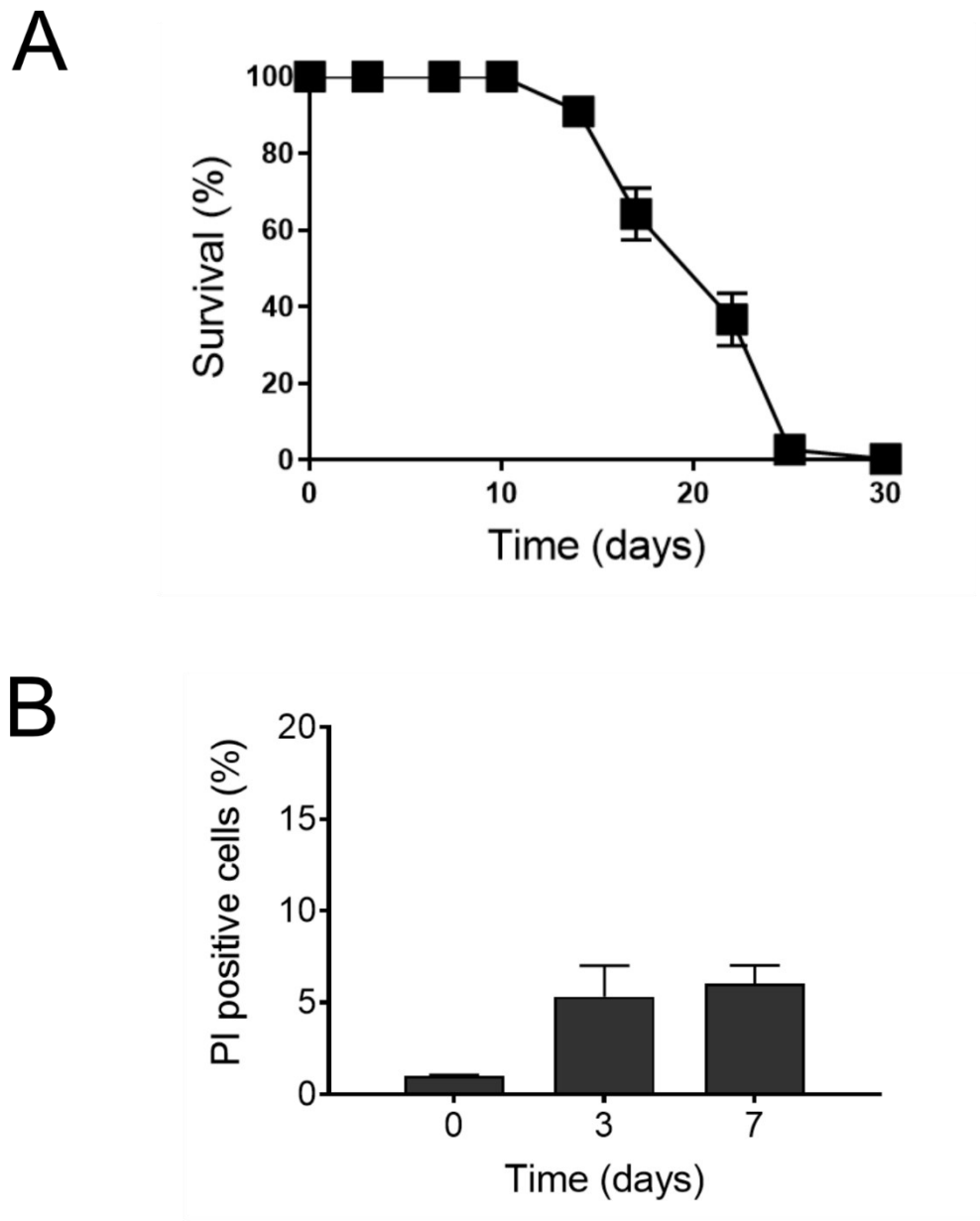


Figure 20. No substantial increase in cell death is observed in the first days of yeast chronological lifespan. (A) Chronological lifespan of stationary-phase yeast strain BY4741. Cell viability was measured at 2–3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%) **(B)** Assessment of membrane integrity analysis of aged yeast cells, by flow cytometry with the propidium iodide (PI) stain. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

3.2.1. Nucleic acids can be detected in yeast culture media during chronological lifespan

Nucleic acids, in particular RNA, have been recently described as carriers of biological information between different cells, through cell-nonautonomous mechanisms [160, 274]. These putative transmissible factors have the potential to influence physiological and molecular processes on the receptor cells and provide novel insights into the progression of several diseases or even the ageing phenomenon [276]. The budding yeast secretome has already been characterised to have small non-coding RNA

fragments, like small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) or messenger RNA (mRNA) species no longer than 250 nucleotides [160, 277].

To understand the potential role of nucleic acids as transmissible factors involved in cell-nonautonomous mechanisms of ageing in the budding yeast CLS, we collected 1 ml culture media from BY4741 cells at days 0, 3 and 7 of CLS and attempted to isolate nucleic acids using an adapted TRIzol reagent-based precipitation method. After nucleic acids extraction, the presence of either RNA or DNA, were assessed with a NanoDrop spectrophotometer in the samples from day 0, day 3 and day 7. At these time points, cell viability was near 100%, as shown in figure 20. The quantification of three independent biological replicas of for each time point displayed the presence of high quantities of both DNA and RNA, with DNA ranging from 7 to 50 ng/µL and RNA amounts up to several hundreds of ng/µL across samples (Fig 21A and B).

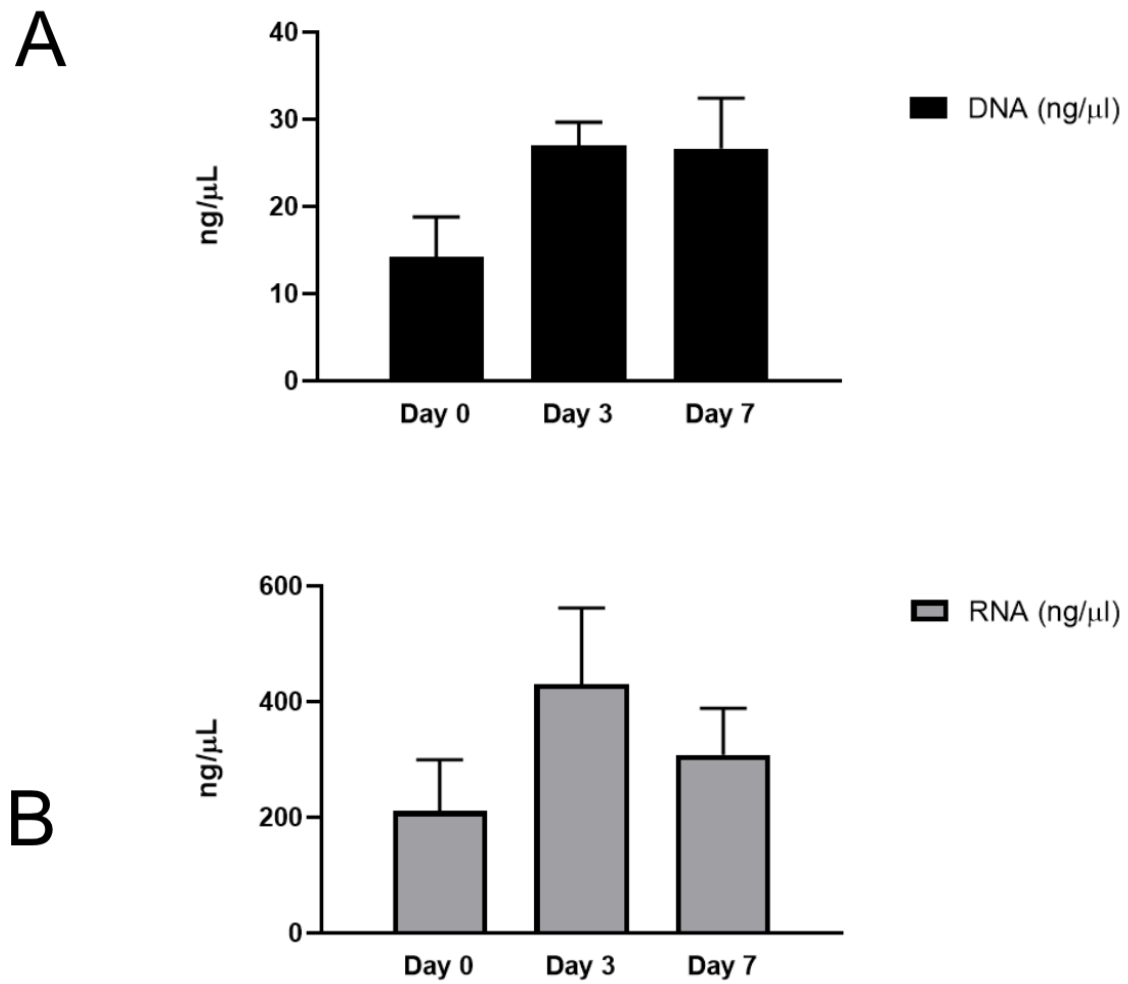


Figure 21. Nucleic acids can be found on the culture media of yeast during chronological lifespan (A) Culture media samples were extracted with a TRIzol based method and quantified via NanoDrop for DNA and RNA **(B)** species. Data represents at least three independent biological replicates Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM)..

DNA has been described to be released as a constituent of the extracellular matrix and biofilms in dimorphic fungi species such as *Candida albicans* or *Aspergillus fumigatus* [278, 279]. This extracellular DNA, eDNA, was found to be identical to genomic DNA [279]. Small RNAs have also been described to be abundant for fungi species' secretomes, often enveloped into EVs [274, 277, 280-282]. Small RNAs have also been described to mediate gene silencing on the receptor cells as a cell-nonautonomous mechanism and on host-pathogen interactions on several fungi species [167, 277, 283]. On our screening, we were able to quantify large amounts of free RNA in the yeast secretome, unlike the most reported EV associated small RNAs. Whether this RNA was originally secreted inside EVs that our protocol damaged is uncertain. To the best of our knowledge, reports of fungal RNA found in secretome are much more commonplace than DNA. We also detected DNA in the samples from the different time points, which could be related to the extracellular matrix constituents such as eDNA [279]. The differences between samples from day 0, 3 or 7 on either DNA or RNA amounts were not statistically significant. Nevertheless, the quantities of nucleic acids, either DNA or RNA, found in these samples seems to show a trend of increasing with the age of cells in culture. The differences between media at day 0 of CLS and later days (3 and 7 days), could point out an increased secretion of nucleic acids on later stages of yeast CLS, potentially involved in cell-nonautonomous mechanisms of ageing.

3.2.2. Proteins can be detected on yeast culture media during chronological lifespan

To understand the role of protein secretion in cell-nonautonomous mechanisms that modulate ageing, and considering the recent findings in several fungi species [159, 162, 167, 280] that point to a multitude of low-molecular weight transmissible factors being present in fungal secretomes, including proteins, we began by assessing the presence of proteins in the culture medium of yeast cells. Protein was precipitated and quantified from approximately 40 ml of culture medium collected from BY4741 yeast cells at days 3 and 7 of CLS. Preliminary data with day 0 of CLS indicated no detectable amounts of proteins (data not shown), so instead we focused on more advanced time points of CLS, 3 and 7 days, where the cumulative effect of putative protein secretion on yeast's secretome could be more easily visualized. By the Ponceau S staining profile (Fig. 22A), the presence of different bands with distinct intensities and molecular weights was detected. Densitometric analysis of the detected bands (Fig. 22B) suggests that culture medium with 7 days, contains a higher amount of proteins than culture media from 3 days, which together with the

absence of proteins in day 0, could point to a cumulative effect of proteins on the yeast secretome with ageing. Protein secretion in fungi is a widely reported phenomenon [152, 283] and yeast prions are a well-known mechanism of epigenetic inheritance [152, 175].

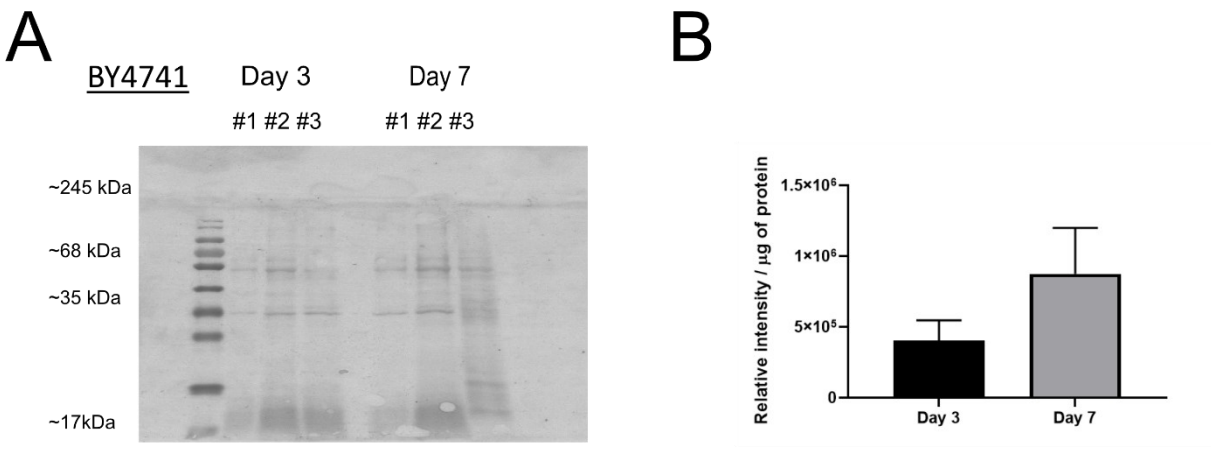


Figure 22. Proteins can be found on the culture media of yeast during chronological lifespan (A) SDS-PAGE electrophoresis protein profile of wild-type BY4741 yeast culture media collected at days 3 and 7 of CLS. **(B)** Graphical representation of the intensity of total protein obtained by densitometric analysis. Data represents mean ± SEM of at least three biological independent replicas. Statistical significance was determined by Student's t-test with Welch's correction (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM).

3.2.3. Lipids can be detected in yeast culture media during chronological lifespan

Until here, we detected the presence of free nucleic acids and proteins in the culture medium of yeast cells, collected at different time points of chronological lifespan. Next, we asked whether lipids could also be present in these media. Several classes of lipids (fatty acids, sphingolipids, glycerol and diacylglycerols) have previously been described as modulators of yeast CLS [143, 284] through nutrient sensing pathways. Increased glycerol concentrations within a cell are described to extend CLS, whilst increased sphingolipids can act as a pro-ageing factor. Accumulation of unoxidized fatty acids and diacylglycerols accelerates liponecrotic regulated cell death in yeast [143]. In addition to this cell-autonomous mechanisms, lipids have also been associated with cell-nonautonomous mechanisms of ageing in several fungi species, including budding yeast [162, 167, 280, 283, 285]. To assess the presence of lipids on the culture medium, we performed a liquid-liquid lipidic extraction from either concentrated freeze-dried media or 6 ml of unprocessed yeast culture medium collected at different time points of CLS and then ran the lipid enriched extract on a thin-layer silica plate by thin-layer chromatography (TLC). Several protocols for TLC running and development were tried, while attempting to increase the efficiency of solvent evaporation, and finally ran unprocessed samples from days 3 and 7 in Petroleum ether/diethyl ether/acetic acid (70:30:1) mobile phase and developing the plate with $MnCl_2$. Charring with $MnCl_2$ allowed for the irreversible staining of several classes of neutral lipids present in the silica plate. With the

samples from day 0 CLS (data not shown) no bands or spots pertaining to lipids were detected in the TLC, unlike what was achieved with samples from culture medium of day 3 and 7 of cells CLS. After charring with $MnCl_2$, multiple bands corresponding to different lipid classes were detected (Fig. 23A), yet to be properly identified. Comparing our data with published results in the literature using the same method, it is plausible to infer that bands closer to the point of origin are likely sterols and those further apart could be either free fatty acids, triacylglycerols or sterol esters. However appropriate standards need to be chosen to unequivocally determine the classes present on the TLC plate. Samples from culture medium with 3 days appeared to have a higher concentration of lipids than samples from day 7, even though there are not statistically significant differences (Fig. 23B). This represented a contrast with the incremental trend observed for nucleic acids and proteins (Fig. 21 and 22). Several classes of lipids are known to activate nutrient-sensing longevity regulators [143] and some classes (like sterol-enriched lipid rafts, saturated fatty acids, sphingolipids) are also abundant constituents of the lipidic bilayer of EVs [184]. EVs have been associated with the transport of lipids in some fungi species [168]. Nevertheless, this data confirms the presence of lipids in the secretome of yeast cells during CLS, which could potentially work as transmissible factors release freely between yeast cells or as carriers of other types of molecular cargo as EVs.

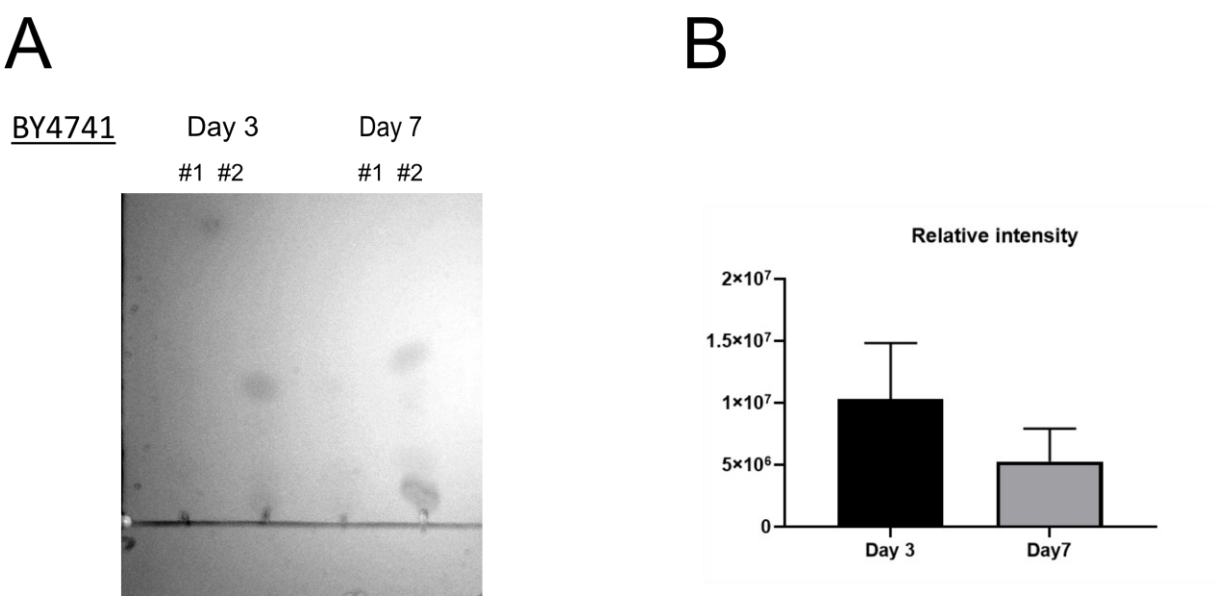


Figure 23. Neutral lipids can be found on the culture media of yeast during chronological lifespan (A) TLC profile of wild-type BY4741 yeast culture media collected at days 3 and 7 of CLS. **(B)** Graphical representation of the intensity of total lipids obtained by densitometric analysis. Data represents mean ± SEM of at least three biological independent replicas. Statistical significance was determined by Student's t-test with Welch's correction (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM).

3.2.4. The role of Extracellular vesicles (EVs) in cell-nonautonomous mechanisms of ageing

Extracellular vesicles (EVs) are lipid bilayer membrane compartments produced by all domains of life [280, 283]. EVs have been associated with cell-nonautonomous mechanisms of ageing in several organisms, mediating the delivery of low-molecular weight transmissible factors between cells [169, 179] both in context of health and disease pathogenesis [174, 183, 286, 287]. EVs have been described to be produced by yeast and other fungi species associated with both conventional and unconventional secretion pathways [162, 163, 277]. EVs can act as a transport vector for different cargo between cells of the same population, shuttling RNA, proteins or lipids within them, which can act as transmissible factors of biological information. In the present study, EVs isolation from chronological aged yeast CM was attempted to understand the role of this type of secretion in cell-nonautonomous mechanisms and the transport of putative molecular transmissible factors between yeast populations during chronological lifespan.

3.2.4.1. EVs can be characterised by their size distribution

Knowing that besides the release of low-molecular weight molecules such as nucleic acids, protein or lipids to the culture, these putative transmissible factors could also be transported enveloped into EVs, we assessed the presence of EVs in the culture medium of BY4741 wild-type cells. Vesicular fractions obtained from BY4741 yeast cells at days 0, 3 and 7 of CLS were analysed by Dynamic Light Scattering (DLS). DLS is a spectroscopy technique based on the principle of light refraction on a liquid suspension of particles. The intensity of the light scattering is measured over time, considering the Brownian motion of suspended particles [288]. With this technique, we were able to determine the particles' diameter distribution (Fig. 24). Overall, the samples' size range was comprised between a minimum of 28 nm and a maximum of 531 nm. The Z-average parameter of DLS is an intensity weighted mean of all the particles detected in the samples [289]. On Table 2, we can see our samples Z-average and standard deviation. As previously reported by Oliveira and colleagues [162], the DLS technique tends to overestimate particles' diameter. The presence of larger particles in vesicular fractions can also be a confounding factor increasing this parameter and masking the abundance of lower sized particles.

Samples from culture medium collected at the 0 days of CLS presented a distribution below 100 nm, while culture medium from day 3 and day 7, showed particles above this level (Fig. 24). These diameter values are compatible with what is found in the literature for either exosomes (<100 nm) or microvesicles (>100 nm) [169, 176] in mammalian cells. Also, this is in accordance with the works of Oliveira and

colleagues that suggest that more than one secretory pathway, that is both the conventional ER-Golgi secretory pathway and the endosomal/MVBs pathways or others, might be involved in yeast extracellular vesicles secretion [162, 163]. This could possibly originate different types of EVs, some of them more similar to exosomes and others to larger microvesicles.

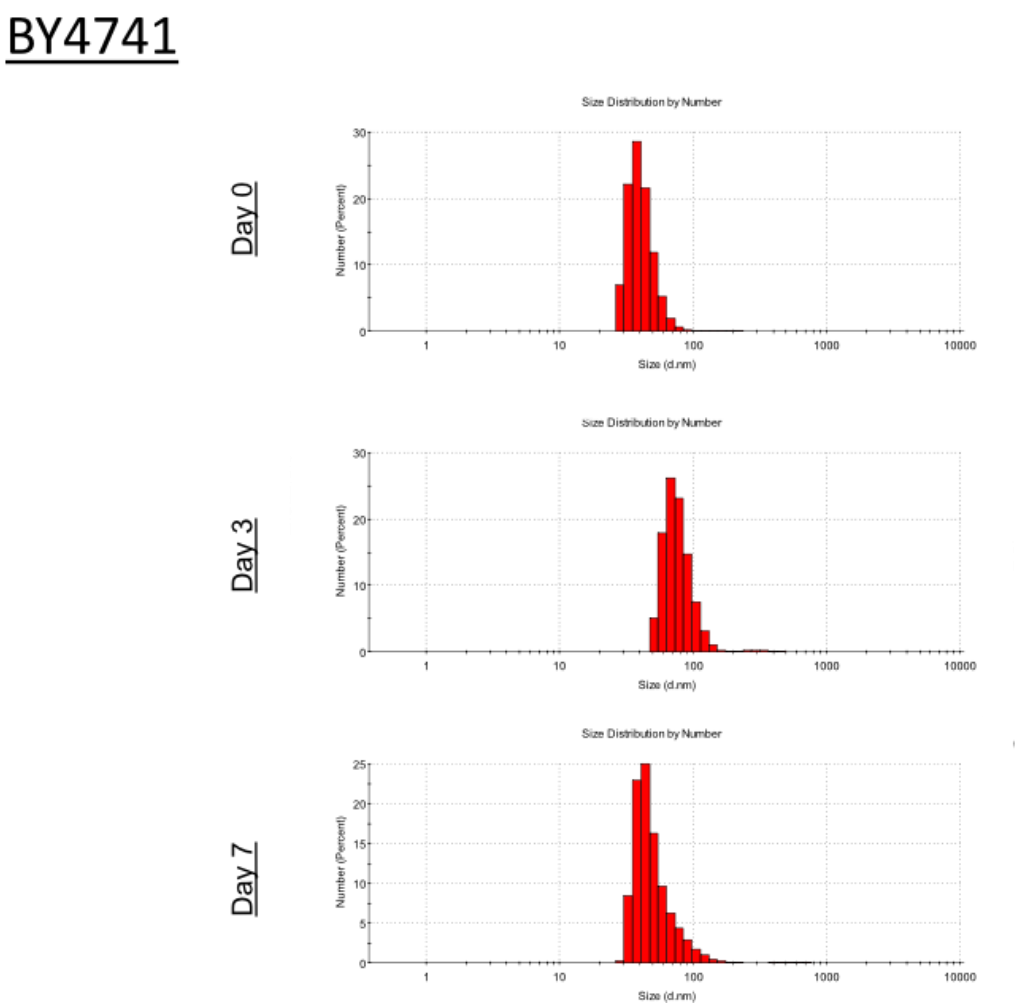


Figure 24. Vesicular fraction isolates analysed by DLS of culture media have particles with diameters on the nanometer scale Vesicular fractions obtained from culture media of BY4741 cells collected at days 0, 3 and 7 of CLS were analysed by DLS. Histograms of diameter distribution of a representative biological replicate shown. Three independent biological replicates were analysed.

Table 2. Average and standard deviation of particle's diameter distribution on vesicular fractions isolates The Z-average parameter is an intensity weighted mean of all the particles detected by DLS. Vesicular fractions obtained from culture media of BY4741 cells collected at days 0, 3 and 7 of CLS were analysed by DLS Three independent biological replicates were analysed.

Sample	Z-average (nm)	Standard deviation (nm)
Day 0	204.03	13.02
Day 3	293	66.97
Day 7	466.73	74.57

The same vesicular fractions were also analysed by Nanoparticle Tracking Assay (NTA). NTA also tracks the Brownian motion of particles in a suspension. In addition to a laser, NTA apparatus have optical camera instruments. With this technique we were able to determine the concentration of particles in addition to their diameter distribution. The particles were grouped into two categories, exosomes-like and microvesicle-like according to the sizes reported in the literature (Fig. 25). The higher particles concentrations detected were on the lower diameters, compatible with the presence of exosomes. We also detected to a smaller extent, some particles compatible with larger microvesicles. The higher concentrations of exosome-like particles were also detected in the older day 7 samples and overall, this NTA data corroborates the previous data obtained by DLS. Both methods strongly support the presence of EVs in the vesicular fractions of yeast culture media, secreted by conventional or unconventional pathways. These EVs could modulate other cells ageing outcomes by cell-nonautonomous mechanisms, by delivering transmissible factors as their cargo.

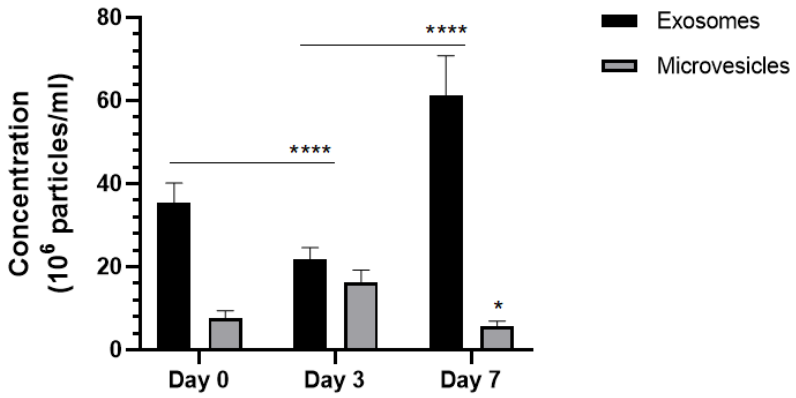


Figure 25. Vesicular fraction isolates of culture media analysed by NTA have particles with diameters mainly in the exosome range Vesicular fractions obtained from culture media of BY4741 cells collected at days 0, 3 and 7 of CLS were analysed by NTA. Histograms of diameter distribution plotted against particle concentration of a representative biological replicate shown. Three independent biological replicates were analysed Significance was determined by two-way ANOVA between cells. Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells The error bars represent the standard error of the mean (SEM).

Then, to better understand the dynamics of EV biogenesis on the budding yeast, we performed genetic abrogation of the unconventional endosomal/MVB secretory pathway with the *SNF7* null mutant. Snf7 is a subunit of ESCRT-III, a key mediator of MVB biogenesis [198]. BY4741 *Δsnf7* cells exhibit a much-abridged lifespan regarding the wild-type strain (Fig. 26). Since survival on this mutant starts to plummet significantly from day 3 of CLS onward, we focused in the day 0 and 3 time points, excluding 7 days of CLS.

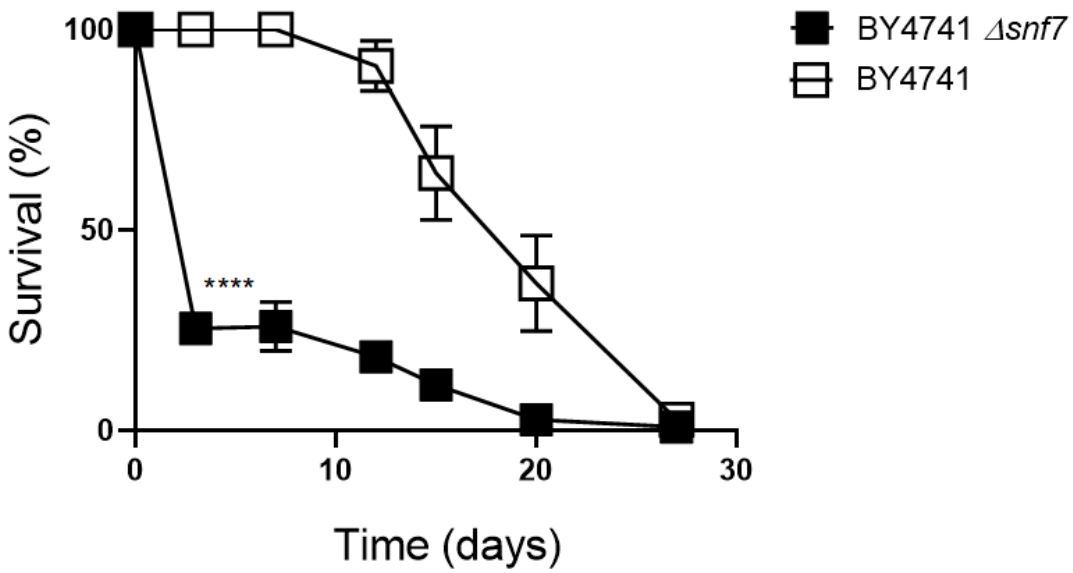


Figure 26. Genetic abrogation of MVB pathway by *SNF7* deletion results in a decreased lifespan Chronological lifespan (CLS) of stationary-phase yeast strain BY4741 and BY4741 *Δsnf7*. Cell viability was measured at 2–3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). Statistical significance was determined by two-way ANOVA (*p≤0.05, ****p≤0.0001) between cells. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

Vesicular fractions obtained from culture medium collected at 3 and 7 days of CLS of BY4741 *Δsnf7* were analysed by DLS and the diameter's distribution plotted (Fig. 27).

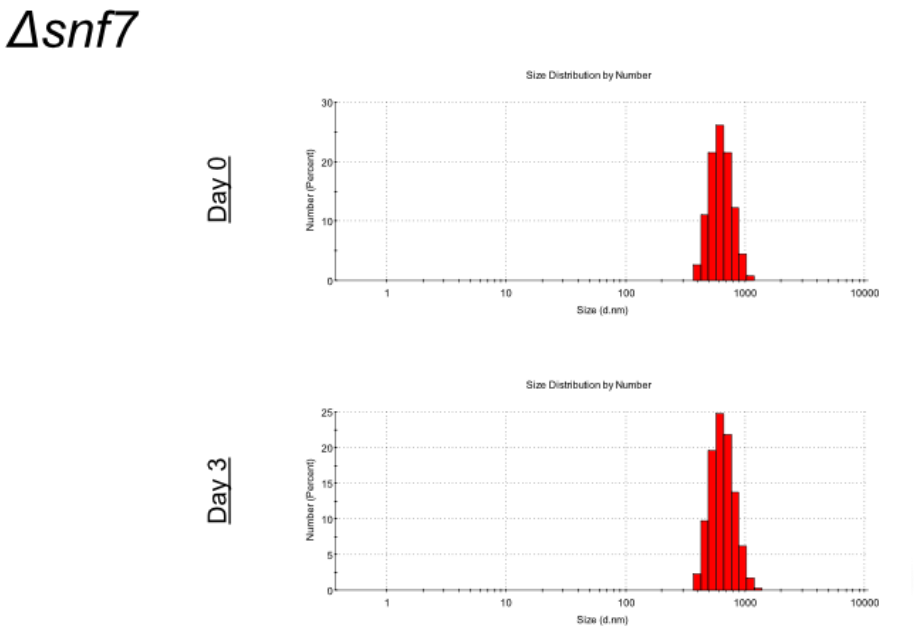


Figure 27. Vesicular fraction isolates of culture media from *Δsnf7* cells have larger diameters Vesicular fractions obtained from culture media of BY4741 *Δsnf7* cells collected at days 0 and 3 of CLS were analysed by DLS. Histograms of diameter distribution of a representative biological replicate shown. Three independent biological replicates were analysed.

Here, the samples diameter distribution appeared to be concentrated way above 100 nm and below 1000 nm, which seem to point out to the presence of larger microvesicles, and none of the smaller (<100 nm) exosome-like particles. Also, the Z-average parameter is considerably higher than what was detected on wild-type BY4741 samples (Table 3).

Table 3 Average and standard deviation of particle's diameter distribution on vesicular fractions isolates from *Δsnf7* cells The Z-average parameter is an intensity weighted mean of all the particles detected by DLS. Vesicular fractions obtained from culture media of BY4741 *Δsnf7* cells collected at days 0 and 3 of CLS. Three independent biological replicates were analysed

Sample	Z-average (nm)	Standard deviation (nm)
Day 0	781.0	109.3
Day 3	707.8	47.19

The overall lack of smaller, exosome-sized particles seems to be a consequence of the effective genetic abrogation the endosomal/MVB pathways. This data also supports the notion that the MVB pathways is essential for the biogenesis of exosome-sized vesicles in yeast. The presence of larger particles (>100 nm) could point out the role of other secretory pathways such as the conventional ER-to-Golgi secretion in EVs biogenesis in yeast. Other potential pathways of relevance to EVs biogenesis could also include secretory autophagy and CUPS associated unconventional secretion. This data agrees with other authors

suggesting that in yeast different types of EVs from different origins are secreted and not one single pathway is responsible for the totality of EV biogenesis [162, 163].

At the onset of this screening study, we hypothesized that yeast cells secrete various classes of low-molecular weight transmissible factors, perhaps nucleic acids, proteins, or lipids, enveloped or not into EVs, that could modulate ageing through cell-nonautonomous mechanisms. We began by characterizing the CLS of the common wild-type BY4741 cells and found no significant increase in cell death in the first days of CLS. Therefore, any molecules found on the extracellular medium of cells during that timeframe, is likely secreted by either conventional or unconventional pathways and not as a byproduct of the breakdown of dying cells. Nucleic acids (DNA and RNA), proteins and lipids were detected in significant amounts in samples from days 3 and 7. Protein and RNA seem to accumulate over time in the secretome, however the lipids at day 7 decreased, regarding day 3. This could mean that at this time point, lipids could already be entering other yeast receptor cells in the vicinity. Also, as EVs have been described in all domains of life, and particularly in yeast and fungi species to act as transport vectors between cells, carrying these same classes of molecules, in addition to being themselves enriched in several lipidic classes, we assessed the presence of EVs in the vesicular fractions obtained from samples collected at days 0,3 and 7 of CLS. Our results suggest that EVs of sizes compatibles with both exosome and microvesicles are present in vesicular fractions of aged cells' secretome, starting at the beginning of CLS (day 0). Genetic abrogation of a key player in unconventional secretion, abrogates the release of exosomes sized particles, though not of microvesicles. The findings of these screening experiments suggest a role for cell-nonautonomous mechanisms of ageing in yeast CLS, with classes of molecules as putative candidates for transmissible factors. In accordance with the literature, it also shed light into the possible involvement of EVs biogenesis and release in the transmission of biological information between chronological ageing yeast.

Section 3.3

Cell-nonautonomous mechanisms and the modulation of yeast chronological lifespan: A proteotoxic stress model (aSyn)

3.3. Conditioned media from prematurely aged cells can modulate ageing progression

In the previous chapter, we endeavoured to search and characterise the diversity of molecules that constitute the secretome of yeast cells during chronological ageing. Cell-nonautonomous mechanisms are suggested to modulate ageing by releasing several molecular classes of transmissible factors [143, 284]. Although many questions remain, we managed to find different classes of molecules present in ageing yeast conditioned media, from lipids to nucleic acids, proteins and ascertained the presence of EVs, likely originating from both conventional and unconventional secretory pathways, as already suggested in the literature [162].

Here, we set out to characterise the role of cell-nonautonomous mechanisms in yeast ageing modulation by employing a yeast expression model and monitoring a specific molecule as a proof-of-concept of this type of intercellular communication. The human aSyn protein, associated with aggregation and synucleinopathies in neurons, can be heterologously expressed in yeast, promoting proteotoxic stress and premature ageing [247]. This model was already well-established to study cellular ageing on yeast [247, 290, 291], as shown previously in **Section 3.1**. In this section, the same yeast model was employed to understand the role of cell-nonautonomous mechanisms on protein secretion during yeast CLS that can potentially influence ageing progression.

aSyn was already shown to be propagated between different neuronal cells in the human brain in a prion-like behaviour [173, 229]. aSyn can be proteolytic degraded in the extracellular medium or transported inside neighbouring cells, which can also be facilitated with exosomes as shuttle vectors [174]. Prions are mechanisms of epigenetic inheritance in yeast, as is the case of the prototypical Sup35 prion, which was recently found to be secreted enveloped in EVs [171]. We also want to understand if by cell-nonautonomous mechanisms during yeast CLS, aSyn known prion-like behaviour is conserved in this ageing model too. And if so, if aSyn horizontal transmission occurs between different cells in an ageing yeast population, with impact on longevity of the receptor cells and whether aSyn is enveloped in exosome-like structures or other types of EVs.

3.3.1. Prematurely aged cells secrete aSyn into the extracellular milieu

Considering the findings obtained in humans about the prion-like horizontal transmission of aSyn between neurons [173], and the association of aSyn transmission with exosomes [174], we set out to understand

if such a horizontal transmission of this pro-ageing protein occurred in yeast. First, we began by characterizing the CLS of aSyn expressing cells and, as expected, we found a significant decrease (about 50% until day 7 of CLS) in lifespan associated with the wild-type aSyn expression in these cells regarding the control vector and the aSyn A30P variant (Fig. 28A and B). Taking advantage from our yeast model expressing aSyn variants and in an attempt to evaluate the horizontal transmission of aSyn between yeast cells, we collected culture medium from 0, 3 and 7 days CLS, and analysed both the proteins present in the extracellular media and the vesicular fractions obtained from these aSyn-expressing cells. We started to evaluate the presence of aSyn in the collected medium. For that, after proteins' extraction from the the medium, we performed an immunoblot assay against aSyn. Data revealed the presence of secreted aSyn into the extracellular milieu (Fig. 29), at days 0, 3 and 7 of CLS. These data suggest that cells have the ability to secrete aSyn to the medium.

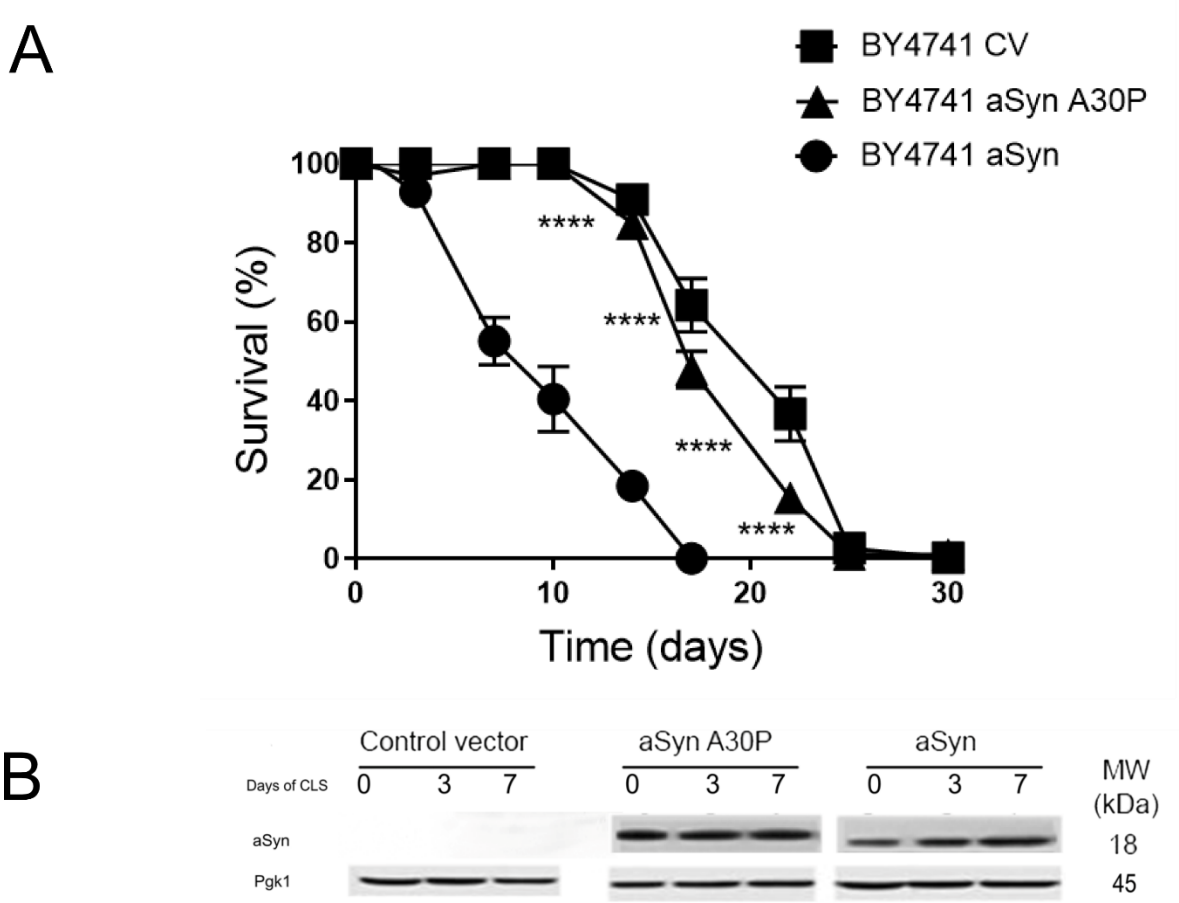


Figure 28. aSyn decreases survival of BY4741 yeast during chronological lifespan (A) Chronological lifespan of BY4741 expressing wild-type aSyn, aSyn A30P or control vector plasmids. Cell viability was measured at 2–3 day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). Significance was determined by two-way ANOVA between cells. Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells The error bars represent the standard error of the mean (SEM). **(B)** aSyn levels of BY4741 cells expressing wild-type aSyn, aSyn A30P or the control vector variant, during days 0,3 and 7 of CLS. At least three biological independent replicas were analysed.

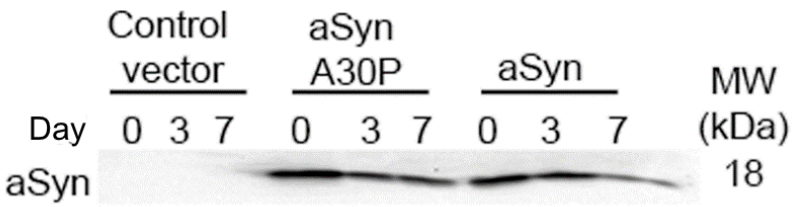


Figure 29. aSyn is secreted to the extracellular media Immunoblot analysis of aSyn precipitated from culture media of BY4741 cells expressing wild-type aSyn, aSyn A30P variants or control vector at days 0,3 and 7 of CLS. At least three biological independent replicas were analysed.

Our next question was related to the mechanism by which cells release proteins such as aSyn and the possibility of EVs being used to shuttle aSyn between cells. Thus, we also attempted the isolation, by ultracentrifugation, of the vesicular fractions present in the culture medium of yeast cells expressing the aSyn variants at days 0, 3 and 7 of CLS. The obtained vesicular fractions were analysed by Dynamic Light Scattering (DLS) (Table 4 and Fig. 30). With this technique, we were able to determine the particles' diameter distribution and the intensity weighted Z-average parameter. Overall, the samples' size range was comprised between a minimum of 28 nm and a maximum of 615 nm. The age appears to have an effect on the particles' size, as with culture medium of cells at day 0 of CLS, all the samples have a distribution below 100 nm, while with at day 3 and 7, most of the particles found in the culture medium presented a size above this level. Culture medium from aSyn and aSyn A30P expressing cells also seem to have larger particles (above 200 nm) than the media from control vector. These trends seem to be corroborated by the differences in Z-average parameter as well (Table 4). These diameter values are compatible with what is found in the literature for either exosomes (<100 nm) or microvesicles (>100 nm) [169, 176], suggesting that more than one secretory pathway might be involved in yeast extracellular vesicles biogenesis.

Table 4. Average and standard deviation of particle's diameter distribution on vesicular fractions isolates from BY4741 aSyn expressing cells The Z-average parameter is an intensity weighted mean of all the particles detected by DLS. Vesicular fractions obtained from culture media of BY4741 cells expressing the wild-type aSyn, aSyn A30P or the control vector at days 0, 3 and 7 of CLS were analysed by DLS. Three independent biological replicates were analysed.

Sample	Z-average (nm)	Standard deviation (nm)
Day 0		
BY4741 CV	204.3	13.02
BY4741 aSyn A30P	151.3	66.48
BY4741 aSyn	269.2	44.75
Day 3		
BY4741 CV	293	66.97
BY4741 aSyn A30P	327.1	30.62
BY4741 aSyn	201.4	54.31
Day 7		
BY4741 CV	466.73	74.56
BY4741 aSyn A30P	575.5	81.88
BY4741 aSyn	493.6	112.4

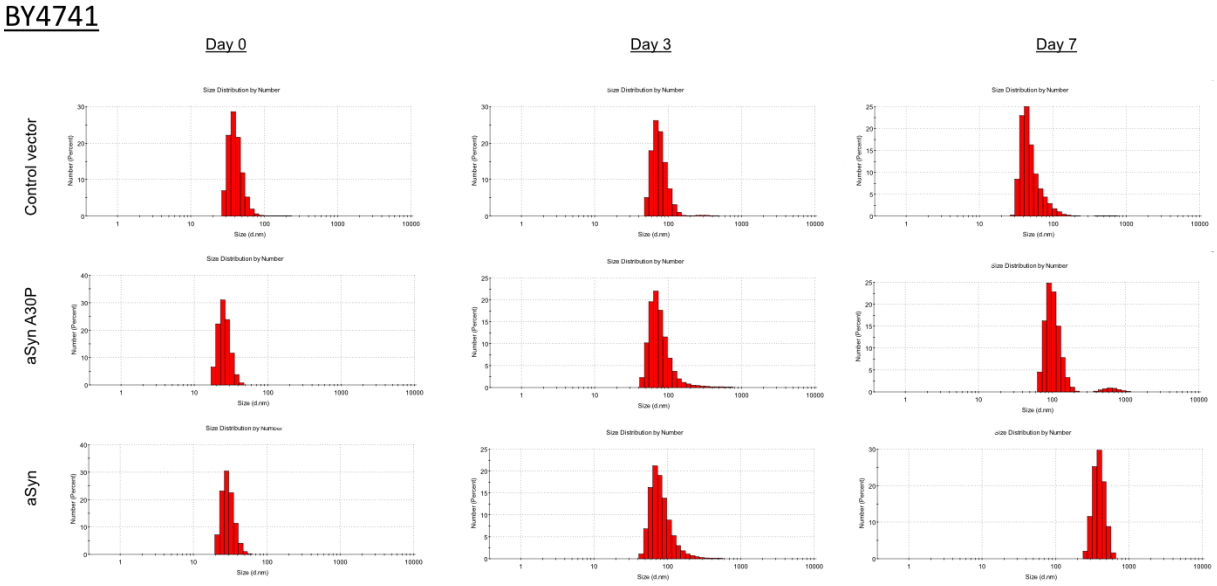


Figure 30. Vesicular fraction isolates of culture media analysed by DLS have particles with diameters on the nanometer scale Vesicular fractions obtained from culture media of BY4741 cells expressing the wild-type aSyn, aSyn A30P or the control vector at days 0, 3 and 7 of CLS were analysed by DLS. Histograms of diameter distribution of a representative biological replicate shown. 3 independent biological replicates were analysed.

The same vesicular fractions were also analysed by Nanoparticle Tracking Assay (NTA) (Fig. 31). With this technique we were able to determine the concentration of particles and their diameter. Particles were grouped by diameter in two categories: exosome-like and microvesicles-like. This corroborated our

previous DLS data that hinted at the presence of both types of EVs in the vesicular fractions and complemented them with information on their concentration in each sample. Results suggested that the higher particles concentration detected were on the lower diameters, compatible with the presence of higher amounts of exosome-like structures.

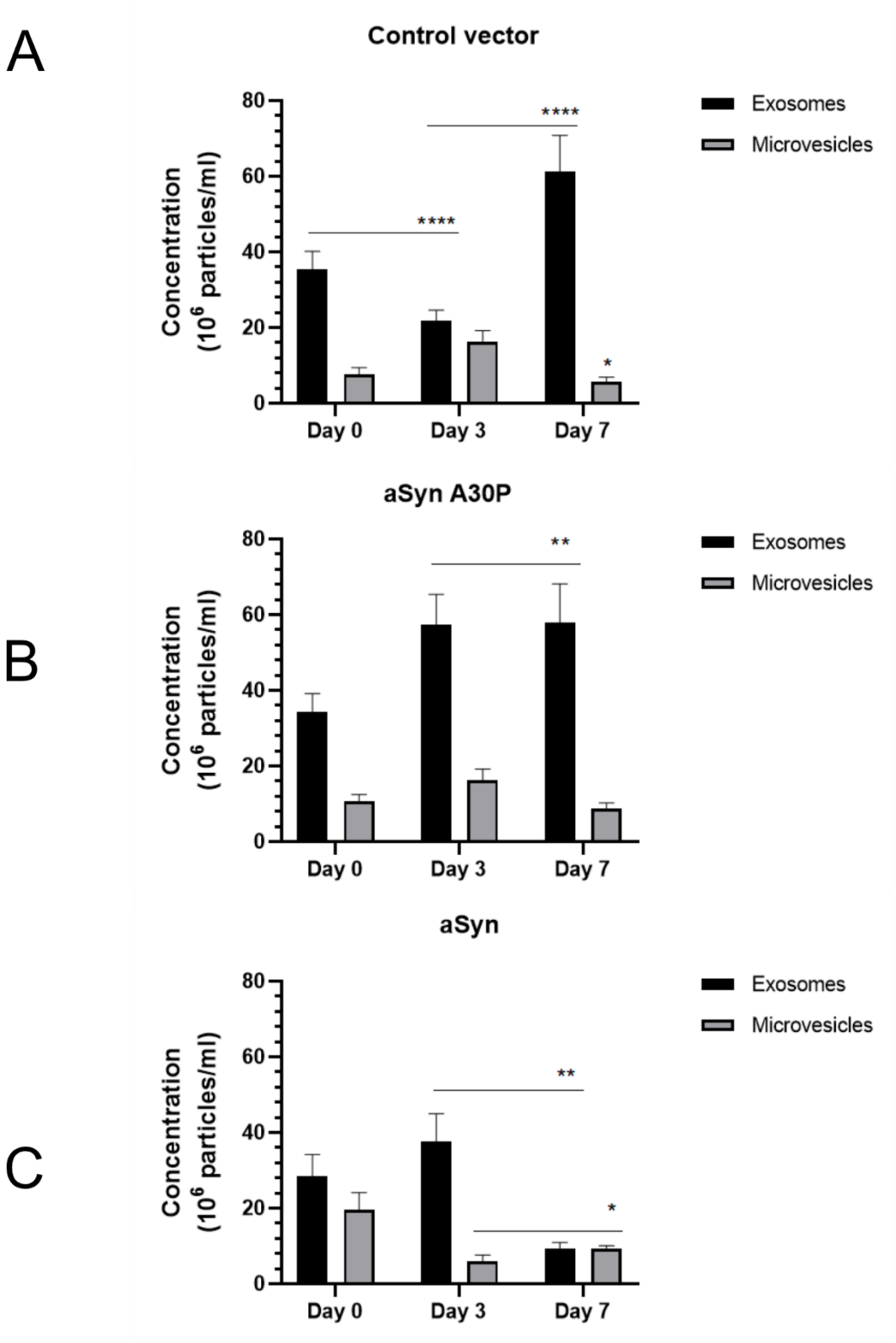


Figure 31. Vesicular fraction isolates of culture media analysed by NTA have particles with diameter mainly in the exosome range Culture media from BY4741 cells expressing the the control vector **(A)**, or the aSyn A30P **(B)** and wild-type aSyn **(C)** variants collected at days 0, 3 and 7 of CLS was analysed by NTA Significance was determined by two-way ANOVA between cells. Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells The error bars represent the standard error of the mean (SEM).

Furthermore, for medium from cells expressing the control vector or the non-toxic aSyn variant (aSyn A30P) exosome-like particles concentration seems to increase with ageing. In contrast, the medium of cell expressing the toxic variant (aSyn) presented lower number of EVs, and a decrease on the number of vesicles during ageing is observed. It is tempting to speculate that a cell-nonautonomous mechanism could be mediating the uptake of EVs by receptor cells in the same population during CLS, in vesicles containing wild-type aSyn proteins. Another possibility is that the aSyn protein blocks EVs secretion over time.

To attempt to elucidate the former hypothesis, of a cell-nonautonomous mechanism involving the transmission of a deleterious protein such as aSyn during yeast CLS, in the same vesicular fractions of isolated from culture media of cells at days 3 and 7 of CLS, we were able to detect and quantify several proteins. We ran 3 µg of protein extracts into SDS-PAGE electrophoresis (Fig. 32A) and visualized several bands corresponding to proteins of different molecular weights mainly in the vesicular fractions from aSyn variants (Fig. 32A and B). The higher band intensities were detected in the culture medium of cells expressing wild-type aSyn at day 3 days of CLS. Finally, to confirm the presence of the human aSyn protein also in these vesicular fractions we performed an immunoblot assay against aSyn (Fig. 32C).

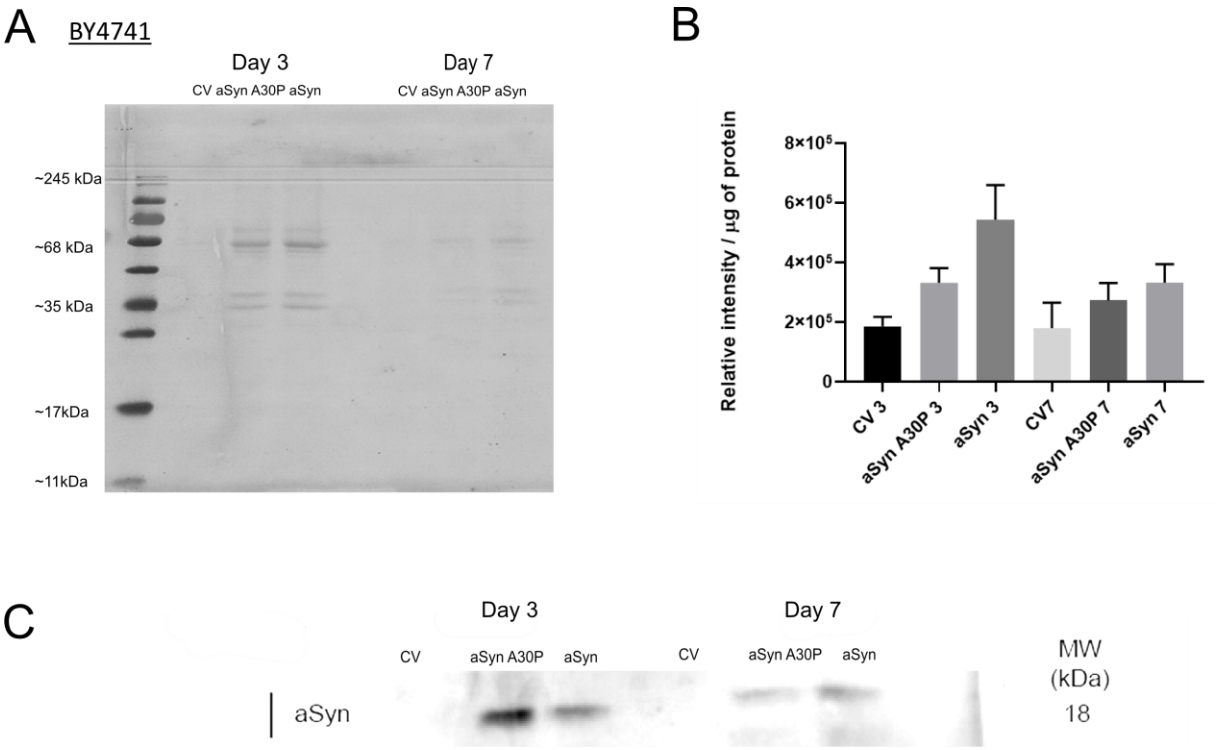


Figure 32. Proteins like aSyn can be found on the vesicular fraction isolates of aSyn expressing yeast during chronological lifespan (A) SDS-PAGE electrophoresis protein profile of vesicular fractions obtained from culture media collected at days 3 and 7 of CLS from BY4741 expressing the wild-type aSyn, aSyn A30P variants and control vector. **(B)** Graphical representation of the intensity of total protein obtained by densitometric analysis. Data represents mean ± SEM of at least two biological independent replicas. Statistical significance was determined by Student's t-test with Welch's correction (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM). (C) Immunoblot of vesicular fractions obtained from culture media collected at days 3 and 7 of CLS from BY4741 expressing the wild-type aSyn, aSyn A30P variants and control vector. aSyn was detected using a aSyn-specific antibody.

Bands of aSyn were detected on vesicular fractions obtained from both aSyn variants' media at days 3 and 7 of CLS.

This data supported the notion that aSyn protein was secreted into the extracellular milieu, not just as a free molecule, but also inside EVs during yeast CLS. This is interestingly analogue to the transmission of aSyn in neurons [229]. Like the above NTA concentration data (Fig. 31), samples from culture medium of cells with 7 days of CLS seem to have lesser amounts of aSyn protein than the culture medium of cells with 3 days of CLS, suggesting a hypothetical cell-nonautonomous clearance or trafficking mechanism taking place. Further research is necessary to address this hypothesis.

3.3.2. Conditioned media containing aSyn modulates yeast chronological lifespan

We had established above that medium from aSyn expressing yeast cells contained aSyn protein free in the medium and in the vesicular fractions. Whether the presence of aSyn in the medium of premature aged cells may influence the CLS of receptor younger (naïve) cells in the same population, was our next question. Thus, we collected media from aged cells expressing aSyn variants (wild-type, aSyn A30P) or the control vector, at distinct time points and supplemented naïve receptor cells with that medium and evaluated their CLS. The medium collected from premature aged cells, which contain all metabolites and molecules secreted by them was designed by conditioned medium (CM). This methodology is detailed below (Fig. 33).

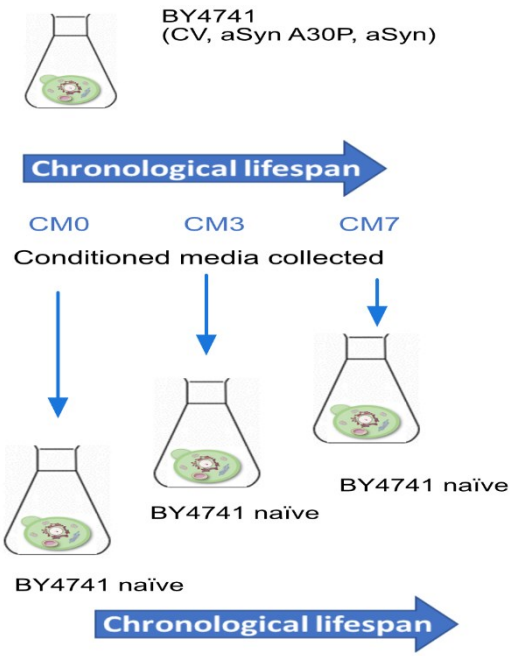


Figure 33. Conditioned media is harvested from BY4741 Conditioned media at days 0,3, and 7 of CLS **(**CM0, CM3, and CM7) is harvested from BY4741 cells expressing wild-type aSyn, aSyn A30P or the control vector by differential centrifugation. CM is then supplemented to naïve cells at day 0 of their CLS.

By the previous analysis of the aSyn-expressing cells CLS (Fig. 28), we set out to collect the CM until day 7 of CLS, as that time point represents a CLS reduction of about 50%. We hypothesised that in later times the percentage of death cells could be a confounder, leading to uninterpretable data. The collected CM from 0, 3 and 7 days of CLS was used to supplement naïve receptor cells (that do not express aSyn variants) at 0 days of CLS. Data revealed that naïve receptor cells displayed decreased CLS when transferred into the CM from older cells. The CLS decrease of receptor cells was more pronounced with CM of cells with 3 and 7 days of CLS.

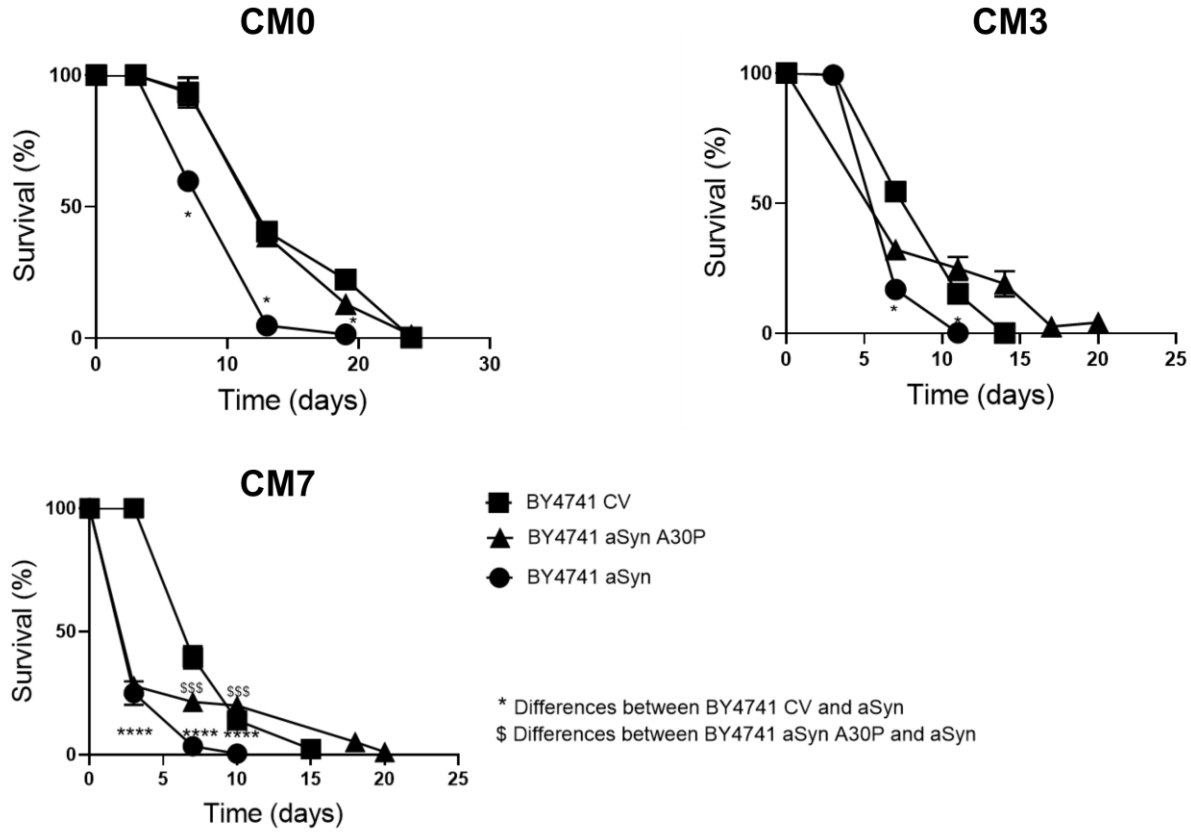


Figure 34. Conditioned media containing aSyn decreases yeast chronological lifespan Chronological lifespan of naïve BY4741 cells supplemented with conditioned media from BY4741 cells expressing wild-type aSyn, aSyn A30P or the control vector collected at days 0 , 3, and 7 (CM0, CM3, CM7) of CLS. Cell 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%). Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

The CLS of receptor cells grown in the CM of cells expressing the vector control or the aSyn A30P variant presented a similar CLS between them. The differences were more pronounced with CM from cells

expressing the aSyn wild-type variant (Fig. 34A, B and C). These results were confirmed by comparing the mean and maximum lifespans of the receptor cells supplemented with the different CM (Fig. 35). Together, the results suggested that during ageing, cells release molecules that might act as transmissible factors modulating organismal longevity by cell-nonautonomous mechanisms, raising the hypothesis that such processes are widespread. In addition, these results also raise the possibility that the CM from cells under proteotoxic stress present different metabolites that contribute to lifespan regulation, such as the aSyn protein detected in the secretome of the aSyn-expressing cells.

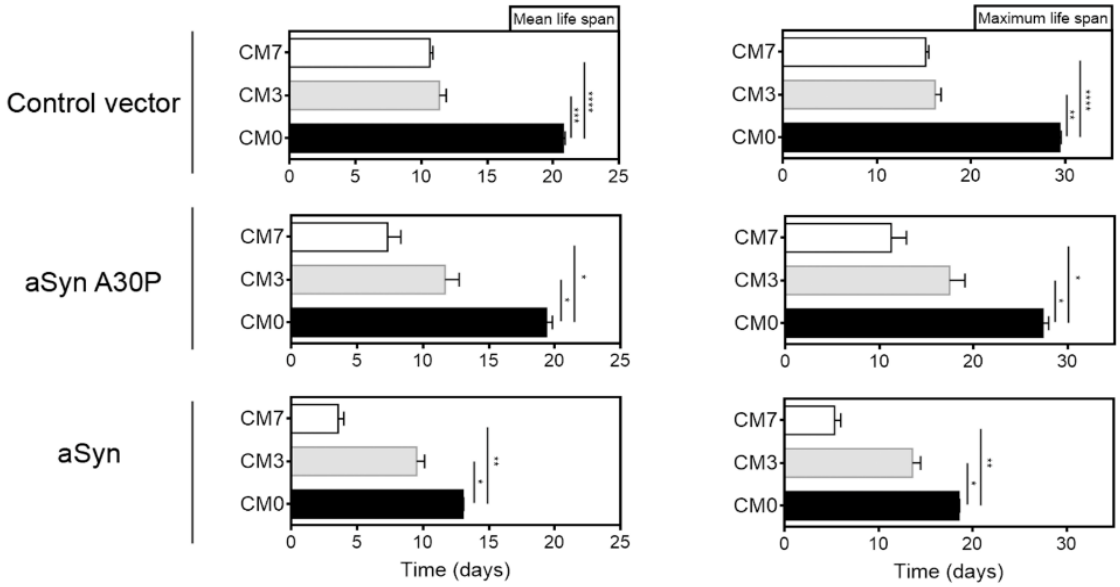


Figure 35. Conditioned media containing aSyn decreases yeast survival Mean (50% survival) and maximum (10% survival) lifespans determined from curve fitting of the survival data from CLS of naïve BY4741 cells supplemented with conditioned media from BY4741 cells expressing wild-type aSyn, aSyn A30P or the control vector collected at days 0, 3, and 7 of CLS. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

3.3.3. Naïve cells uptake aSyn from the conditioned media

Given the aforementioned results that aSyn is secreted by yeast during ageing and that CM from prematurely aged aSyn-expressing cells decreases the lifespan of naïve cells, we hypothesized that these prematurely aged cell expressing aSyn release toxic molecules to the medium, likely aSyn itself or other pro-ageing factors, that promote cell death on neighbouring cells. aSyn could conceivably be involved in cell signalling pathways or being internalised by the naïve receptor cells to induce premature ageing. The latter hypothesis bears some resemblance with the recently uncovered prion-like horizontal transmission of aSyn described for human neurons [173, 229]. This fact, coupled with the notion that prions in yeast

are epigenetic elements of inheritance that induce phenotypic alterations on the receptor cells [175], lead us to wonder whether and how aSyn protein released from aged a-Syn expressing cells entered naïve receptor cells. To that end, we start to evaluate if naïve receptor cells uptake aSyn from the CM. Thus, we collected whole-cell protein extracts from the naïve receptor cells supplemented with CM (at days 0, 3, 7 and 10 of their CLS) and ran immunoblots against aSyn (Fig. 36). We were able to detect the presence of aSyn protein on naïve receptor cells co-cultured with CM0, CM3, and CM7 of both aSyn variants. The uptake and accumulation of aSyn within naïve receptor cells appear to be a gradual and cumulative process that occurs during CLS: with little to no protein detected at day 0 of naïve cells' CLS on either CM0, CM3 or CM7 supplementation; steadily increasing throughout the timeframe we monitored from day 3 until day 10 of naïve cells' CLS (Fig. 36).

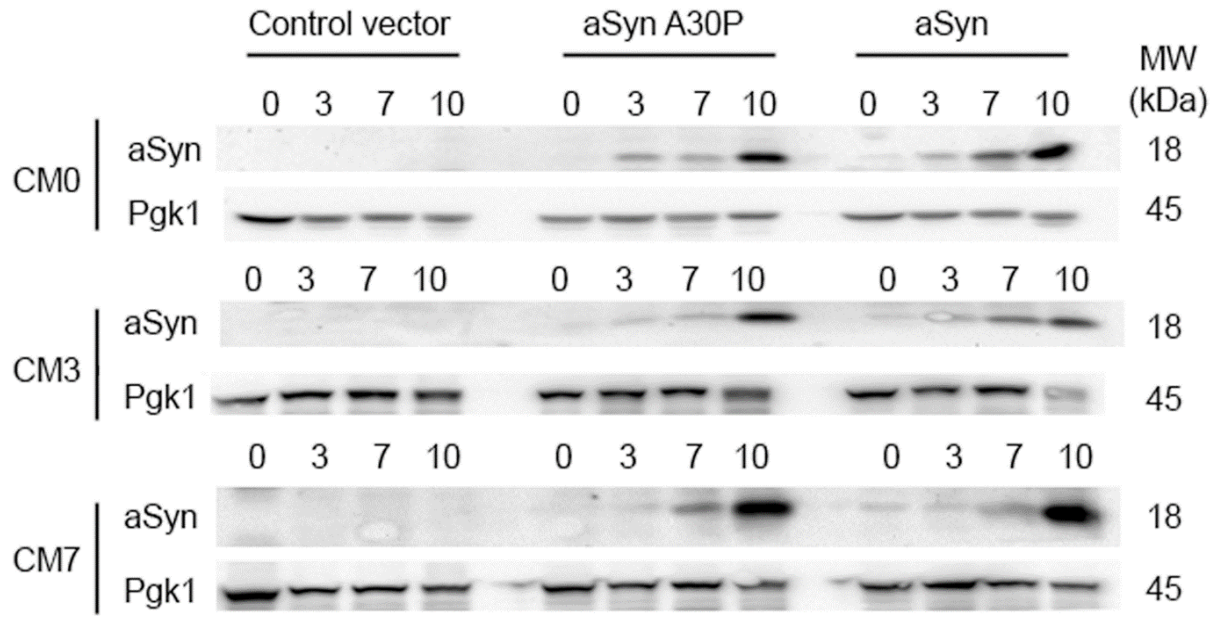


Figure 36. aSyn secreted to yeast conditioned media can enter naïve receptor cells Immunoblot analysis of aSyn extracted from wild-type naïve receptor cells grown on conditioned media collected from BY4741 cells expressing wild-type aSyn, aSyn A30P variants or control vector at days 0, 3 and 7 of CLS. Media was supplemented to naïve receptor cells at day 0 of their CLS. At least three independent biological replicates were analysed.).

To corroborate these findings, we also attempted to visualize aSyn entering naïve receptor cells through immunofluorescence microscopy. Cells were observed at day 3 and 7 of their CLS, in bright field (BF), with a DAPI nucleic acid stain and on FITC fluorescence to detect aSyn protein through the AlexaFluor 488 conjugated aSyn antibody. We were able to visualize aSyn-associated fluorescence in naïve receptor cells both at days 3 and 7 (Fig. 37 and 38). The number of aSyn A30P fluorescent cells we could detect

was visibly greater than those of the wild-type aSyn variant. This could be due to different intracellular localization of both variants; aSyn A30P tends to accumulate within the cytoplasm, while wild-type aSyn initially localizes to the plasma membrane [224].

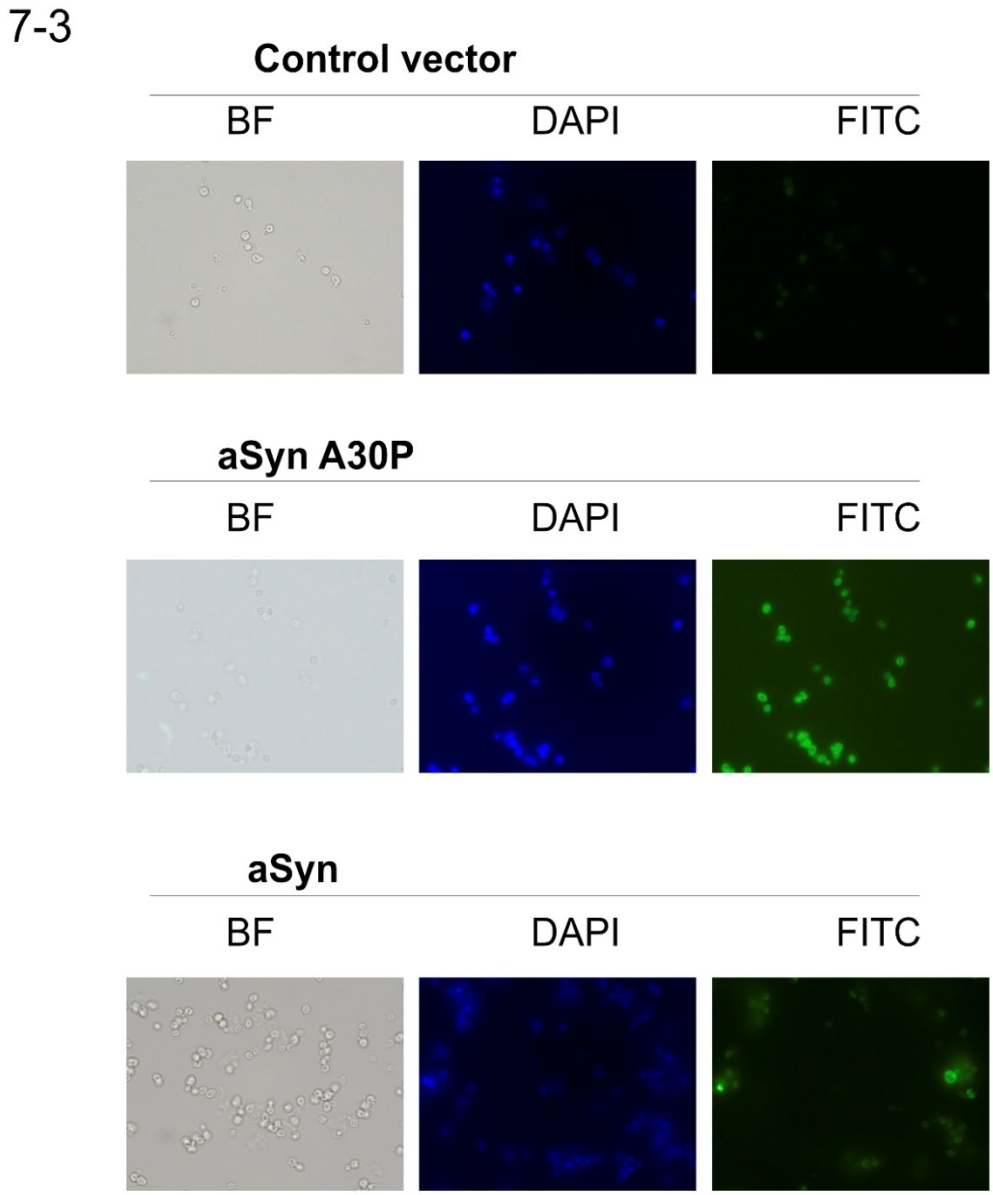


Figure 37. aSyn secreted to yeast conditioned media can enter naïve receptor cells Representative immunofluroescence microscopy images on bright field (BF), nucleus staining (DAPI) and aSyn-conjugated antibody of naïve receptor cells grown to day 3 of CLS supplemented with CM collected from BY4741 cells expressing wild-type aSyn, aSyn A30P variants or control vector at day 7 of CLS.

7-7

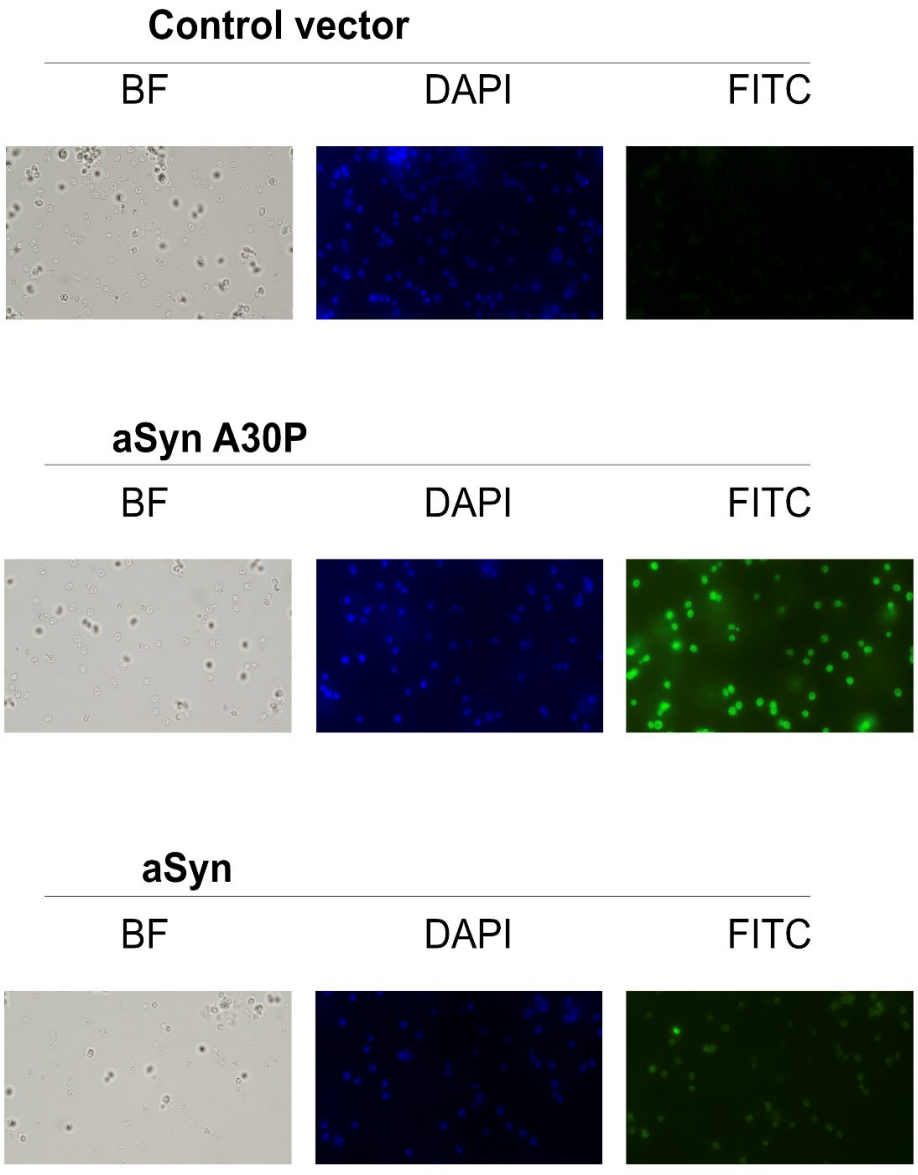


Figure 38. aSyn secreted to yeast conditioned media can enter naïve receptor cells Representative immunofluroescence microscopy images on bright field (BF), nucleus staining (DAPI) and aSyn-conjugated antibody of naïve receptor cells grown to day 7 of CLS supplemented with CM collected from BY4741 cells expressing wild-type aSyn, aSyn A30P variants or control vector at day 7 of CLS.

Overall, our results support the notion that aSyn can work as a proof-of-concept model for horizontal transmission of transmissible factors between prematurely ageing yeast cells. This protein expression in yeast decreases CLS and the protein can be found on either CM from aSyn expressing cells or vesicular fractions isolates from CM, pointing to a possible role of EVs in its secretion. Supplementation with CM containing aSyn of naïve receptor cells also promotes premature ageing and decreased survival during chronological lifespan. The aSyn can also be detected inside these naïve receptor cells during chronological lifespan, suggesting a mechanism for its uptake. The precise mechanisms of aSyn secretion and uptake in yeast remained open to further investigation.

Section 3.4

Cell-nonautonomous mechanisms and vesicle trafficking: Mechanistic links between retrograde and anterograde trafficking in a proteotoxic stress model (aSyn)

3.4 Cell-nonautonomous mechanisms modulating yeast chronological lifespan are impacted by vesicle trafficking

In the previous section, our data established human aSyn protein as a proof-of-concept model for the horizontal transmission of low-molecular weight factors between yeast cells during CLS. Not only was aSyn secreted into the extracellular milieu, but it also entered naïve receptor cells and altered the ageing progression outcomes.

The human aSyn yeast expression system, already extensively employed to study yeast ageing, proteotoxic stress and pathogenesis of synucleinopathies [29, 224, 247], was chosen here to understand the dynamics of cell-nonautonomous mechanisms of ageing. This is an interesting model also considering the findings in humans about the prion-like propagation of aSyn [173, 174] and its association with EVs. Fungal EVs have been described in several studies in the literature [159, 162-164, 168], and on this thesis. We presented data that suggests that the budding yeast secretes distinct types of EVs during its chronological lifespan, partially through unconventional secretory pathways such as the endosomal/MVBs pathways.

Remarkably, aSyn was detected in the same vesicular fractions as EVs. All the above data points to a scenario where aSyn seems to travel across different cells on a yeast population, potentially enveloped in EVs of different origins via several trafficking pathways. However, the precise mechanisms underlying the secretion and uptake of aSyn remained unknown. In the following section, data will be presented on our efforts to understand the role of the unconventional secretory pathway of MVBs on aSyn secretion and clathrin-mediated endocytosis on its uptake during yeast chronological lifespan.

3.4.1 Effects of the abrogation of the multivesicular bodies pathway on aSyn secretion

Considering the aSyn secretion and uptake by yeast cells described above, many hypotheses remained open to elucidate the process. Yeast cells have been described in this thesis (**Sections 3.2 and 3.3**) and elsewhere [152, 163, 175] to produce extracellular vesicles. Whether aSyn is secreted enveloped in EVs or not, and by which pathways does the EVs' biogenesis occur is a topic that still needed research. One possibility is the involvement of the endosomal/MVB pathway [152, 163] and to that end we obtained a null mutant for *SNF7*. Snf7 is a subunit of the ESCRT-III complex, essential for MVB biogenesis [198]. We began by characterizing the *Δsnf7* cells expressing aSyn variants and their survival during CLS. All *Δsnf7* cells had a much-abridged lifespan, regarding wild-type BY4741 (Fig. 39). Nevertheless, we could

not detect any difference in the CLS, independently of aSyn expression. This data could however suggest that abrogation of the endosomal/MVB pathway also abrogates the aSyn mediated toxicity.

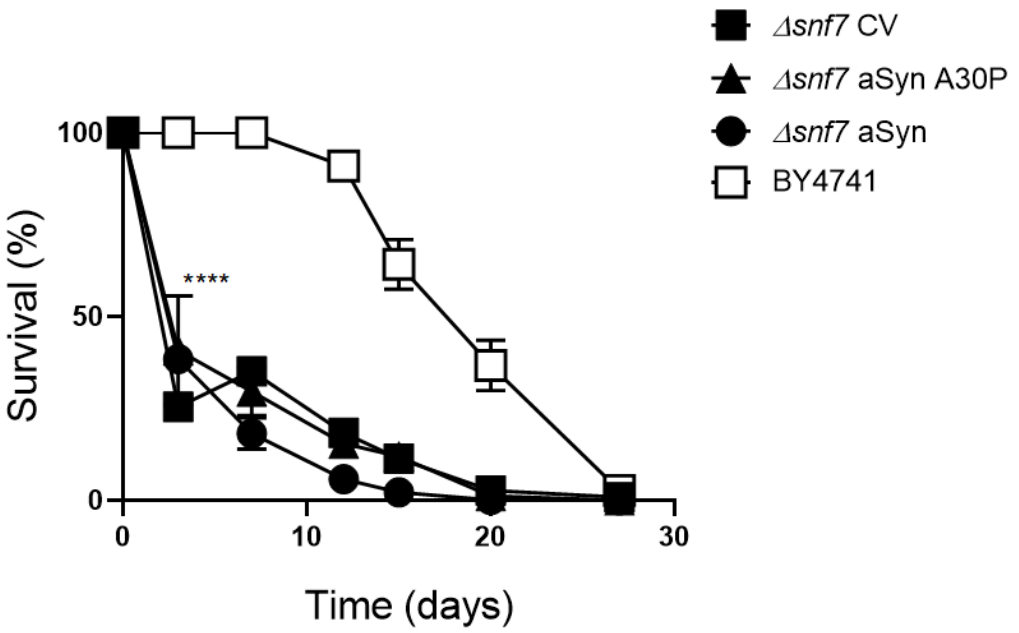


Figure 39. Genetic abrogation of MVB pathway by *SNF7* deletion results in a decreased lifespan irrespective of aSyn variants expression
Chronological lifespan (CLS) of stationary-phase wild-type yeast strain BY4741 and BY4741 Δsnf7 expressing the wild-type aSyn, aSyn A30P variants and control vector. 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%). Statistical significance was determined by two-way ANOVA (*p≤0.05, ****p≤0.0001) between cells. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

Then, to understand if the abrogation of the endosomal/MVB pathways influenced cell-nonautonomous mechanisms of ageing during yeast CLS, we supplemented naïve wild-type cells at day 0 of CLS with CM collected from *Δsnf7* cells expressing aSyn variants and control vector at days 0, 1 and 3 of CLS, and the CLS of wild type naïve receptor cells were evaluated (Fig. 40).

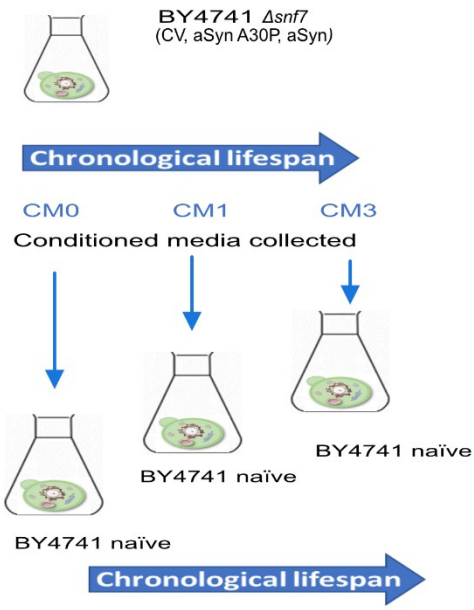


Figure 40. Conditioned media is harvested from BY4741 *Δsnf7* CM0, CM1, and CM3 is harvested from BY4741 *Δsnf7* cells expressing wild-type aSyn, aSyn A30P or the control vector by differential centrifugation. CM is then supplemented to naïve cells at day 0 of their CLS.

As described in the **Section 3.3** (Fig. 33, 34 and 35), these wild-type cells are naïve cells, as they do not express aSyn, and are receptor cells, since they will be conditioned with CM from older cells. The naïve receptor cells supplemented with CM from *Δsnf7* cells expressing aSyn variants presented increased CLS in comparison with the CLS obtained by naïve receptor cells conditioned with CM of the wild-type strain aSyn-expressing cells (Fig. 41A). Furthermore, these results were also confirmed by the analysis of the mean and maximum lifespans of naïve receptor cells with supplemented with CM from *Δsnf7* cells expressing aSyn variants: CM from *Δsnf7* cells promoted higher mean and maximum lifespans regarding the wild-type cells (Fig. 41B). Nevertheless, the presence of aSyn still reduced the mean and maximum lifespans of the cells with the wild-type aSyn variant, when comparing with aSyn A30P and control vector, which could suggest that a hypothetical cell-nonautonomous mechanisms involving aSyn as a transmissible factor between yeast cells is not entirely inhibited by the abrogation of the MVB pathway.

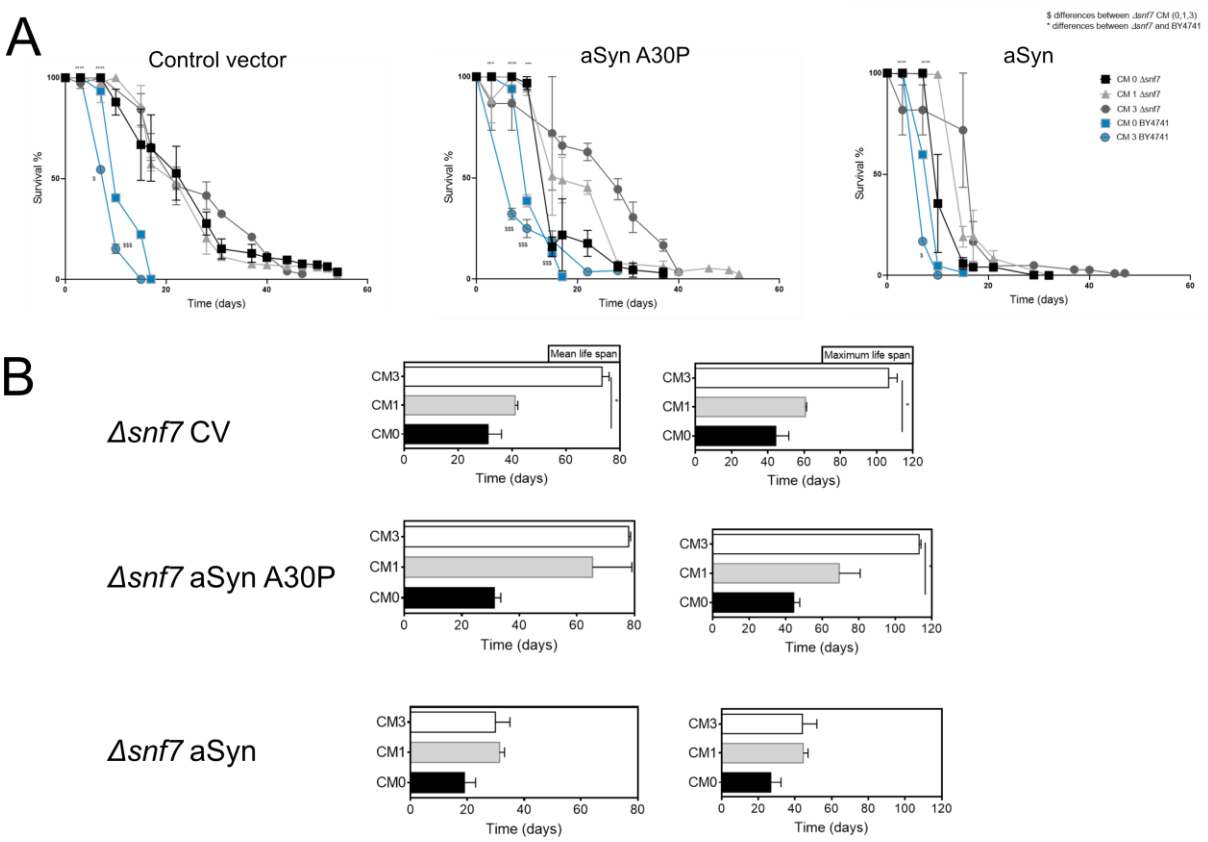


Figure 41. Conditioned media from aSyn-expressing *Δsnf7 cells* extends yeast chronological lifespan (A) Chronological lifespan of naïve BY4741 cells supplemented with conditioned media from either BY4741 *Δsnf7* cells (shades of grey) or BY4741 wild-type cells (light blue) expressing the control vector, aSyn A30P or wild-type aSyn variants collected at days 0, 1 and 3 of CLS. 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%). **(B)** Mean and maximum lifespans determined from curve fitting of the survival data from CLS of wild-type cells expressing the control vector or aSyn variants. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. Data represents mean ± SEM of at least three biological independent replicas. The error bars represent the standard error of the mean (SEM).

Given the current knowledge about aSyn horizontal transmission in other organisms via exosomes [174], and our data pointing to the release of EVs by aSyn-expressing cells of the wild-type BY4741 cells, we also attempted to characterise the profile of EVs secretion on aSyn-expressing cells with the genetic abrogation of the endosomal/MVB pathways, essential for exosome biogenesis. With DLS, we analysed vesicular fractions obtained from CM of endosomal/MVB pathway compromised cells, BY4741 *Δsnf7,* collected at days 0 and 3 of CLS. Here, the samples diameter distribution and weighted averages appeared to be concentrated way above 100 nm and below 1000 nm, which seem to point out to the presence of larger microvesicles, and not as much of the smaller (<100 nm) exosome-like particles. The only notable exception to this pattern is the conditioned media from aSyn expressing cells at day 3 of CLS, with most particles detected around 100 nm (Fig. 42A and Table 5).

Table 5. Average and standard deviation of particle's diameter distribution on vesicular fractions isolates from aSyn expressing *Δsnf7* cells The Z-average parameter is an intensity weighted mean of all the particles detected by DLS. Vesicular fractions obtained from culture media of BY4741 *Δsnf7* cells expressing the control vector, aSyn A30P or wild-type aSyn variants, collected at days 0 and 3 of CLS Three independent biological replicates were analysed

Sample	Z-average (nm)	Standard deviation (nm)
Day 0		
Δsnf7 CV	781.0	109.3
Δsnf7 aSyn A30P	530.0	76.67
Δsnf7 aSyn	878.4	69.22
Day 3		
Δsnf7 CV	707.8	47.19
Δsnf7 aSyn A30P	911.1	57.55
Δsnf7 aSyn	253.1	7.80

A lipidic characterisation of these vesicular fractions was attempted with an adapted TLC protocol [257]. Despite the standards employed for free fatty acids (FFA) and sterols, we were not able to detect these classes of lipids on any sample. The presence of lipids, identified as triacylglycerols and sterol esters by their comigration patterns in relation to the standards [257], can be detected on either wild-type strain and the *Δsnf7* mutant (Fig. 42B). Overall, no triacylglycerols (TAG) can be seen on the mutant, alongside with increased sterol esters (SE) levels, as assessed by densitometry analysis (Fig. 42C).

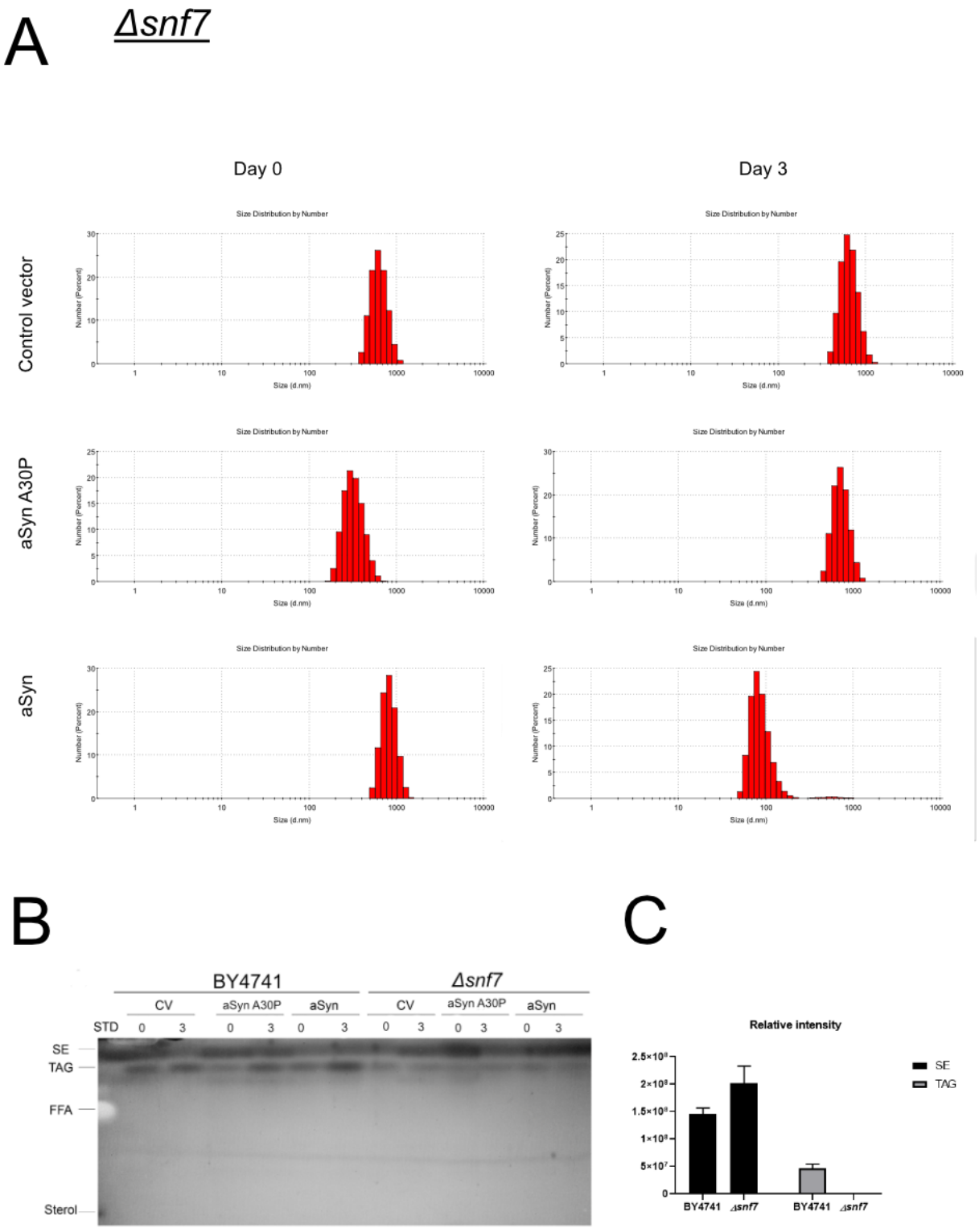


Figure 42. ***SNF7* genetic abrogation alters yeast EVs secretion profile**. **(A)** Vesicular fractions obtained from culture media of BY4741 *Δsnf7* cells expressing the wild-type aSyn, aSyn A30P or the control vector at days 0 and 3 of CLS were analysed by DLS. Histograms of diameter distribution of a representative biological replicate shown. **(B)** Lipids were extracted from the vesicular fractions and ran on a TLC silica plate. **(C)** Graphical representation of the relative intensity of total sterol ester (SE) and triacylglycerol (TAG) lipids obtained by densitometric analysis. The error bars represent the standard error of the mean (SEM).

Whether the genetic abrogation of *SNF7* also impact the composition of EVs, as well as their size and biogenesis pathways are open hypotheses, that our data so far cannot answer. This data is in accordance to what others authors described for budding yeast [162], with EVs biogenesis being reliant on both mechanisms of conventional and unconventional secretion. Still, while the genetic abrogation of the MVB pathway does not inhibit EVs biogenesis, it significantly alters the secretion profile, that is, the types of EVs released.

Strikingly, when we precipitated protein from the conditioned media of *Δsnf7* cells expressing aSyn variants, we still were able to detect aSyn in the media (Fig. 43), in a similar pattern to the wild-type cells, pointing that secretion of aSyn is not inhibited by the endosomal/MVB pathway abrogation.

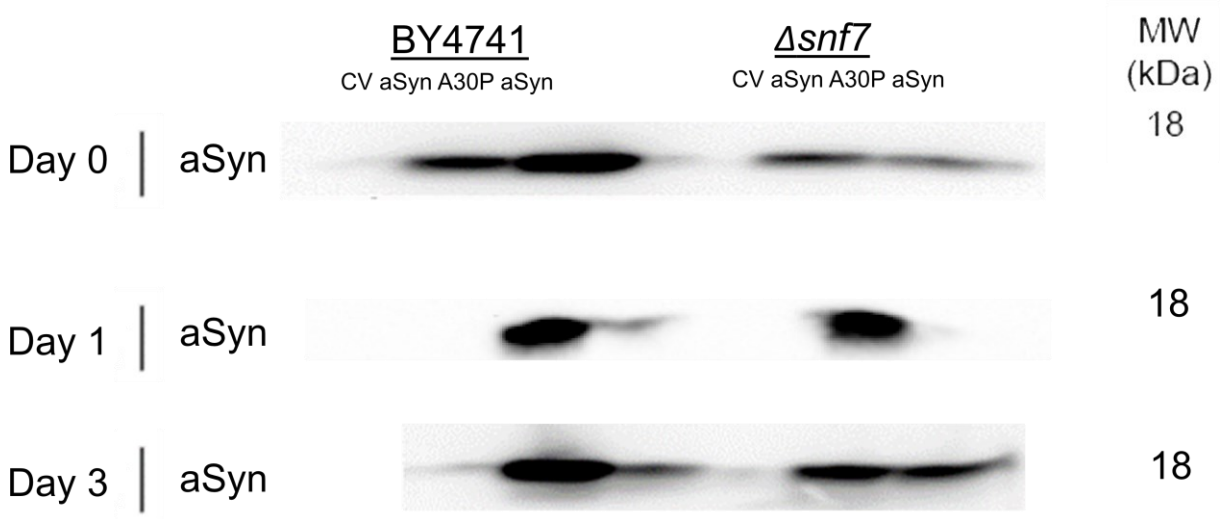


Figure 43. MVB pathway abrogation doesn't appear to impact on the secretome of aSyn expressing cells Immunoblot analysis of conditioned media precipitated from BY4741 cells and *Δsnf7* cells expressing the wild-type aSyn, aSyn A30P and control vector plasmids at days 0,1 and 3 of CLS. At least 3 independent biological replicates were analysed.

However, when we attempted to ascertain the uptake of aSyn into naïve receptor cells from the conditioned media of *Δsnf7* cells expressing aSyn variants collected at days 0, 1 and 3 of CLS, we could not detect any aSyn via immunoblot at any timepoint of CLS (0,3,7 and 10) (Fig. 44).

Likewise, we could not visualize any fluorescence up until day 3 of CLS via the immunofluorescence protocol against aSyn on receptor cells supplemented with conditioned media from *Δsnf7* cells expressing aSyn variants at day 0 of CLS (Fig. 45).

This intriguing set of results made us wonder what where the exact mechanisms that would regulate the aSyn uptake in yeast cells. And if aSyn horizontal transmission between yeast cells is only partially dependent on the MVB pathway. Previous data suggested that abrogation of MVB pathway by *Δsnf7* mutant does not seem to inhibit the secretion of particles with sizes compatible with EVs, although it changes the profile of secretion significantly, *i.e.*, overall lack of particles in the exosome size range.

Perhaps, while the secretion of proteins like aSyn in yeast can by tackled by both conventional and unconventional secretory pathways, its uptake is, at least preferably, more reliant in the budding/uptake of exosome-like structures from MVB biogenesis on the naïve receptor cells.

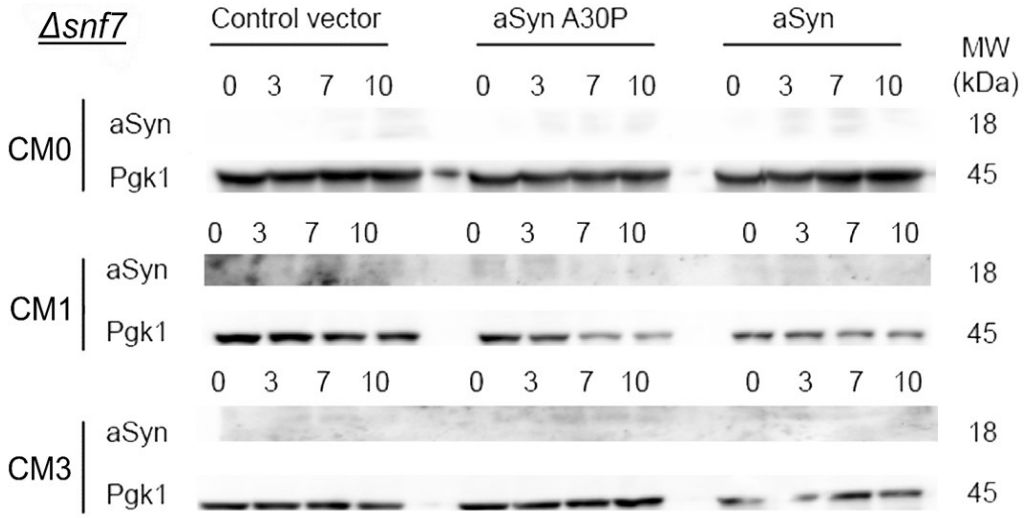


Figure 44. No aSyn uptake detected on naïve receptor cells supplemented with conditioned media from *Δsnf7* cells Immunoblot analysis of aSyn extracted from wild-type receptor cells grown on conditioned media collected from *Δsnf7* cells expressing wild-type aSyn, aSyn A30P or control vector plasmids at days 0, 1 and 3 of CLS. Media was supplemented to naïve receptor cells at day 0 of their CLS. At least 3 independent biological replicates were analysed.

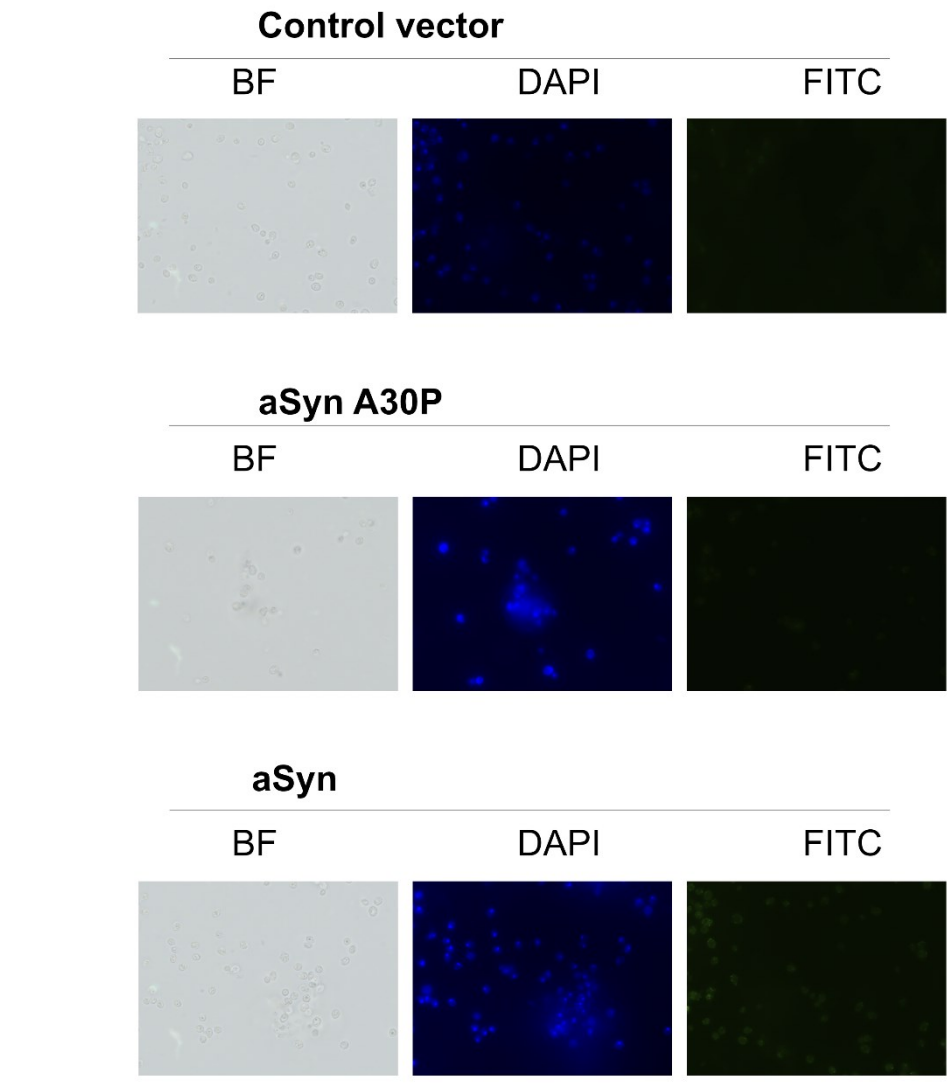


Figure 45. No aSyn uptake visualized on naïve receptor cells supplemented with conditioned media from *Δsnf7* cells Representative immunofluroescence microscopy images on bright field (BF), nucleus staining (DAPI) and aSyn-conjugated antibody of naïve receptor cells grown to day 3 of CLS supplemented with conditioned media collected from *Δsnf7* cells expressing wild-type aSyn, aSyn A30P variants or control vector at day 0 of CLS.

3.4.2 Effects of the abrogation of the endocytic pathway in the uptake and secretion of aSyn

Our data with the genetic abrogation of the endosomal/MVB pathways on aSyn expressing yeast suggested an inhibition of the deleterious effects on cells survival seen on the wild-type cells, as well as

an inhibition of the aSyn uptake by naïve receptor cells. We hypothesized that the uptake of this protein could be tied to specific unconventional secretion by MVB-derived exosomes. To elucidate the molecular mechanisms behind the cell-to-cell horizontal transmission of aSyn, we obtained a null mutant for a protein, End3, essential for the endocytosis pathway. Clathrin-mediated endocytosis (CME), herein simply endocytosis, is a retrograde vesicular trafficking pathway known to regulate uptake of nutrients and responses to extracellular stimuli within cells. During endocytosis cells deform the plasma membrane to form small vesicles (60-120 nm) that transport several types of cargo molecules into the cytoplasm [292]. Clathrin-coated vesicles are directed to fuse with the endosome [293]. Actin filament recruitment is critical for the coating of the vesicles and the endocytic process. End3 is a small 40kDa protein essential for this step of endocytosis [294-296]. Giardina and colleagues have described the *END3* gene as essential for the modulation of EVs release in response to nutrient availability in yeast [165]. Gluconeogenic enzymes are secreted when yeast is under nutrient limiting conditions, and rapidly internalized when yeast is switched to high glucose availability medium. The authors found that *END3* is necessary for the uptake of EVs containing gluconeogenic enzymes and some other non-gluconeogenic enzymes in yeast such as glyceraldehyde-3-phosphate or cyclophilin A [165]. Hence, with these findings in mind we wanted to the role of endocytosis on aSyn uptake and secretion, potentially enveloped in EVs, in a prion-like mechanism. If endocytosis regulates the uptake of EVs containing several enzymes, the possibility remained open to other protein cargos such as the aSyn protein to be internalized in a conserved endocytic mechanism.

The exact role of endocytosis on ageing progression remains elusive and its potential influence in cell-nonautonomous mechanisms of ageing, could provide insights into the wider ageing field, when coupled with the aSyn expression model in yeast.

To explore the involvement of the endocytic pathway in the mechanisms of cell-to-cell transmission of aSyn, we heterologously expressed the aSyn variants in the null mutant cells for the *END3* gene. As a first approach, we set out to characterise its growth curve compared with the wild-type cells. Particularly, we intended to find if the expression of aSyn on these mutant cells lead to differences in growth rates. We did not observe major differences between cells expressing or not the aSyn variants concerning the growth curves profiles (Fig.46). Furthermore, the growth curves are similar between mutant and wild type cells.

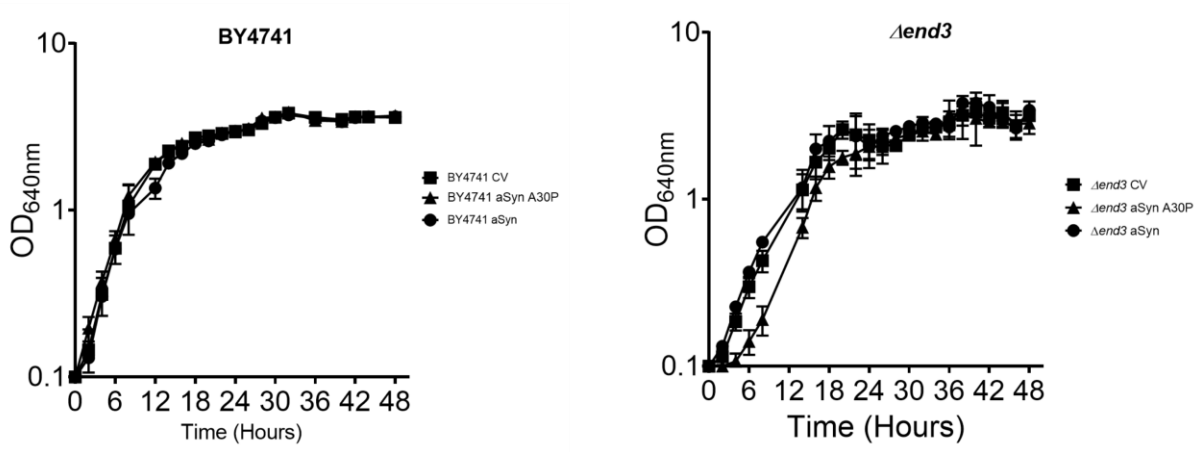
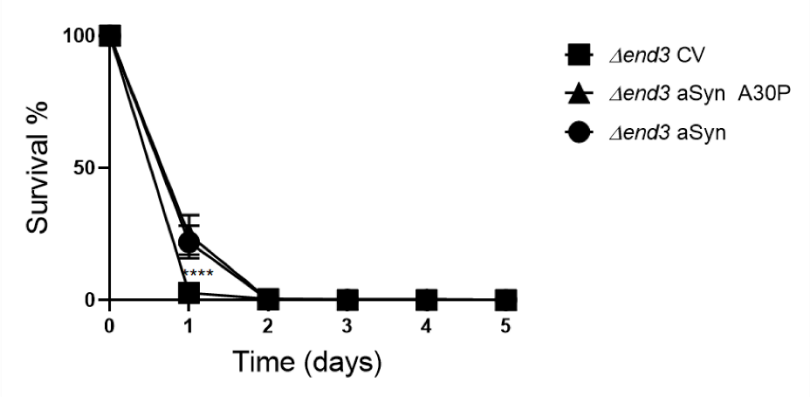


Figure 46. *Δend3* cells and BY4741 show no significant difference in growth rate during the first 48 hours Growth curve rates of the wild-type strain and the *Δend3* mutants expressing wild-type aSyn, aSyn A30P or control vector plasmids. Significance was determined by two-way ANOVA between cells. The error bars represent the standard error of the mean (SEM).

Regarding the cells' CLS, as already described, *Δend3* cells presented a very short lifespan, regarding the wild-type cells (Fig. 47A). Furthermore, data also revealed that in the mutant cells, the expression of aSyn is not toxic regarding the control vector expressing cells. The heterologous expression of aSyn by the constitutive promoter was confirmed during CLS (Fig. 47B).

A



B

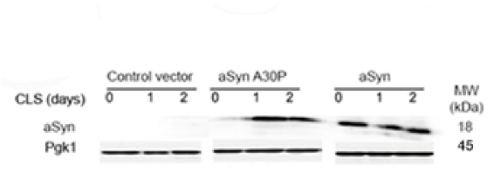


Figure 47. *Δend3* has a short chronological lifespan (A) Chronological lifespan of *Δend3* cells expressing aSyn A30P, wild-type aSyn variants and control vector. Significance was determined by two-way ANOVA between cells. Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (*p≤0.05, **p≤0.01,***p≤0.001, ****p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM). **(B)** aSyn levels of BY4741 cells expressing wild-type aSyn, aSyn A30P or the control vector variant, during days 0,1 and 2 of CLS. At least three biological independent replicas were analysed.

To understand whether endocytosis influences downstream anterograde trafficking, we designed an experiment with the yeast aSyn expression model. Endocytosis-compromised cells transformed with the aSyn plasmids were grown into days 0 and 1 of their CLS, their conditioned media was harvested and supplemented to naïve receptor cells (Fig. 48A).

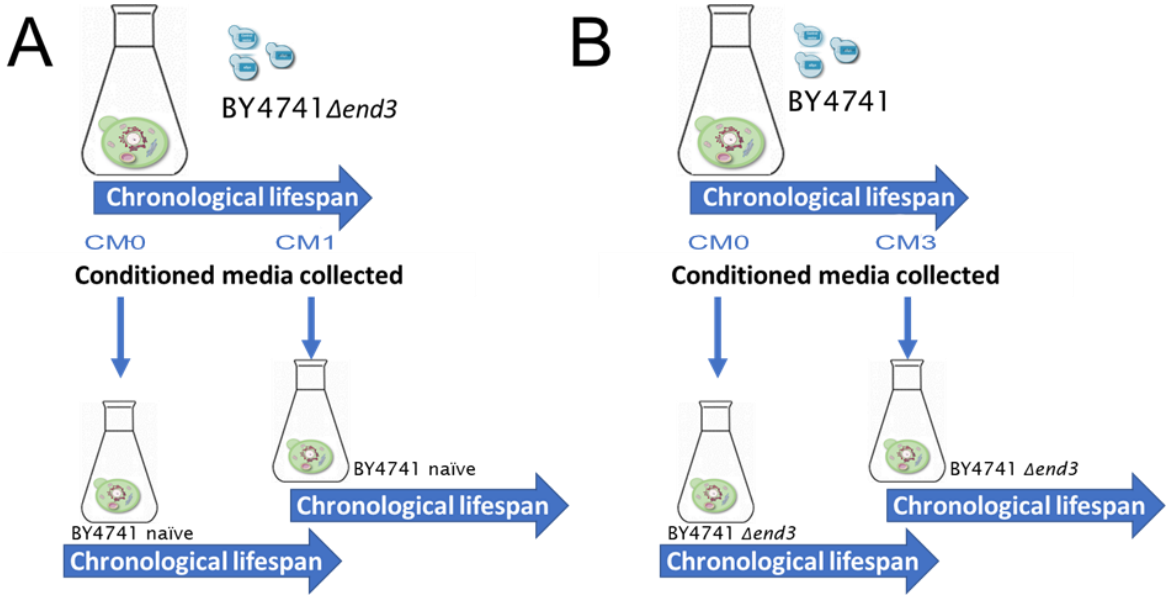


Figure 48. Conditioned media is harvested from BY4741 *Δend3* and wild-type BY4741 (A) CM0 and CM1 are harvested from BY4741 *Δend3* cells expressing wild-type aSyn, aSyn A30P or the control vector by differential centrifugation. CM is then supplemented to naïve cells at day 0 of their CLS. **(B)** CM0 and CM3 are harvested form BY4741 expressing wild-type aSyn, aSyn A30P or the control vector by differential centrifugation. CM is then supplemented to BY4741 *Δend3* cells at day 0 of their CLS.

Naïve cells were monitored through their CLS, but no significant decrease or increase in survival was found (Fig. 49), unlike what was seen earlier in the wild type and *Δsnf7* cells (Fig. 34 and 41), where conditioned media from aSyn cells promotes a shorter lifespan of the receptor cells.

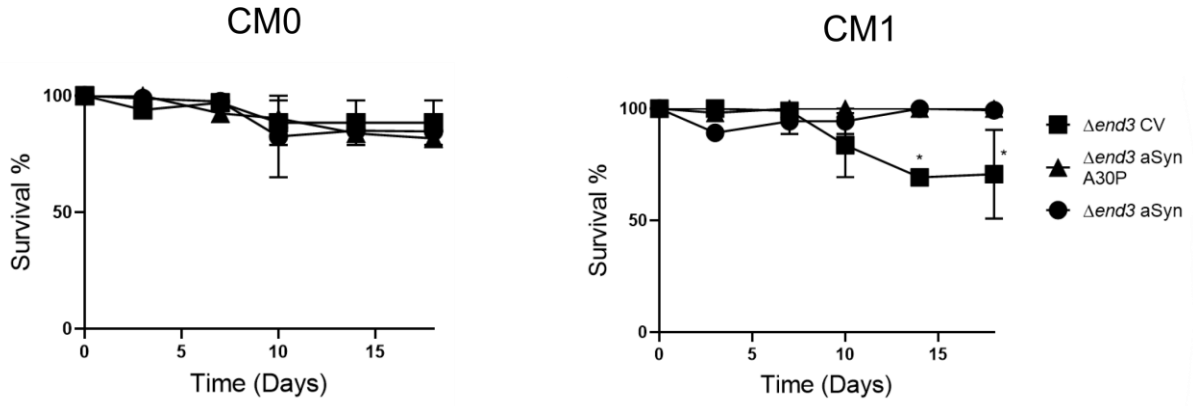


Figure 49. Conditioned media from *Δend3* cells has no effect on yeast chronological lifespan Chronological lifespan of naïve BY4741 cells supplemented with conditioned media from *Δend3* cells expressing wild-type aSyn, aSyn A30P and control vector collected at days 0 **(A)** and 1 **(B)** of CLS. Significance was determined by two-way ANOVA (*p≤0.05,) between cells. Data represents mean ± SEM of at least two biological independent replicas The error bars represent the standard error of the mean (SEM).

Next, to explore if the abrogation of the endocytic pathway interferes with the release of aSyn to the medium, we explore the presence of aSyn in the CM from *Δend3* cells. For that end, as described above, we precipitated the proteins presented in the media collected at days 0 and 1 of CLS. As a control, we also precipitated the proteins from conditioned media of wild type cells expressing the aSyn variants and the control vector, at the same days of CLS (Fig. 50). In conditioned media from aSyn A30P variant we transiently detect the presence of aSyn, which can be due to the different localization of this aSyn variant in yeast cells. A30P aSyn is disperse throughout the cytoplasm, while the wild-type aSyn variant initially localizes in the plasma membrane [9]. In contrast, in the media from *Δend3* aSyn-expressing cells, we did not detect aSyn.

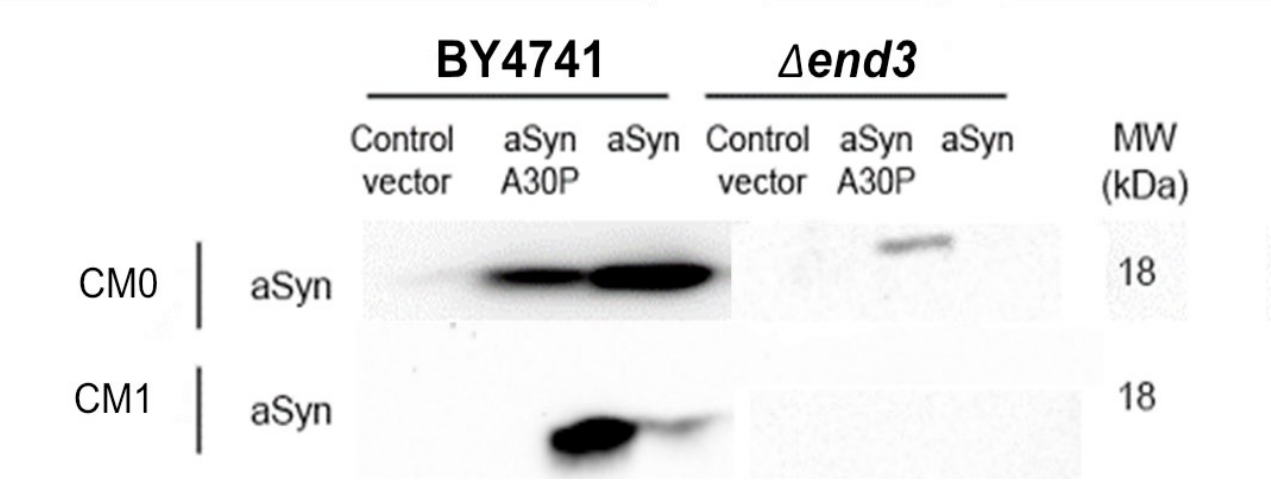


Figure 50. Endocytosis abrogation impacts on the secretome of aSyn expressing cells Immunoblot analysis of conditioned media precipitated from BY4741 cells and *Δend3* cells expressing the wild-type aSyn, aSyn A30P and control vector plasmids at days 0 and 1 of CLS.

As above, we also supplemented conditioned media collected from endocytosis-abrogated *Δend3* cells to wild-type naïve cells at day 0 of CLS. To further ascertain if inhibition of endocytosis leads to a blockage of the cell-to-cell transmission of aSyn, we collected whole-cell protein extracts from the naïve receptor cells supplemented with *Δend3* CM at 4 timepoints (0, 3, 7, 10 days) on their CLS and performed an immunoblot against the aSyn protein (Fig. 51). Though various bands from different sizes can be seen, none matches the molecular weight of aSyn (roughly 18 kDa). Moreover, we did not observe any difference in the immunoblot analysis of cells expressing or not aSyn. This corroborates the previous observation that apparently *Δend3* cells do not secrete aSyn to the conditioned media (Fig. 50). We were able to monitor the uptake of aSyn into naïve cells from the wild type cells expressing aSyn (Fig. 36), so our findings may point out a role of the abrogation of endocytosis in the modulation of yeast cell's secretome

by inhibiting the secretion of aSyn. This could be tied with the role of endosomes produced by the endocytic pathway in anterograde trafficking pathways such as the endosomal/MVB pathway.

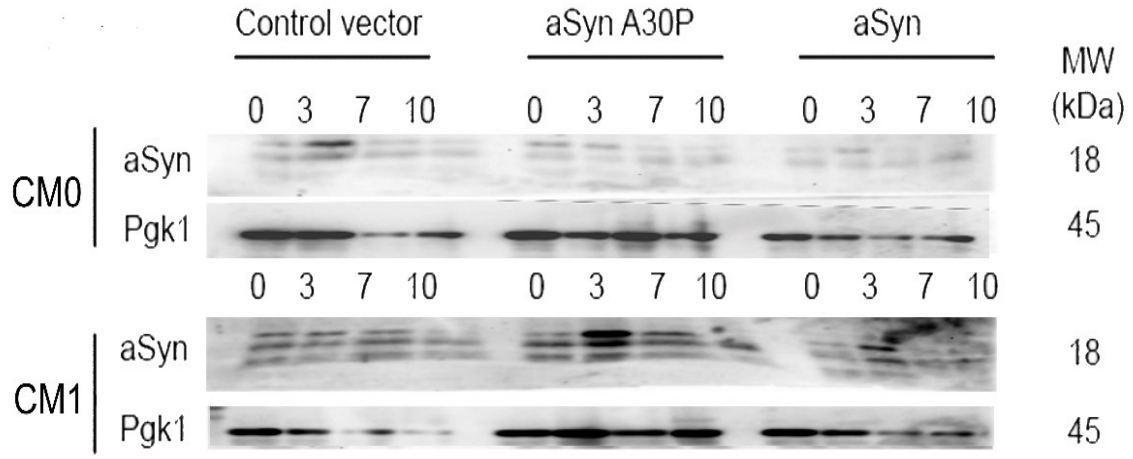


Figure 51. No aSyn uptake detected on conditioned media from *Δend3* endocytosis-compromised cells Immunoblot analysis of aSyn extracted from wild-type receptor cells grown on conditioned media collected from *Δend3* cells expressing wild-type aSyn, aSyn A30P or control vector at days 0 and 1 of CLS. Media was supplemented to naïve receptor cells at day 0 of their CLS.

To understand if the uptake of aSyn into wild-type yeast cells is mediated by endocytosis, we performed the reverse experimental design (Fig. 48B). We collected conditioned media from wild-type cells expressing the aSyn variants and control vector at days 0 and 3 of CLS and supplemented endocytosis-abrogated *Δend3* cells with it. Due to their short lifespan, we monitored CLS over a period of 24 hours (Fig. 52). Cells' survival plummeted steadily after just 6 hours of CLS. However, we cannot at this point attribute causality to the CM effect. Then, we collected whole-cell protein extracts from the naïve *Δend3* cells supplemented with conditioned media from the wild type cells expressing aSyn at 4 timepoints over the course of 24 hours of CLS (0, 6, 12 and 24 hours) and ran an immunoblot against aSyn (Fig. 53). No bands matching the molecular weight of aSyn can be detected. The positive control (BY4741 aSyn), ran on the same electrophoresis gel, worked as intended. This result seems to point out there is no uptake of aSyn by endocytosis-compromised cells, whereas the wild-type cells show uptake of aSyn by the naïve cells (Fig. 36) highlighting the importance of this trafficking pathway to the cell-nonautonomous mechanisms of premature ageing promoted by aSyn.

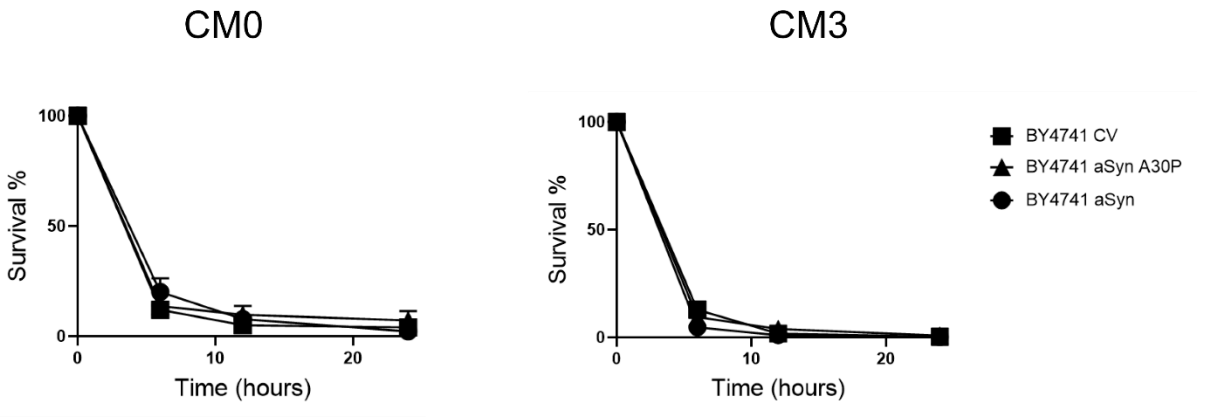


Figure 52. Conditioned media from BY4741 and the short chronological lifespan of *Δend3* cells Chronological lifespan of naïve *Δend3* cells supplemented with conditioned media from BY4741 cells expressing wild-type aSyn, aSyn A30P or control vector collected at days 0 and 3 of CLS. Significance was determined by two-way ANOVA. Data represents mean ± SEM of at least two biological independent replicas The error bars represent the standard error of the mean (SEM).

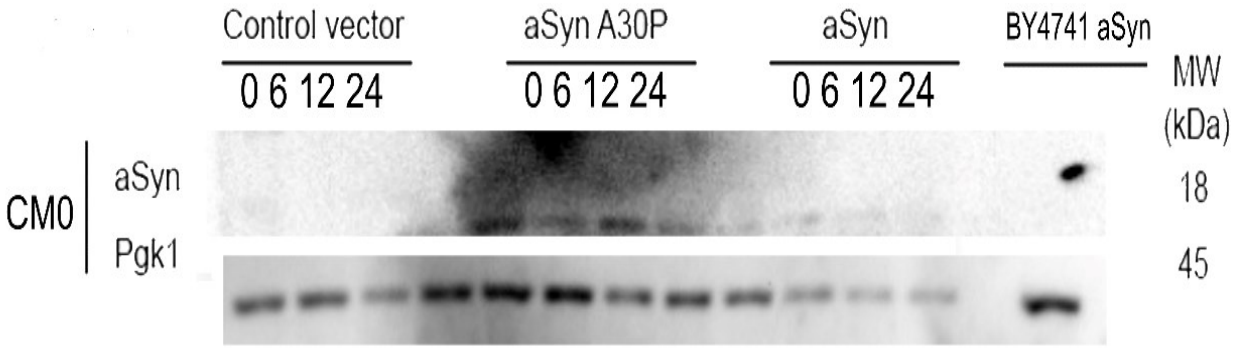


Figure 53. No aSyn uptake detected when endocytosis is abrogated Immunoblot analysis of aSyn extracted from *Δend3* receptor cells grown on conditioned media collected from BY4741 cells expressing wild-type aSyn, aSyn A30P variants and control vector at days 0 and 3 of their CLS supplemented to naïve *Δend3* cells at day 0 of CLS. Whole-cell protein extracts were taken from the naïve cells from 0 hours until 24 hours of its CLS. Whole-cell protein extract from BY4741 aSyn shown here as a positive control.

Our data with the aSyn yeast expression model point out that the secretion and uptake of this protein by chronologically ageing yeast cells is partially dependent on the anterograde endosomal/MVB pathway and the retrograde endocytic process. The endosomal/MVB pathway might be involved in aSyn secretion and ageing modulation in yeast. Naïve receptor cells supplemented with conditioned media from endosomal/MVB abrogated cells' (the *Δsnf7* mutant) lifespan significantly increased regarding the wild-type cells. Vesicular fractions from *Δsnf7* mutant yeast expressing the aSyn variants were significantly altered in their size and potentially lipidic composition. Regarding wild-type cells, these *Δsnf7* vesicular fractions contained less particles in the exosome range. CM still showed the presence of aSyn, but this protein was unable to be up taken by naïve receptor cells in a significant manner. One hypothesis is that the horizontal transmission of aSyn in yeast cells occurs at least preferentially through exosome-like structures produced by the MVB pathway, mimicking the mechanisms known to occur in humans [174]. Endocytosis also seems to be a mechanism responsible for the uptake of aSyn into yeast cells. Its

abrogation seems to have broader effects on the anterograde endosomal/MVB trafficking as well. Abrogation of endocytosis seems to prevent the secretion of aSyn into yeast extracellular medium and its uptake also by wild-type strain naïve receptor cells. These findings provide novel insights into the mechanistic links between vesicle trafficking pathways and cell-nonautonomous mechanisms of ageing, which could potentially involve several anterograde and retrograde pathways from conventional to unconventional forms of secretion (CUPS, MVBs, secretory autophagy) and endocytosis.

CHAPTER 4

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

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Ageing is a stochastic, complex biological process affecting living organisms from the simpler unicellular yeast to higher eukaryotes [1, 42]. Ageing in the broadest sense represents a decline of cell or organismal function, accumulation of damage, and increased susceptibility to death [7]. Ageing was found to be regulated by highly conserved nutrient and energy sensing cell signalling networks, orchestrated by master longevity regulators, such as sirtuins, TOR, Sch9 or Ras/PKA [4, 28, 41, 48, 56, 120, 144, 297]. Genetic, pharmacological, or dietary interventions on these conserved signalling pathways have allowed for a better understanding of the ageing biological phenomenon, as well as successful lifespan extension on several model organisms [23, 28, 49, 298]. CR is the most successful dietary intervention that promotes lifespan extension across several model organisms, though the exact mechanisms are not clearly understood. CR is reported to promote longevity through cell signalling networks and the proteolytic systems of the proteostasis network [23, 28, 67, 68, 97]. Ageing progression is also impacted by the loss of proteostasis associated with the functional decline in the major proteolytic systems, UPS and ALP [67, 68, 87, 107]. These mechanisms are constrained to the boundaries of individual cells and are called cell-autonomous mechanisms of ageing [49].

Here, to study the role of these cell-autonomous mechanisms on cell signalling and the regulation of homeostasis by the proteolytic systems during ageing, the yeast model was employed with heterologous expression of the aggregation-prone aSyn protein under CR conditions. Expression of the wild-type variant of aSyn induces phenotypical changes in yeast, akin to premature ageing and constitute a proteotoxic burden on the proteolytic systems, UPS and ALP. The yeast "humanized" expression model allows for the elucidation of the physiological and molecular underpinnings behind aSyn toxicity and is already well-established both as a model for ageing and study of proteins associated with neuronal pathogenesis, such as synucleinopathies [299].

Our data corroborates previous reports by Guedes and co-workers [23] showing that CR promotes chronological lifespan extension [97]. Moreover, CR rescued yeast lifespan from wild-type aSyn toxicity nearing control vector levels, while maintaining autophagy at homeostatic levels. As the activities of both the UPS and ALP proteolytic systems are reported to decrease with ageing and that the CR beneficial effects are dependent on both proteolytic systems, even more so considering the crosstalk between them [266, 267, 300], these systems were monitored under CR intervention. The crosstalk between UPS and ALP is described in several models but remained poorly understood [266]. Nevertheless, the crosstalk is poised as essential to achieve protein homeostasis, and consequently, increased longevity [86, 266, 300]. In accordance with the effects described in the literature for other ageing models [235, 301], aSyn expression was found to significantly decreased UPS catalytic activity during chronological lifespan and

the accumulation of ubiquitinated substrates under normal dietary regimen. However, under CR regimen UPS catalytic activity is recovered. This points out a CR-driven recovery of the UPS activity during chronological ageing and under proteotoxic stress conditions.

To explore the crosstalk between the two major proteolytic systems, UPS and ALP, each was individually inhibited under CR condition during yeast chronological lifespan. Autophagy inhibition upregulated UPS catalytic activities under CR intervention, as a compensatory mechanism. Autophagy inhibition decreases the lifespan of yeast under CR, however it is still above the lifespan of yeast expressing aSyn on normal growth conditions. Hence, CR beneficial effects without autophagy are diminished but not completely abrogated, in part due to UPS activity compensation.

On the other hand, UPS inhibition by both pharmacological and genetical approaches, resulted in no major discernible impacts in the autophagy activity. This observation apparently contradicts some literature reporting that autophagy is upregulated to compensate for UPS inhibition [267, 270, 302]. Inhibition of UPS does, however, reduce chronological lifespan in regard to non-inhibited cells under CR, demonstrating the importance and contribution also of this proteolytic system to the CR associated beneficial effects. We hypothesized that the apparently stability in autophagic flux levels could be related with the formation of PSGs during yeast chronological lifespan. PSGs protect inactive proteasomes from organelle-specific autophagic degradation, proteophagy, during carbon starvation [99]. Chronological ageing yeast cells expressing aSyn are both under carbon starvation and proteasomal impairment. While autophagy flux could be increased to degrade inactive proteasomes, at the same time due to carbon starvation, proteasomes are protected from degradation by PSGs. While autophagy is important for the beneficial effects of CR on homeostatic levels, exacerbated autophagy levels could be deleterious and degrade intact proteasomes. The beneficial effects of CR on chronological ageing yeast under aSyn induced proteotoxic stress could be partially dependent on proteophagy inhibition and storage of proteasomes in PSGs. Cue5 is an ubiquitin receptor that targets inactivated proteasomes for autophagic degradation [269]. Lifespan of *Δcue5* (proteophagy abrogated) cells under aSyn induced proteotoxic stress, under normal growth conditions increased significantly regarding to wild-type cells. However, under CR conditions this proteophagy inhibition show no significant differences from normal growth conditions. This could suggest that the inhibition of proteophagy and formation of PSGs are necessary for the beneficial effects promoted by CR. This hypothesis needs still further research in the future to be validated. Overall, this set of data suggests that during yeast chronological ageing and under proteotoxic stress, conditions elicited by aSyn expression, the beneficial effects attributed to the CR are associated with the coordinated response of both major proteolytic systems. Under CR conditions, UPS activity is upregulated,

and autophagy maintained at homeostatic levels leading to the rescue of yeast from aSyn proteotoxicity and increasing longevity. Each proteolytic system is important and contributes to the CR-driven beneficial effects. A compensatory mechanism of crosstalk upregulates UPS, when autophagy is inhibited. Inhibition of either proteolytic system results in reduced lifespan. Maintenance of autophagy at homeostatic, non-deleterious levels seems to be the central pathway promoting the rescue of chronological ageing yeast cells from aSyn-induced proteotoxicity through a cell-autonomous mechanism of ageing, as can be summarized on Figure 54.

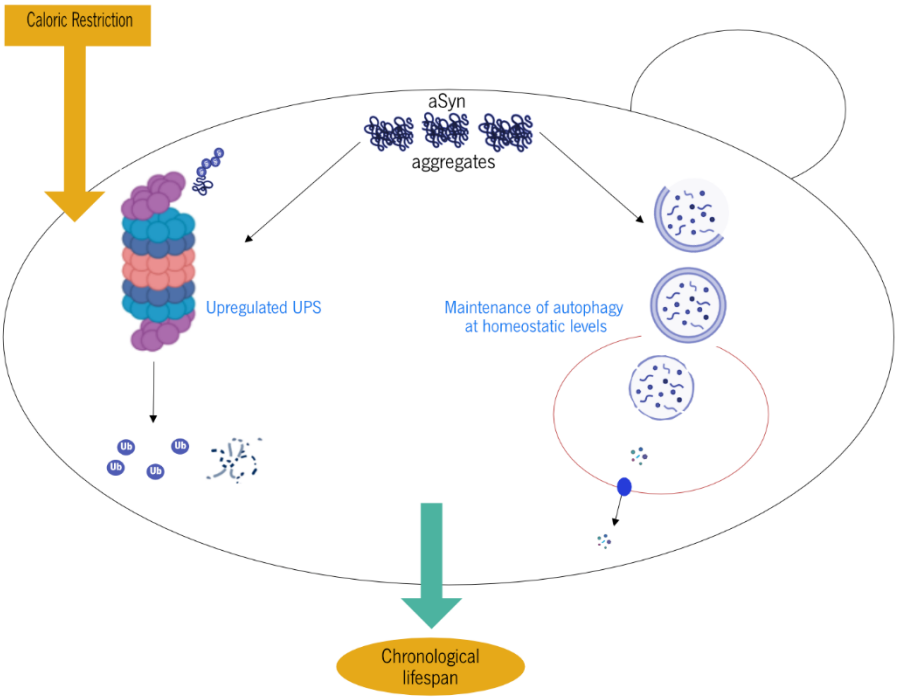

Figure 54. CR rescues ageing yeast from aSyn toxicity through autophagic control of proteostasis Our data supports the notion that during CLS, CR upregulates UPS activities while maintaining autophagy at homeostatic levels, in order to promote increased lifespan.

Recently, it has been increasingly reported that cell-nonautonomous mechanisms involving a signalling network between multiple cells within an organism or population, can also contribute to modulate the complex ageing biological phenomenon. Different classes of molecules could transmit biological information between cells as low-molecular weight transmissible factors that can modulate ageing. Recent findings in the budding yeast and other fungi species [149, 160, 164, 168, 235, 303-305], that point to the role of this type of signalling in modulating lifespan through the release of these compounds to the extracellular milieu, where they can be uptake by neighbouring cells in their vicinity [159]. Protein, lipids, nucleic acids were reported to be exported by several fungi species [159, 167, 306]. Moreover, mounting evidence suggest that these molecules could be secreted through both conventional and unconventional

pathways into the extracellular medium inside extracellular vesicles (EVs). In the present work, a screening was attempted to isolate and characterise different classes of molecules in the secretome of chronological aged yeast. Nucleic acids, lipids and proteins presence on the culture media was assessed, as well as the presence and characterisation of EVs.

Small noncoding RNAs were found on several fungi species including the budding yeast, through high-throughput RNA sequencing [160, 274]. Most of the secreted RNA consisted in species no longer than 250 nucleotides. In our screening, we were able to quantify appreciable amounts of both DNA and RNA in yeast secretome. Regarding RNA, we were not able to visualize it on agarose electrophoresis, despite the quantification results. Other high-throughput techniques might be necessary [307]. It should also be taken into account that more precise methodologies might be required to maintain RNA integrity. Nevertheless, our data still points to the presence of nucleic acids on the ageing yeast culture media, which have the potential to be involved in cell-nonautonomous mechanism of ageing and horizontal transmission of biological information, through hitherto unknown molecular pathways.

Some classes of lipids (sphingolipids, fatty acids, or diacylglycerols) are described as lifespan modulators on chronological yeast ageing [143]. These lipids can modulate lifespan through nutrient sensing pathways and master longevity regulators within a cell and have been associated with cell-nonautonomous mechanisms of ageing in yeast and other fungi species [162, 167, 280, 283, 285]. In addition, sterol-enriched membrane subdomains called lipid rafts are common on EVs. Saturated fatty acids and sphingolipids are also more common on EVs composition [184] and have been proposed as EVs markers. We were able to visualize neutral lipids profile in small volumes CM samples from different timepoints (days 3 and 7) of chronological ageing. It is equally plausible that the lipids detected are themselves molecular transmissible factors of cell-nonautonomous mechanisms or, instead they could be components of EVs membranes.

The secretome from chronological ageing yeast was also found to contain different proteins. These molecules could be secreted in free form or enveloped into extracellular vesicles [274, 285]. Through Ponceau S protein staining of precipitated culture media, we saw different protein secretion profiles from ageing yeast cells at different timepoints (days 3 and 7 of CLS). Proteins from vesicular fraction isolates of culture media also displayed similar profiles on Ponceau S staining. This preliminary data hints at the possibility that some proteins can be secreted by yeast through cell-nonautonomous mechanisms within EVs. It is however unclear if the detected proteins are secreted as free molecules through conventional protein secretion, or if the isolation protocols used can damage and disrupt EVs lipidic bilayers. The same question remains open for the other classes of molecules detected.

Vesicular fractions were also characterised by their size distribution to ascertain their most likely biogenesis pathway in yeast, on wild-type and *Δsnf7* cells. Snf7 is a subunit of the ESCRT-III complex essential for MVB biogenesis and the formation of MVB derived exosomes [198, 203]. The interval of the distribution is overall compatible with the sizes of EVs described in the literature [169, 179, 198], either for exosomes (40-100 nm) or microvesicles (>100 nm). Vesicle size seems to increase over time, *i.e.*, at day 7 of CLS it is more common to find microvesicles-like particles than exosome-like. With the genetic abrogation of *SNF7*, the size distribution on the vesicular fractions is altered, and the lower sized particles compatible with exosomes are not present. Instead the distribution is focused on the >100 nm range, compatible with the presence of microvesicles, but also some apoptotic bodies [189]. This suggests that, also as other authors have hinted [162, 163], both conventional and unconventional secretory pathways are involved in EVs' biogenesis in yeast. Blocking one secretory pathway is not enough to completely abrogate EVs' biogenesis, as it is a complex and interconnected process between various types of trafficking machinery [163, 167, 285].

Taken as a whole, the data set from this chapter represents the efforts to screen for the presence of molecules of yeast secretome that could conceivably be used as transmissible factors on cell-nonautonomous mechanisms of ageing, and the fine tuning of experimental methodologies to isolate and characterise them. To that end, protein, lipids, and nucleic acids were successfully detected on chronologically ageing yeast CM. Moreover, vesicular fraction isolates were obtained by ultracentrifugation and the presence of lipids, proteins and EVs of various sizes were determined. Further characterisation of these molecules still needs to be performed to better understand how these secretions tie to cell-nonautonomous mechanisms of ageing modulation during yeast chronological lifespan, but so far these findings are promising and coherent with the consensus in the literature [160, 162, 179, 274, 285].

Then, after the data presented in the previous section, a proof-of-concept for the horizontal transmission of biological information capable of ageing modulation through cell-nonautonomous mechanisms was performed. To do so, an established model on proteotoxic stress and the pathogenesis of synucleinopathies was employed: heterologous expression of human aSyn in the budding yeast [247]. With the yeast aSyn "humanized" expression model, it was found that prematurely aged cells expressing aSyn protein, secreted it to the extracellular milieu. Furthermore, these aSyn expressing cells were found to release EVs compatible with the size of exosomes and microvesicles. Exosome-like particles concentration seems to increase incrementally with the age, barring the aSyn expressing samples of CM7, where particle concentration was remarkably low. Cells expressing the aSyn variants also resulted in vesicular fractions with higher concentrations of particles than the control vector. It could be theorized

that the proteotoxic stress and premature ageing induced by aSyn expression drive a cell-nonautonomous response to secrete this protein into the extracellular milieu. As already reported in human neuronal cells, aSyn has a prion-like behaviour and is transmitted between different cells in the human brain either in free oligomers or encapsulated into exosomes [229] The protein can be shuttled from cells under severe proteotoxic burden and internalised by endocytosis in neighbouring cells, to be degraded via autophagy. It is tempting to speculate that a cell-nonautonomous mechanism could be behind the clearance or uptake of EVs at 7 days of CLS, in vesicles containing wild-type aSyn in yeast [173, 174]. Secretory autophagy in yeast mediates the unconventional secretion of unwanted molecules like aggregation-prone proteins similar to aSyn [110]. Secretory autophagy is tied to other forms of unconventional secretion such as CUPS or endosomal/MVB pathways [110, 192, 204]. Yeast prions are mechanisms of epigenetic inheritance and the prototypical yeast prion Sup35 was found in EVs produced by yeast [171, 175]. Moreover, in the same vesicular fractions, we detected proteins matching aSyn.

aSyn-expressing cells CM significantly reduced chronological lifespan of naïve receptors cells supplemented with it, and moreover, naïve receptor cells of the wild-type strain were found to contain the aSyn protein inside them. This uptake was confirmed both by immunoblot and immunofluorescent microscopy on CM from cells expressing aSyn variants. The uptake appears to be gradual and cumulative over the course of naïve receptor cells' CLS.

The aforementioned data suggested a cell-nonautonomous mechanism involving both anterograde trafficking, coordinating the secretion of the protein from the donor cells, and retrograde trafficking, for the uptake into the naïve receptor cells. However, the mechanisms of trafficking remained largely unknown. aSyn has been recently described to be propagated in neurons in a prion-like manner enveloped in extracellular vesicles of endosomal nature, *i.e.* exosomes [173, 174], and the budding yeast is also described to secrete extracellular vesicles [162, 167, 280, 304] in the literature. On **sections 3.2** and **3.3** of the present thesis, we obtained data strongly supporting EVs production in yeast. To understand the contribution of the unconventional endosomal/MVBs pathways to the aSyn transport, *Δsnf7* cells were used. Snf7 is a subunit of the ESCRT-III complex essential for MVB biogenesis [198]. Genetic abrogation of *SNF7* on yeast resulted in a significantly shorter survival regarding the wild-type cells, regardless of aSyn variants expression. Naïve receptor cells supplemented with CM from *Δsnf7* cells expressing aSyn variants survival increased, as evidenced by the mean and maximum lifespans, regarding cells supplemented with wild-type CM. At the same time, the aSyn protein was still detected in *Δsnf7* CM, but we could not confirm its uptake into naïve receptor cells neither by immunoblot or immunofluorescence techniques. CM from the *Δsnf7* cells does not share the same pro-ageing effects of conditioned media

from the wild-type cells, but aSyn is still secreted and detected on it. Based on data from EVs characterisation shown in the previous section of this work, it seems that EVs' biogenesis is partially, albeit not entirely, reliant on the endosomal/MVB pathways. It also appears that aSyn could conceivably be secreted enveloped in EVs, both from the conventional and unconventional secretory pathways (Fig. 55).

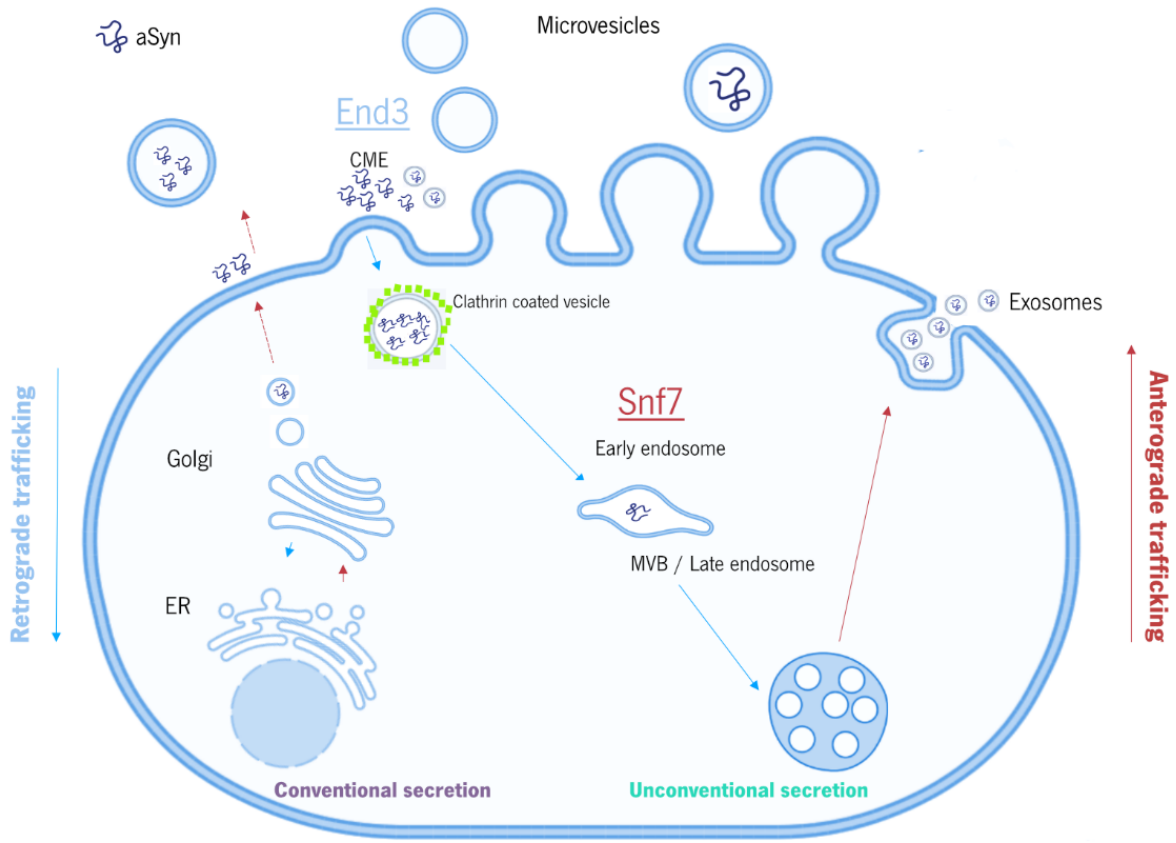

Figure 55. A proposal for aSyn trafficking in yeast through cell-nonautonomous mechanisms The bulk of our data supports the notion that aSyn can be secreted via Snf7 dependent endosomal/MVBs unconventional pathways, enveloped into EVs or directly to the extracellular medium. aSyn secretion through conventional secretion cannot also be ruled out. aSyn uptake also seems to be dependent on End3 dependent CME. Adapted from [111, 176]. .

This is supported by the observation of higher exosome-like particles on vesicular fractions isolated from aSyn expressing cells. One hypothesis is based on the observation that the genetic abrogation of the MVB pathway, while not completely blocking EVs' biogenesis and release, significantly alters its profile. The EVs produced in this situation, with larger sizes than those observed in the wild-type BY4741, could be less oriented for aSyn transport. Other is that the uptake of aSyn by naïve receptor cells is reliant on a specific cargo recognition that only occurs with EVs derived from the endosomal/MVBs or other unconventional pathways. Vesicular fractions were also analysed for neutral lipids, on wild-type and *Δsnf7*. Lipids were

found on both mutants and wild-type cells, but they could not be identified. It is uncertain if these lipids are components of the EVs membrane bilayer or cargo carried inside it. Nevertheless, as discussed above, the abrogation of the endosomal/MVBs pathways does not seem to be enough to inhibit EVs' biogenesis and release on chronological aged cells, rather just altering the secretion profile. In recent years, EVs with similarities to mammalian exosomes have been reported in fungi and putative biogenesis pathways alluded [163, 274]. The precise mechanisms ruling EVs' biogenesis, the secretion of aSyn, and its uptake into naïve receptor cells in yeast remain to be determined. Still, the data herein brought advancements in yeast EV biogenesis and helps consolidate the notion that unconventional secretion of exosome-like structures also are likely to occur in yeast, in addition to more conventional pathways.

To better elucidate the uptake of aSyn into naïve receptor cells during yeast chronological lifespan and its impacts on cell-nonautonomous mechanisms behind its horizontal transmission, genetic abrogation of clathrin-mediated endocytosis was performed with the *END3* yeast null mutant cells [165, 295, 308]. Endocytosis is a retrograde vesicular trafficking pathway known to regulate uptake of nutrients and responses to extracellular stimuli within cells. The exact role of the endocytic pathway on ageing progression and its influence in cell-nonautonomous mechanisms of ageing remains unknown. Giardina and colleagues demonstrated that *END3* is essential not only for retrograde processes, but also for some anterograde trafficking, like the secretion of gluconeogenic enzymes to the extracellular medium in EVs [165]. *Δend3* cells demonstrated no major differences on their growth rate regarding the wild-type cells, however their survival during CLS was much abridged, pointing out the importance of endocytosis to cellular homeostasis. CM from aSyn expressing *Δend3* cells did not reduce CLS of naïve receptors cells supplemented with it in any significant manner, unlike what was observed in the wild-type cells. Also, we could not consistently detect aSyn in the *Δend3* cells CM nor consequently, any uptake of aSyn by naïve receptor cells. Conversely, we could not detect any alterations on survival during CLs or in aSyn uptake by *Δend3* cells supplemented with CM from wild-type cells expressing the aSyn variants. These findings point out that endocytosis abrogation impacts the secretome of cells expressing the aSyn variants, which suggests the interconnectedness of the mechanisms regulating vesicle trafficking in both directions, retrograde and anterograde (Fig.55). It remains to be proven exactly how this occurs and if endocytosis abrogation affects the endosomal/MVB pathways of EVs' biogenesis. It's possible that the abrogation of endocytosis compromises the availability of early endosomes to mature through the MVB pathway. No uptake of aSyn by endocytosis-abrogated Δ*end3* cells also supports the notion that endocytosis is the mechanism mediating aSyn uptake into chronological aged yeast cells. The "humanized" yeast expression model of aSyn provided some valuable data as a proof-of-concept of the horizontal transmission of proteins

and EVs as low-molecular weight factors involved in ageing modulation through cells-nonautonomous mechanisms of ageing.

In summary, the ensemble of the data presented in this thesis brings new data on the role of cell signalling during yeast chronological lifespan, be it by cell-autonomous or cell-nonautonomous mechanisms that regulate ageing. On the cell-autonomous dimension, the role of exquisite protein quality control coordinated by the proteolytic systems and modulation of nutrient sensing pathways by CR intervention during yeast chronological lifespan was investigated. The data points to a role of both UPS and autophagy proteolytic systems in the beneficial effects promoted by CR, namely in the increased UPS activity and maintenance of autophagy at homeostatic levels. Interestingly, CR beneficial effects have not only been well-established in cell-autonomous mechanisms, but recently have been implicated also in cell-nonautonomous mechanisms of ageing modulation as well, via amino acid transmissible factors [139]. At the cell-nonautonomous dimension of yeast ageing, a screening for putative signalling molecules was performed and in accordance with previous literature, protein, lipids, nucleic acids, and EVs were detected and characterised during chronological lifespan. A proof-of-concept model for horizontal transmission of biological information through cell-nonautonomous mechanisms was designed with the human aSyn yeast expression system. Tying with the reported aSyn prion-like transmission via exosomes, this protein was found to be secreted into the conditioned media and vesicular fractions of chronological aged cells, entering naïve receptor cells, and modulating their lifespan. The secretion of aSyn, potentially through EVs, appeared to be partially dependent on the endosomal/MVB pathways and endocytic machinery, while its uptake was found to depend on endocytosis. Further research needs to be conducted to elucidate both the cell-autonomous and cell-nonautonomous mechanisms at play during yeast chronological lifespan. However, the data herein answers a few questions and poses interesting new ones about the precise mechanisms and pathways that interact to modulate ageing.

4.1. Future perspectives

Ageing is a complex biological process affecting living organisms, that can be fundamentally characterised by a time-dependent functional decay of cellular functionality and physiological integrity. Ageing can be modulated by master longevity regulators in response to nutrient and energy sensing pathways. These pathways in turn can be regulated by both intrinsic or extrinsic stimuli, in cell-autonomous or cell-nonautonomous mechanisms in complex network of interactions and cell signalling. The results presented in this thesis attempt to provide new insights into the role of cell signalling in the modulation of ageing

and lifespan through both cell-autonomous and cell-nonautonomous mechanisms. Regarding cell-autonomous mechanisms modulating ageing during yeast chronological lifespan, the data presented shows that CR intervention promotes beneficial effects on rescue of proteotoxic damage and lifespan extension through the coordinated crosstalk of the proteolytic systems from the proteostasis network. The maintenance of autophagy at homeostatic levels seems to be the main driving pathway enabling beneficial effects. It is plausible that those protective effects are partially dependent on the inhibition of proteophagy and relocation of inactive proteasomes to PSGs. During yeast chronological lifespan, yeast is both under nutrient starvation and proteasomal impairment, enabling the formation of PSGs. It is also plausible that, with exacerbated levels of autophagy, such as those promoted by proteotoxic stress and ageing progression, intact, functional proteasomes could be degraded as well as inactive one, further aggravating the proteotoxic vicious cycle. With the impairment of proteophagy by *CUE5* deletion, the CR beneficial effects are lost. Further research needs to be conducted to tie the deleterious effects of exacerbated autophagy with increased proteophagy and the degradation of functional proteasomes outside PSGs. The autophagic degradation of proteasomes could be monitored *in vivo* during yeast chronological lifespan under CR and normal growth conditions with the GFP-Atg8 processing assay and fluorescence microscopy on *Δcue5* cells.

More recently, the beneficial effects of CR intervention, well-established and studied as a cell-autonomous phenomenon modulating cell signalling networks and stress responses, have been implicated in intercellular communication and cell-nonautonomous mechanisms of longevity modulation. Enriquez-Hesles and colleagues recently described the secretion of higher concentrations of specific amino acids into the extracellular medium by yeast cells under CR intervention. Indeed, the authors found that concentrated CM increased the longevity of yeast cells in normal growth conditions to levels near those promoted by CR. It would be interesting to attempt to replicate this data in our aSyn yeast expression model, with both CR and normal growth conditions conditioned media supplemented to naïve receptor cells during chronological lifespan. Moreover, the analysis of the cell-autonomous responses within the naïve receptor cells, namely the activity of the proteolytic systems could be monitored as before to check whether the naïve receptor cells mimic the cellular response of the donor cells under CR.

Other molecules found in our screening for transmissible factors of longevity could be further characterised. One of the shortcomings of our survey for nucleic acids, particularly RNA, was the lack of a visual confirmation of their presence which could be achieved with a Bioanalyser (Agilent) or similar micro-capillary based electrophoresis devices. Small noncoding RNA species, already described in fungal

secretomes, are not visible with conventional agarose gel electrophoresis. Other high throughput molecular techniques such as RNA sequencing or RT-PCR could also be better suited to perform a thorough characterisation of RNA found in media from aged cells and provide clues about its role on cell signalling and cell-nonautonomous mechanisms too. For the characterisation of lipids from both CM and vesicular fractions samples, appropriate standards for the migration pattern on silica TLC plates must be chosen. During this screening, lipids representative of the sterols and fatty acids classes were used. However, this was not enough to characterise the lipidic diversity found on vesicular fractions. Standards for other classes of lipids such as sterol esters, di- or triacylglycerols or even sphingolipids or ceramides could provide more information on the secretome of yeast cells in these ageing yeast CM samples, and the composition of yeast EVs. Regarding EVs' biogenesis, our data suggests that both conventional and unconventional secretory pathways are involved in this process. Abrogation of MVB pathway, alters the secretory profile significantly, decreasing the occurrence of particles with lower sizes in vesicular fractions, compatible with exosomes. To understand the contribution of the conventional secretory pathways to EVs' biogenesis, either genetic or pharmacological abrogation of these pathways should be performed. Other potential pathways of relevance to EVs' biogenesis that could be targeted could also include secretory autophagy and CUPS associated unconventional secretion. Both processes are still novel in yeast and their precise mechanisms and connections with other pathways are a matter of debate.

The proof-of-concept human aSyn expression system on yeast model provided invaluable data to this thesis on cell-nonautonomous mechanisms modulating chronological lifespan. aSyn containing conditioned media induces premature ageing phenotypes on naïve receptor cells, presumably entering cells enveloped into EVs. The abrogation of the endosomal/MVB pathways annuls these pro-ageing effects, and no aSyn uptake was detected in those conditions. Endocytosis is suggested to be the pathway mediating aSyn entry into the naïve receptor cells. It would be interesting to follow these findings with the abrogation of other secretory pathways, as mentioned in the above paragraph (conventional, CUPS, secretory autophagy) and monitoring if aSyn propagation in a prion-like manner occurred as before. The possible mechanistic links between retrograde endocytosis and anterograde endosomal/MVB pathways need also to be explored further. The status of several signalling pathways within the naïve receptor cells (such as TOR, PKA, Sch9) and the coordinated activities of the proteostasis network to cope with the proteotoxic burden of aSyn could also provide valuable insights into the topic of cell signalling during yeast chronological lifespan modulation, either by cell-autonomous or cell-nonautonomous dimensions. The present thesis aimed to unveil some of those mechanisms, in the hope that a better understanding of

them could eventually leap and transcend the yeast model and allow for advances in tackling ageing and ageing-associated diseases, such as synucleinopathies, on humans as well.

This thesis is but a humble and honest contribution to a novel and enticing research field that still produces knowledge and changes each passing day.

CHAPTER 5

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