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Master dissertation
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Work supervised by
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Acknowledgment

"In my view, all that is necessary for faith is the belief that by doing our best, we shall succeed in our aims: the improvement of mankind."
-Rosalind Franklin 1940

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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.

Abstract

The term "aging" refers to the gradual accumulation of changes that take place progressively over time. These changes are either connected with or responsible for the ever-increasing vulnerability to disease and mortality that comes with growing age. The process of aging is responsible for these changes that occur as a result of the passage of time. Notably, cells, in order to maintain their own viability in the face of threats to the integrity of their proteomes, such as those posed by factors like environmental damage or the natural aging process, have evolved very sophisticated systems of adaptability. Thus, aging can be modulated by cell-non-autonomous mechanisms, which comprise a set of pathways that allows for the coordinated response of several cells to a potentially lethal stimulus. A plethora of findings suggests that cells in the vicinity may communicate with one another by releasing and taking up small molecules. However, there are still many unknowns related to the mechanisms that regulate this intercellular communication, it seems that the unconventional autophagy of cells is a key factor behind the release of small transmissible molecules. Synucleinopathies, like Parkinson's disease, is caused by the spread of alpha-synuclein (aSyn) aggregates across neural networks, which disrupts cellular homeostasis. According to research conducted on neurons, aSyn has the capability to behave in a manner similar to that of a prion and to be released into the extracellular space, where it may subsequently be taken up by the cells that are located nearby. Due to conserved cellular processes from yeast to humans, in this thesis, *Saccharomyces cerevisiae* was used as a cellular model to investigate the secretion of aSyn to the extracellular environment during chronological aging. We have shown that the secretion of aSyn to the extracellular milieu and chronological aging have a strong correlation with the aSyn expression levels. We also evaluated the mechanism that may be responsible for the secretion of aSyn during the chronological lifespan. Based on our findings, we may deduce that inhibition of the biogenesis of MVBs, as well as the ESCRT machinery, can strongly block the secretion of aSyn while they relatively have a small impact on the chronological lifespan of cells. In conclusion, in this thesis, we went through yeast aging, secretion of aSyn with different expression levels, and the mechanism behind it, while we have designed a novel system for further screening of horizontal transmission of the aSyn with the yeast cells.

Keywords: aging, cell-non-autonomous mechanism, *Saccharomyces cerevisiae*, alpha-synuclein

Resumo

O termo "envelhecimento" refere-se à acumulação gradual de danos que ocorrem progressivamente ao longo do tempo. Estas mudanças estão ligadas ou são responsáveis pela vulnerabilidade, cada vez maior, à doença e à mortalidade que advém da idade. Notavelmente, as células, para manter a sua própria viabilidade face a ameaças à integridade do seu proteoma, dirigidas por diversos fatores, tais como alterações ambientais ou o processo de envelhecimento natural, desenvolveram sistemas de adaptabilidade muito sofisticados. Neste sentido, o envelhecimento pode ser regulado por mecanismos celulares-não autónomos, que envolvem um conjunto de processo que permitem uma resposta coordenada de várias células a um estímulo potencialmente letal. Evidências sugerem que células próximas podem comunicar umas com as outras, através da secreção e captação de pequenas moléculas. Embora existam ainda muitas incógnitas em relação aos mecanismos associados a esta comunicação entre células, é sugerido que a autofagia não convencional é um dos fatores chave que controlam a libertação de moléculas transmissíveis. As sinucleínopatias, como é exemplo a doença de Parkinson, são causadas pela propagação de agregados de alfa-sinucleína (aSyn) através das redes neuronais, perturbando a homeostase celular. De acordo com estudos realizados em neurónios, a aSyn tem a capacidade de se comportar de uma forma semelhante à de um prião e ser libertada no espaço extracelular, onde pode ser posteriormente ser captada pelas células que estão localizadas nas proximidades. Devido à conservação dos processos celulares, desde a levedura até aos humanos, nesta tese, a levedura *Saccharomyces cerevisiae* foi o modelo celular usado para estudar os mecanismos relacionadas com a secreção de aSyn para o ambiente extracelular, durante o envelhecimento cronológico. Os resultados aqui apresentados mostram que os níveis de expressão de aSyn têm uma forte correlação com a sua secreção para o meio extracelular, bem como com o envelhecimento cronológico. Também avaliámos o mecanismo que pode ser responsável pela secreção de aSyn durante o tempo de vida cronológico. Com base nos nossos resultados, podemos deduzir que a inibição da biogénese dos MVBs, bem como a maquinaria ESCRT podem bloquear a secreção de aSyn e regular o envelhecimento cronológico das células. Em suma, nesta tese, avaliámos o envelhecimento cronológico da levedura, a sua correlação com a secreção de aSyn com diferentes níveis de expressão, e os mecanismos associados a essa mesma secreção, enquanto desenhamos um novo sistema para posteriormente avaliar a transmissão horizontal da aSyn, usando como modelo a levedura.

Palavras-chave: envelhecimento, mecanismos celulares-não autónomos, *Saccharomyces cerevisiae*, Alfa-sinucleína.

Table of Contents

List of Abbreviations and Acronyms

%	**Percentage**
AD	**Alzheimer disease**
aSyn	**α-Synuclein**
BSA	**Bovine serum albumin**
CFRT	**Transmembrane conductance regulator**
CFTR	**Cystic fibrosis transmembrane conductance regulator**
CLS	**Chronological lifespan**
CR	**Caloric restriction**
CUPS	**Compartment for unconventional protein secretion**
CV	**Control vector**
ER	**Endoplasmic reticulum**
ESCRT	**Endosomal sorting complex required for transport**
EVs	**Extracellular vesicles**
GFP	**Green Fluorescent Protein**
GRASP	**Golgi reassembly and stacking protein**
HD	**Huntington disease**
Hsp150	**Yeast heat-shock150 protein**
ILVs	**Intraluminal Vesicles**
mg	**Milligram**
MVBs	**Multivesicular bodies**
OD	**Optic density**
PCR	**Polymerase chain reaction**
PD	**Parkinson's disease**
PM	**Plasma membrane**
rDNA	**Ribosomal DNA**
RLS	**Replicative lifespan**
rpm	**Rotation per minute**
TGN	**Trans-Golgi network**
TM	**Melting temperature**
UPS	**Unconventional protein secretion**

Vps	**Vacuolar protein-sorting**
WT	**Wild type**
YNB	**Yeast nitrogen base medium**
YPD	**Yeast extract peptone dextrose medium**

List of Figures

List of tables

CHAPTER 1

Introduction

1. The yeast *Saccharomyces cerevisiae* as a model for aging and age-related diseases

Aging is a complex and multidimensional process influenced by genetic, environmental, and random variables that can cause abnormalities at the genomic and proteomic levels to be incorporated cumulatively into a multifaceted process.(Sampaio-Marques, Burhans, et al., 2019). Each of mentioned parameters that have been caused by aging is merely able to let severe dysfunctions emerge. Thus, the physiological integrity gradually deteriorates with age, impairing function and making a person more susceptible to mortality (López-Otín et al., 2013). For example, protein aggregation is a result of protein misfolding which may have been caused by aging. In contrast, the accumulation of these aggregates is thought to be the primary cause of neurodegenerative disorders. Prion, tau, β-amyloid, and α-synuclein, are a few examples of natural proteins in the brain that go through structural changes which extremely influence not only their roles but also their toxicity towards the cells (Ciechanover & Kwon, 2015; Hansen et al., 2007; Tutar et al., 2013).

The majority of misfolded and aggregated proteins in the human proteome can be degraded by the control system of the cells at the cellular level (Hansen et al., 2007; Tutar et al., 2013); however, in some cases, the buildup of protease-resistant misfolded and aggregated proteins, underlines misfolding protein disorders, including neurodegenerative diseases like Huntington's disease (HD), Alzheimer's disease (AD), and Parkinson's disease (PD) (Blagosklonny, 2008). For instance, cancer is the result of an abnormal increase in cellular fitness, whereas aging is associated with a decline in fitness. Thus, at first glance, they seem to be opposed processes. However, cancer and aging are related at a more fundamental level. Accumulated cellular damage over time is generally accepted as the root cause of aging, whereas in the case of cancer, certain damaged cells may gain undue advantage from the cellular damage, leading to the development of cancer. As a result, it is logical to see cancer and aging as two distinct but related expressions of the same underlying process: the progressive buildup of cellular damage (Blagosklonny, 2008; Gems & Partridge, 2013; López-Otín et al., 2013). As a result, although the hallmarks of aging are already known and accepted (Fig. 1), the core factors concerning aging remain unresolved, and the area is alive with lively discussions even about how we should conceptualize the process, also what form of damage might be responsible for this deterioration destiny if there was an accumulation of damage (Gems & Partridge, 2013)?

Due to the studies, each eukaryotic species has its own collection of age-related diseases, and the defining characteristics of cellular aging are remarkably conserved (Sampaio-Marques, Burhans, et al., 2019). The study of aging has made remarkable strides in recent years, thanks in large part to the

discovery that the rate of aging is controlled by a set of genetic pathways and metabolic processes that have been conserved through evolution (López-Otín et al., 2013).

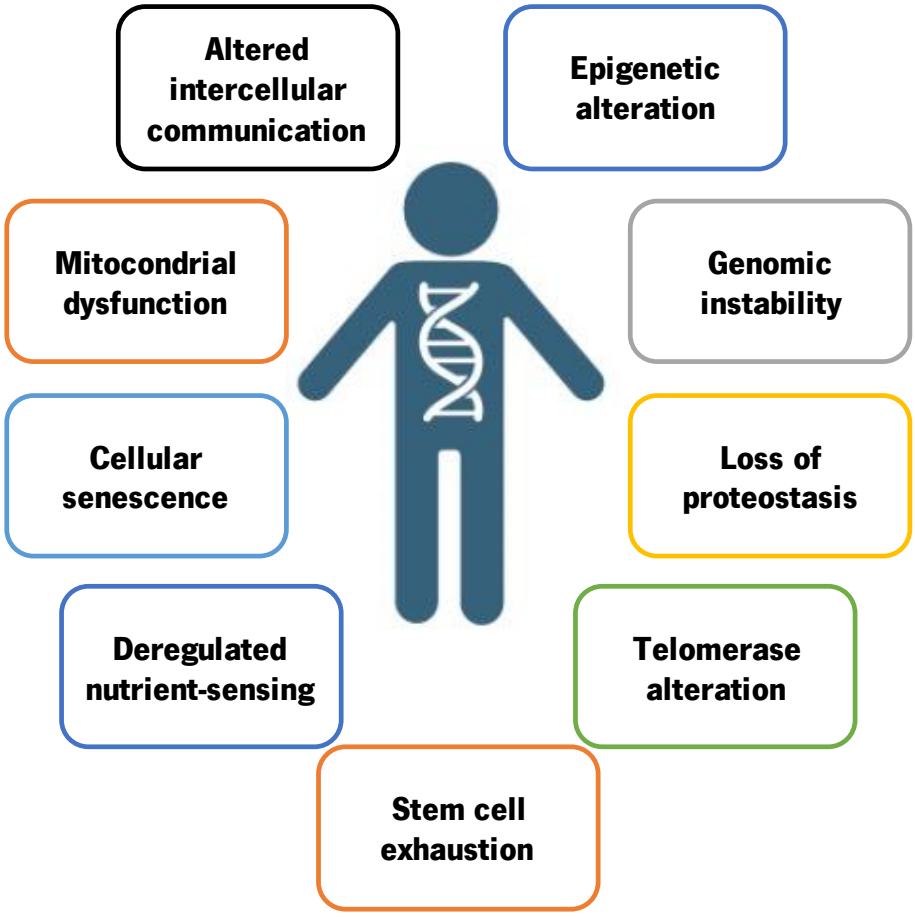

Figure 1. Hallmark of aging epigenetic changes, cellular senescence, telomere attrition, loss of proteostasis, unregulated nutrient sensing, mitochondrial failure, Genomic instability, stem cell fatigue, and altered intercellular communication are the main indicators of aging, adapted from(López-Otín et al., 2013).

Previous research using the budding yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse have revealed important information about the laboratory model organism for aging (Hansen et al., 2007). These organisms all experience aging and death but on incredibly varied timescales: three weeks for *C. elegans*, three months for *Drosophila*, three years for mice, and about 3-7 days for buddying yeast (Gems & Partridge, 2013; Kenyon, 2010; Minois et al., 2005). The finding that these organisms' lifespans can be increased and their health throughout aging may be enhanced by straightforward environmental and genetic manipulations has been a major advance. Researchers suggest that when the aging process has been

targeted, a shorter life expectancy may alternatively be a result of a unique disease rather than an informative acceleration of normal aging (Kenyon, 2010).

Since many years ago, *S. cerevisiae* cells have been regarded as archetypal eukaryotic cells, perfectly adapted to research and revealing many of the fundamental aspects of eukaryotic life (Breitenbach et al., 2012). A fully developed system of sexual reproduction with stable haploid and diploid phases enabling complementation and recombination analysis, a small genome size (12 Mbp), and a short doubling time (80 min on complex media) all contribute to yeast's unmatched ease and speed of genetic and molecular genetic analysis (Breitenbach et al., 2012; Dilova et al., 2007). Whole genome screening procedures have developed into a potent tool for aging research thanks to the knowledge of the yeast whole genome sequence, the functional annotation of yeast genes that have occurred over the last 15 years, high throughput methods, and the numerous publicly available mutant and gene collections, including cDNA microarrays (Kenyon, 2010).

One of the aging research's most basic model species, yeast, is responsible for a significant portion of its recent accomplishments (Dilova et al., 2007). *S. cerevisiae* is an excellent model species for exploring the molecular processes of cellular lifespan control. In addition to the aforementioned facts, it is notable that due to the short life cycle and well-established molecular genetic procedures in this system, it is extremely simple to generate yeast mutants and the accompanying genes that modify lifespan (Longo & Fabrizio, 2012). Additionally, recent research has shown that several longevity factors and pathways are substantially conserved from mammalians to yeast (Dahiya et al., 2020). For instance, the sirtuin pathway and the TOR signaling system are two of the principal pathways investigated in the context of aging and age-related disease, and yeast played a key role in their discovery (Longo et al., 2012). Additionally, recent research has demonstrated that a number of longevity-related components and processes are largely conserved from yeast to humans (Dilova et al., 2007). Therefore, this unicellular eukaryote is a potent system for identifying novel components in longevity-regulating pathways and studying these processes at the molecular/genetic level (Dahiya et al., 2020).

Replicative lifespan (RLS) and chronological lifespan (CLS) are two separate methods for measuring and studying yeast lifespan. Several longevity factors/pathways have been uncovered separately by both modes of research (Dilova et al., 2007). The number of divisions a cell makes before senescence determines the replicative lifespan. In view of the asymmetrical nature of *S. cerevisiae* cell division, the RLS of mother cells may be precisely determined by physically separating the little daughters from the mother cell under a microscope (Kenyon, 2001). Depending on the background of the strain and the culture circumstances, yeast mother cells cease dividing after an average of about 22-25 divisions

(Dilova et al., 2007; Kenyon, 2010). In general, to reach quiescence and have mitotic division stopped, yeast typically needs two to three days (Longo et al., 2012). The divisional age of a mother cell is an indicator of its age and the Hayflick limit of a cell's longevity due to the accumulation of endogenous DNA damage and ribosomal DNA (rDNA) instability (Knorre et al., 2018). An ample number of replicative aging genes have already been identified. As described before, the sirtuin family of NAD-dependent protein deacetylases from which *Sir2* is one of the best well-known yeast aging genes (Fukuda et al., 2021). In recent years, sirtuins have been thoroughly investigated for their possible function as conserved aging modulators in different species, including mammals (Finkel et al., 2009). Ribosomal DNA homologous recombination is prevented by Sir2 as one of the significant flaws causing aging on RLS is ribosomal DNA instability (Fukuda et al., 2021). The modulation of lifespan during RLS also involves other pathways, including *TOR* or *Sch9,* as it seems reductions in *Ras/PKA*, *TOR*, and *Sch9* signaling during CR-intervention lengthen lifespan (Dilova et al., 2007; Fukuda et al., 2021; Kenyon, 2001).

On the other hand, CLS is the period of time cells survive in a non-proliferating condition (stationary phase or post-diauxic phase) (Longo et al., 2012). Yeast cells in the stationary phase display several phenotypes that resemble the G0 stage (post-mitotic) of higher eukaryotic cells (Fig. 2). In other words, the CLS paradigm may be used to examine aging after yeast cells lose their ability for replication and reach a stationary phase in a condition of quiescence (a reversible state of growth arrest in G0/G1) (Weinberger et al., 2013). As an example, in yeast, where sirtuin 2 (Sir2) was discovered for the first time, *SIR2* overexpression prolonged mitotic (replicative) lifetime. It seems that Sir2 plays a vital role in promoting ribosomal DNA stability by preventing the accumulation of extrachromosomal rDNA circles caused by recombination between repetitive regions. Notably, it is shown that the TOR pathway also increases the interaction of Sir2 with rDNA to improve the stability of rDNA, which prolongs replicative lifespan (Ha & Huh, 2011). On the other hand, studies elucidated that, Sir2 is associated with exacerbated protein toxicity with post-mitotic (chronological) lifespan (López-Otín et al., 2013; Sampaio-Marques, Burhans, et al., 2019; Weinberger et al., 2013). It has been discovered that Sir2 is a crucial autophagy promoter involved in protein toxicity. In stationary phase cells, Sir2 mediates autophagy and mitophagy by controlling the transcription of ATG8 and ATG32 (Sampaio-Marques et al., 2012; Weinberger et al., 2013).

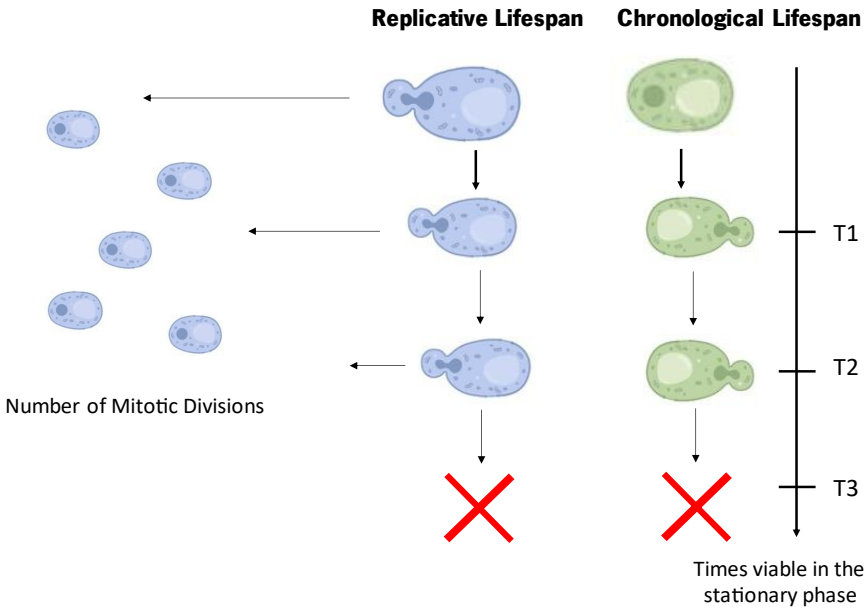

Figure 2. The two different aging models provided by yeast *S. cerevisiae*, Replicative (RLS) and Chronological lifespan (CLS). Adapted from (Chadwick et al., 2016)

Yeast cells have two separate populations during CLS: one that is essentially homogeneous and comprises quiescent, viable cells, and the other that is heterogeneous and consists of dying non-quiescent cells as a result of stress and cellular senescence processes (Longo et al., 2012; Weinberger et al., 2013). On yeast CLS, senescence is an irreversible condition, and ultimately, cell death will happen due to regulated cell death mechanisms. Seemingly, yeast cells, after the stationary phase, may undergo regulated cell death due to the exhaustion of carbon sources (Fabrizio & Longo, 2003).

A straightforward clonogenic experiment is used to measure the chronological age of yeast under laboratory conditions (Arlia-Ciommo et al., 2014). This assay measures the percentage of yeast cells that remain viable in liquid cultures at various time points following the entry of a cell population into the non-proliferative stationary phase. Cell viability in the clonogenic assay is determined by observing a cell's ability to form a colony on the surface of a solid nutrient-rich medium (Arlia-Ciommo et al., 2014).

In general, replicative and chronological yeast aging models have proven valuable tools for studying the dynamics of evolutionarily conserved lifespan regulation, enabling the identification of genes and signaling networks. In this thesis, we shall focus on the notion of post-mitotic, chronological aging of the cells, which will be focused on in the following sections.

1.2. Yeast and the regulation of cellular aging by the cell-autonomous and non-autonomous mechanism

It appears that cell-autonomous and cell-non-autonomous intraspecies processes have developed through natural selection within an ecosystem to regulate yeast lifespan (Arlia-Ciommo et al., 2014; Lamming et al., 2004). It is believed that some compounds in the environment can cause and promote hormetic and/or cytostatic responses in yeast. Cell-autonomous and non-autonomous mechanisms are thought to be fueled by yeast cells' ability to undergo certain pro-survival modifications in their metabolism and physiology in response to certain chemical compounds after being released into the environment by other groups of organisms (Howitz & Sinclair, 2008; Lamming et al., 2004).

1.2.1. Cell-autonomous and non-autonomous mechanism

It is believed that replicative aging in yeast resembles the aging of dividing, mitotically active cells in multicellular eukaryotes (Arlia-Ciommo et al., 2014). The application of robust assays for revealing longevity regulation in chronologically or replicative aging yeast under controlled laboratory settings has substantially expanded our understanding of cell-autonomous systems that coordinate lifespan-defining cellular processes inside a unicellular eukaryotic organism (Arlia-Ciommo et al., 2014; Fontana et al., 2010; Longo et al., 2012). Cell-autonomous processes, which can influence how quickly organisms age and how long they live, are made up of a variety of interconnected cell signaling networks that exist inside cells. These cell-autonomous mechanisms are necessary for modulating some important molecular, physiological, and cellular processes, including metabolism, growth, proliferation, stress resistance, cellular homeostasis, survival, and death. These systems are regulated by evolutionarily conserved nutrition and energy-sensing pathways, which culminate in the control of the so-called master longevity regulators of aging (Arlia-Ciommo et al., 2014).

Studies demonstrated that single gene mutations could severely influence the longevity of different organisms, which makes finding the story behind it more accessible. Many of these master longevity regulator genes were first discovered in *S. cerevisiae.* Due to conserved evolution, a detailed description of the genes and regulators in yeast may greatly aid its comprehension in Mammals, including humans. (Dali-Youcef et al., 2007). As an example of these regulators, The TOR/S6K pathway and sirtuins were found for the first time with the help of yeast cells as they are the homolog of Sch9 in *S. cerevisiae* (Dilova et al., 2007; Sampaio-Marques et al., 2012).

It is described that amino acids and other nutrients activate TOR, while glucose activates another pathway which is Ras/Protein Kinase A(PKA), as it seems that changes in the availability of glucose trigger

the Ras/PKA pathway (Breitenbach et al., 2005). Multiple sensors, including numerous hexokinases and glucokinases, the adenylate cyclase Cyr1, the G protein-coupled receptor Gpr1, the Gpa2 proteins, and others, detect these changes (Dali-Youcef et al., 2007; Kaeberlein et al., 2007). When there is a high level of glucose availability inside the cell, hexokinases (Hxk1/2) or glucokinases (Glk1) phosphorylate the glucose. When activated by the GTP/GDP-binding Ras proteins (Ras1, Ras2), Cyr1 generates cyclic AMP (cAMP), activating PKA (Cherkasova et al., 2010; Dali-Youcef et al., 2007; Deprez et al., 2018; Kaeberlein et al., 2007). PKA is made up of a heterotetrametric composed of three catalytic subunits (Tpk1/2/3) and a regulatory subunit (Bcy1). PKA is activated when cAMP binds to Bcy1, the regulatory subunit of PKA, separating it from the catalytic subunits. When PKA is active, it blocks the Msn2/Msn4 transcription factors, increasing the lifespan and promoting stress tolerance in post-mitotic cells. Sir2 is inhibited on mitotic (replicative) cells by an increased PKA. This cascade of protein interactions is prevented by reduced glucose levels, which may be brought on by starvation or dietary changes, and this results in the production of the Msn2/Msn4 transcription factors, a stress resistance response, and a longer lifespan (Dali-Youcef et al., 2007; Deprez et al., 2018; Kaeberlein et al., 2007). Studies revealed that although these two pathways, along with sirtuins, act on the stress-resistance regulon of Rim15, Msn2/Msn4, and Gis1 and regulate longevity, the target of them are distinct, while PKA and TOR pathways mostly induce longevity and aging in buddying yeast cell as they are in post-mitotic steps whereas sirtuins target the mitotic and actively dividing cells (Fig. 3) (Dali-Youcef et al., 2007; Deprez et al., 2018). In addition, it is vital to note that the Snf1 protein (homolog of mammalian AMP-activated protein kinase, AMPK) is a yeast energy-sensing protein that can change glucose-repressed gene transcription and promote a stress response (Deprez et al., 2018). Snf1 interacts with TOR, Sch9, or PKA (Cherkasova et al., 2010; Dali-Youcef et al., 2007; Dilova et al., 2007).

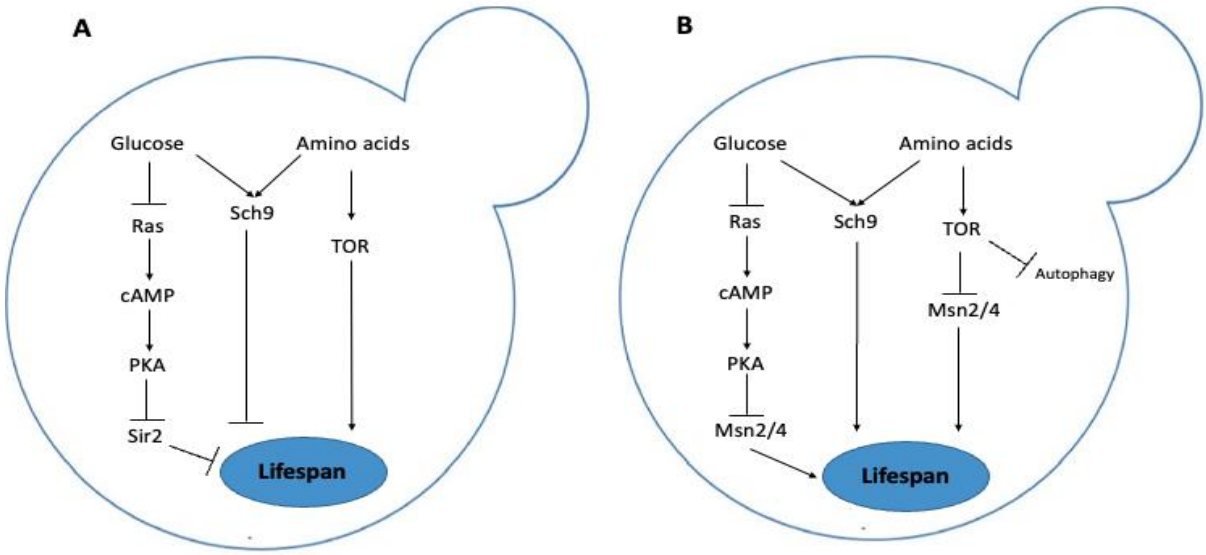

Figure 3. Caloric restriction (CR) and nutrient-sensing longevity-regulating pathways in yeast. (A) represent replicative lifespan while (B) represents chronological lifespan. Reducing signaling through Ras/PKA, Sch9, and TOR pathways extends the lifespan in budding yeast. Adapted from (Dilova et al., 2007).

As previously mentioned, specific longevity-defining genetic, dietary, and pharmacological interventions can modulate a specific set of nutrient energy-sensing signaling pathways that regulate certain cellular processes. All of these distinguishing characteristics of cell-autonomous mechanisms underlying biological aging are conserved in eukaryotes across phyla (Beach & Titorenko, 2011; Medkour et al., 2016). Emerging evidence also suggests that in more distantly related eukaryotes, the aging process is tightly regulated by cell-non-autonomous systems, including bidirectional connections between cells, tissues, and organisms (Heintz & Mair, 2014; Hine & Mitchell, 2015; Medkour et al., 2016).

Research in *S. cerevisiae* has contributed to a deeper understanding of the non-autonomous intraspecies processes that control lifespan. These mechanisms might function in organized populations of yeast cells that are attached to solid surfaces to form a colony or a biofilm; these cells: (1) interact with one another and cells in nearby colonies or biofilms; (2) age chronologically and reproduce; and (3) go through spatially organized growth, differentiation, aging, or death depending on their position within the colony (Longo et al., 2012; Váchová et al., 2012). Growing data suggest that isolated yeast cells may produce and secrete small chemicals that can influence the aging and lifespan of other cells in the same population. Laboratory studies on cell populations grown in liquid medium or on solid surfaces have demonstrated the yeast cells' capacities to produce, secrete, and/or react to such low molecular weight transmissible lifespan factors (Burtner et al., 2011; Medkour et al., 2016; Mei & Brenner, 2015). These spreading longevity factors (1) may either shorten or lengthen the lifetime of cells that generate them and cells that are exposed to them within the population, and (2) work in a cell-non-autonomous way to affect

the other yeasts' chronological and/or replicative age. Acetic acid and ethanol transmission are two excellent examples of a unique yet mystifying process by which low molecular weight transmissible longevity factors control aging and determine cell lifetime in yeast populations (Dumas et al., 2013; Medkour et al., 2016). Both ethanol and acetic acid have been shown to reduce the lifespan of yeast in a transmissible manner. It is notable that, ethanol inhibits the yeast cells' capacity to make TCA cycle intermediates, as it is known that yeast cells are unable to produce TCA cycle intermediates in the stationary phase. On the other hand, it is shown that the Ras/PKA nutrient-sensing pathway can be promoted by acetic acids as this acidic molecule is known to induce intercellular acidification (Mohammad et al., 2018). Both of these byproducts of glucose fermentation are discharged into the liquid culture medium in the stationary phase, post-mitotic yeast (Mohammad et al., 2018). In general, proteins involved in cell-autonomous mechanisms are likely to be involved in signal reception, signal transduction, and do not participate in processes involving cell-cell interactions, while proteins participating in cell non-autonomous mechanisms are signaling molecules or needed for the synthesis of signaling molecules (Arlia-Ciommo et al., 2014).

The mechanism underlying cells' non-autonomous responses were unclear, but recent studies have illustrated the probable role of extracellular vesicles (EVs), which are the lipid-bound vesicles produced by cells into the extracellular milieu (Doyle & Wang, 2019; Rodrigues et al., 2016). Microvesicles (MVs), exosomes, and apoptotic bodies are the three forms of EVs, each with its own unique biogenesis, release mechanisms, size, composition, and function. Cargo of EVs include lipids, nucleic acids, and proteins, in particular plasma membrane proteins, cytosolic proteins, and proteins involved in lipid metabolism (Doyle & Wang, 2019; Yáñez-Mó et al., 2015). One interesting characteristic of EVs is their possible role in the horizontal transmission of proteins in some human-associated diseases. One of the greatest examples is related to the alpha-synuclein (aSyn) protein. aSyn is found in human neurons, and its accumulation is implicated in the pathogenesis of PD, a neurodegenerative disorder that often affects older people, illustrating the true association of this disease with aging (Liu et al., 2016).

1.2.2. Autophagy and secretory pathways

The proteolytic quality control system known as autophagy is crucial for metabolic and cellular homeostasis, and it is considered an effective cellular response to stress. The reduction in autophagy that occurs with age is thought to contribute to several features of the aging phenotype (Sampaio-Marques et al., 2011). Thermal, physical, chemical, and oxidative stresses all contribute to molecular damage during a cell's lifespan. Different parts of the cell, such as the cytosol, the endoplasmic reticulum (ER), the

nucleus, the mitochondria, and the plasma membrane, have developed distinct but cooperative quality control systems to detect and repair this damage (Babst, 2014; Brodsky, 2012; Desdín-Micó & Mittelbrunn, 2017). Unfolded proteins are identified by a complex network of chaperones that then facilitate their refolding. On the other hand, if refolding is unsuccessful, chaperones promote protein degradation via the ubiquitin-proteasome or autophagy-lysosomal pathways (Desdín-Micó & Mittelbrunn, 2017; McClellan et al., 2005). Cells may protect themselves against proteotoxicity by secreting hazardous protein products to the extracellular medium, either in association with lipid vesicles or independently of them. The following secretion can occur when a load of proteins slated for destruction exceeds the capability of the proteolytic systems (Chen et al., 2011; Desdín-Micó & Mittelbrunn, 2017; Tyedmers et al., 2010). In general, in mammalian cells, as well as yeast cells, the bulk of secretory proteins follow the same route, from the endoplasmic reticulum (ER) to the Golgi apparatus. Some membrane proteins need an internal start-transfer signal, whereas others require an N-terminal signal peptide that is broken off during translocation into the ER. Following their passage through the Golgi and the trans-Golgi network (TGN), proteins are then directed to either the plasma membrane (PM) and/or the vacuoles, depending on their destination (Wang et al., 2017). Unexpectedly, several secreted proteins have been found to lack a signal peptide, suggesting the presence of unconventional protein secretion (UPS) pathways. Unconventional secretion pathways that bypass the Golgi complex have been discovered for various proteins in a variety of species, including yeast cells. In other words, to reach the ER and the Golgi apparatus, proteins that are secreted by the so-called "conventional mechanisms" must have an N-terminal peptide that functions as a leader or signal peptide. On the other hand, several important cytosolic proteins do not contain these signal peptides and hence ought to be secreted through a variety of methods that are not conventional or "canonical"(Gonzalez et al., 2020; Ponpuak et al., 2015; Wang et al., 2017). Cystic fibrosis transmembrane conductance regulator (CFTR) in mammalians which is one of the membrane's proteins, yeast heat-shock150 protein (Hsp150) and yeast Ist2 protein are examples of proteins that are delivered to the plasma membrane in an unconventional secretion by avoiding the Golgi. Typically, in mammalians, proteins are secreted after being transported via the ER and the Golgi complex. However, their releasing mechanisms are not well known. It seems that Golgi reassembly and stacking protein (GRASP) in mammalians and its homolog Golgi tethering factor (Grh1) in yeast, along with autophagy, are playing critical roles in these trafficking and secretory pathways (Ng & Tang, 2016; Wang et al., 2017). The fact that this unusual kind of secretion is linked to the autophagy pathway led to the term "secretory autophagy" being applied to it. The autophagosome is the prototypical organelle of this process, and ATG proteins control its biogenesis. Standard macro-autophagy requires

autophagosomes to fuse with lysosomes to degrade their contents before they may be secreted, while the secretory autophagy route avoids this degradation step (Gonzalez et al., 2020). Seemingly, not only amphisomes formation but also multivesicular bodies (MVBs) are so crucial for secretory autophagy (Wang et al., 2017). MVBs are a kind of endosome that store intraluminal vesicles generated by restricting membrane invagination and budding. MVBs can also be considered late endosomes that may fuse with the PM and either release their contents into the extracellular environment or degrade them by lysosomal/vacuolar fusion (Li et al., 2018).

Studies have demonstrated that the difference in the size of EVs can directly relate to their roles. EVs can be classified as apoptotic bodies (>800 nm), Microvesicles (100 to 1000 nm), or exosomes based on their size and the putative biogenesis route (ranging from 40 to 150 nm) (Battistelli & Falcieri, 2020; Lawson et al., 2016). Furthermore, the fusion of MVBs with the plasma membrane can produce exosomes as tiny endosome-derived EVs involved in cell-cell communication, which was previously mentioned with the cell-non-autonomous mechanism. It seems that when endosomal compartments fuse with the plasma membrane, microscopic lipid vesicles called exosomes are secreted into the extracellular environment (Tyedmers et al., 2010). Exosomes, once released into the extracellular environment, may be picked up by neighboring cells or, if they make it into the bloodstream, by cells further away, where they might alter the behavior and destiny of receptor cells (Desdín-Micó & Mittelbrunn, 2017). So, exosomes are now known to play crucial roles as mediators of cell-to-cell communication in a wide range of physiological and pathological processes, such as the immune response, the development and spread of cancer, the exchange of information between neurons, cardiovascular diseases, and the progression of neurodegenerative disorders, although, there is still much to discover about the role of EVs in aging and cell-non-autonomous processes regulation (Kalluri, 2016; Lawson et al., 2016).

Furthermore, as mentioned, exosomes' function as mediators of intercellular communication has been the subject of intensive study in recent years. However, the role of exosome biogenesis and secretion has received comparatively little study. Controlling cellular homeostasis may involve exosomes, which play a role in this process by facilitating the release of potentially damaging and hazardous proteins, lipids, or nucleic acids from inside of the cell to the extracellular space (Tyedmers et al., 2010). There is evidence suggesting that EVs carry out a number of tasks in many physiological processes in mammals and yeasts (Choi et al., 2018). Interestingly, it has been shown that cancer cells secrete EVs, which are the primary factor in the growth of tumors (Chang et al., 2021). Both neurons and microglia are responsible for the release of EVs that carry pathogenic proteins. Many of these proteins are linked to age-related neurodegenerative disorders, such as prions, mutant superoxide dismutase, β-amyloid

peptide, and α-synuclein (aSyn) (Choi et al., 2018; Emmanouilidou et al., 2010). Seemingly, in the case of aSyn, EVs are released by both neuronal and microglial cells, and the mechanism behind it may be related to the secretory and unconventional autophagy (Choi et al., 2018; Emmanouilidou et al., 2010).

Also, a lot of questions are still left unanswered, but numerous studies have suggested that exosomes originate from invaginating buds that are found on the outer layers of cell membranes. At first, primary endocytic vesicles are formed by endocytosis of extracellular proteins, lipids, metabolites, and cell membrane proteins. Early endosomes are formed when vesicles merge, while late endosomes are the result of EEs maturing. Then, Intraluminal Vesicles (ILVs) are formed from LEs by encapsulating with specific proteins and nucleic acids and eventually mature into MVBs. Depending on the proteins that are expressed on their surface, the MVBs have different outcomes. Certain of them move to the plasma membrane, where they fuse with the membrane and release ILVs (release of ILVs considered as exosomes) to the cell-extracellular environment (Fig. 4) (Xu et al., 2022). Despite the fact that the mentioned mechanism may explain the secretion mechanism by the Endosomal/MVBs pathway, other studies also demonstrated that vacuolar protein-sorting (Vps) proteins Vps23 and Snf7, as well as the ESCRT (endosomal sorting complex required for transport) compartment, including ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, have distinct roles in a variety of processes, specifically including MVBs formation, cell shrinkage, and viral budding, which were all found to partly impact the composition of EVs and/or their kinetics of extracellular release and secretion. In research conducted with *S. cerevisiae,* however, genetic impairment of these genes may not consistently terminate the formation of the EVs and MVBs' biogenesis completely (Katzmann et al., 2001; Oliveira et al., 2013)

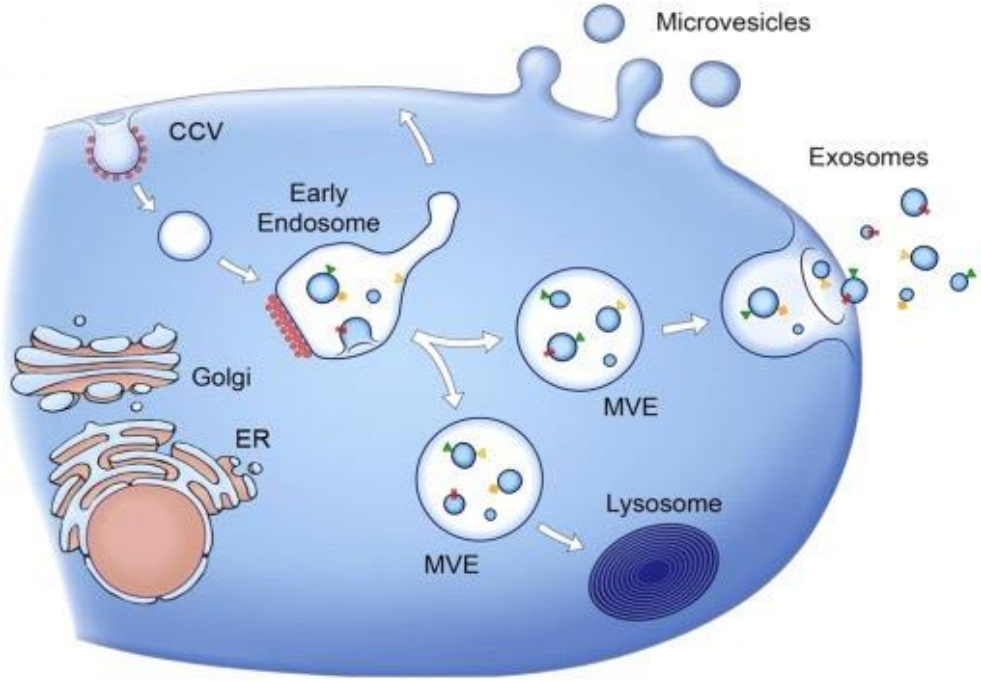

Figure 4. Intervesicular bodies (ILVs) are formed by inward budding of the limiting membrane of MVBs, a process that also initiates the creation of exosomes (30-150 nm in size). By fusing with the plasma membrane, MVB can release the ILVs. Picture from (Gholizadeh et al., 2017). Reusing this picture is granted by license number (5416220171988) from Elsevier.

While the aforementioned players are responsible for the secretion of damaged or hazardous proteins such as aSyn, namely based on unconventional protein secretion with Endosomal/MVB pathways, it seems that the ER-Golgi mechanism and GRASP (Grh1)-mediated machinery indeed play crucial roles as a part of the conventional and unconventional release of EVs, respectively (Oliveira et al., 2013).

Secretion of Acb1 proteins in yeast is one of the known examples of unconventional secretion, and it is accomplished by a combination of the dedicated membrane-bound compartments (called CUPS) compartment for unconventional protein secretion and the GRASP (Grh1 is a non-essential GRASP homolog found in the yeast *S. cerevisiae* (Levi et al., 2010)) machinery for secretory autophagy (Fig. 5) (Abrahamsen & Stenmark, 2010). The highly conserved *ACB1* gene is responsible for encoding the Acyl-CoA-binding protein, which is required for fatty acid transportation (Schjerling et al., 1996). A lot of studies have illustrated that the secretion of Acb1 inside the CUPS requires not only secretory autophagy but also MVB formation. The biggest difference here is related to the presence of Acb1 only in mature CUPS and not on the MVBs. Grh1, which is required for Acb1 secretion, is moved from the Golgi to the bigger CUPS (Curwin et al., 2016). It has been postulated that in eukaryotic cells, the coat proteins COPI and COPII orchestrate the movement of proteins and membranes through the first stages of the secretory route.

These coat proteins not only determine which cargo proteins to transport but also shape the lipid bilayer of donor membranes into buds and vesicles (Duden, 2003). Interestingly, it seems that neither COPII nor COPI-mediated transport is required for CUPS production and CUPS-mediated secretion. As the next step, the Snf7 (ESCRTIII subunits), bind to and stabilize this compartment. It has been hypothesized that CUPS may fuse with autophagosomes to produce amphisomes, allowing Acb1 to be secreted by secretory autophagy (Curwin et al., 2016).

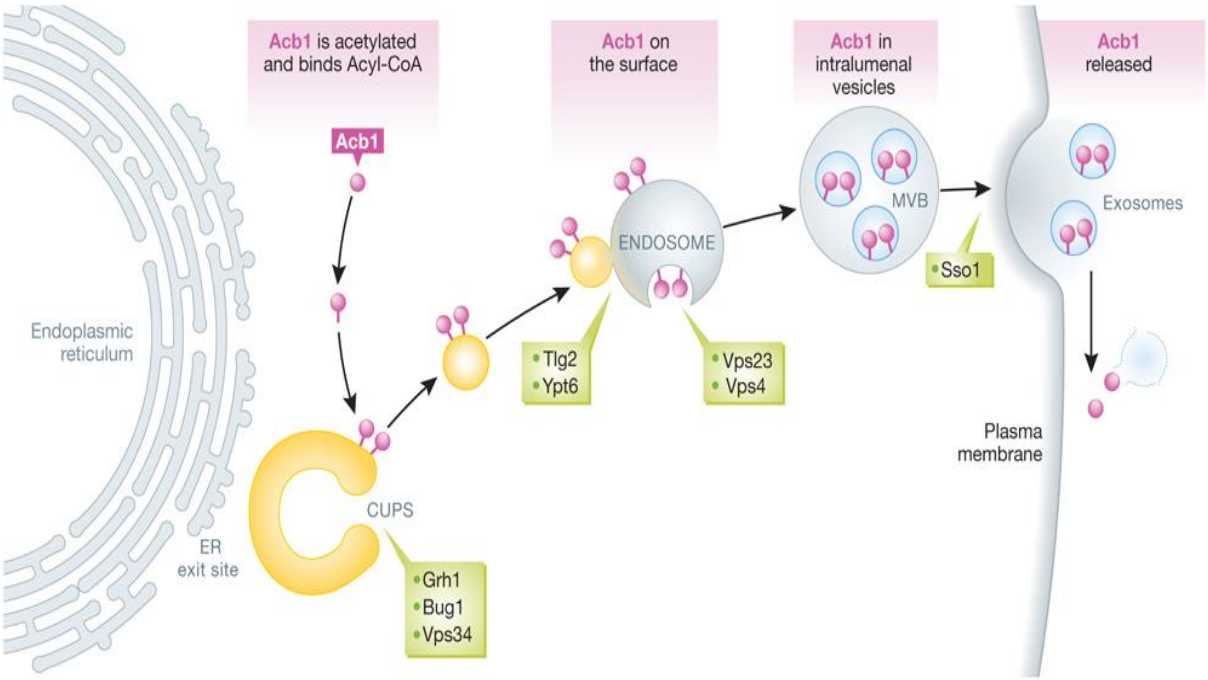

Figure 5. Acb1's unconventional protein secretion mechanism. When yeast is starved, CUPS gathers at the ER exit sites. The proteins Grh1, Bug1, and Vps34 are essential for CUPS biosynthesis (in green). At the CUPS, a COPII-independent mechanism generates a novel class of vesicular intermediates. Upon lysing, the resultant exosome-like vesicles with Acb1 are released into the extracellular space through the t-SNARE Sso1, which mediates the fusion of the MVB with the plasma membrane. Picture from (Malhotra, 2013). Reusing this picture is granted by license number (5416220541014) from John Wiley and Sons publisher.

The process of secretory autophagy was shown to be crucial for amphisomes fusion with the plasma membrane. Many issues about secretory autophagy remain unanswered as still not enough studies have been performed. As described previously, The Grh1 protein in yeast (and its homolog GRASP in humans), the ESCRT complexes involved in MVBs synthesis, and the autophagic machinery directing autophagosome formation are all required to enable this spectacular machinery to keep the homeostasis inside of the cells stable (Ponpuak et al., 2015).

1.3. Yeast and the study of synucleinopathies as Parkinson's' disease

Most neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), have advanced age as their principal risk factor (Hou et al., 2019). The natural process of aging is linked to a low level of physical health, which in turn relates to an increased risk of illness and mortality. The loss of proteostasis is a hallmark of most neurodegenerative illnesses in humans. This is because, as we age, the protein quality control mechanisms that destroy misfolded proteins also lose efficiency. Due to the fact that the incidence of neurodegenerative diseases is much higher in the elderly population, many researchers have begun to refer to these conditions as age-associated diseases (Hou et al., 2019; Sampaio-Marques, Burhans, et al., 2019).

Only a little over two centuries have passed since PD was first recognized as a clinical entity; now, it is the second most prevalent neurodegenerative disease, affecting around two percent of the over-65 population (Singh & Muqit, 2020). Idiopathic Parkinson's Disease is characterized by neuronal Lewy bodies and the pathophysiological loss or degeneration of dopaminergic neurons in the substantia nigra of the mid-brain in addition to the formation of Lewy Bodies, which is a pathologic signature in dopaminergic neurons (Gazewood et al., 2013), and it is linked to variables such as age, family history, pesticide exposure, and environmental pollutants (e.g., synthetic heroin use). Although the root of the problem is still the biggest mystery of PD (Hou et al., 2019; Singh & Muqit, 2020).

Studies elucidated that there may be a gap of twenty years or more between the onset of pathologic alterations and the manifestation of evident symptoms. Motor control is significantly hampered as a consequence of this preferential loss of dopamine-producing neurons. Some proteins, like as aSyn and ubiquitin, are found in the Lewy Bodies (Hayes, 2019; Mutch, 1991). These aggregates disrupt normal neuron activity. In particular, persistent low-level inflammation in the brain ("Inflammaging") is promoted by exposure to environmental pollutants (e.g., pesticides), substance misuse, or the stress of aging. Neurons in the brain undergo cellular senescence as a result of this inflammatory process over time (Gazewood et al., 2013).

There is a connection between the genetic variations that code for brain proteins and the death of neurons. aSyn, in particular, develops abnormalities and forms self-aggregates. These clumps of insoluble aSyn are a prominent component of what is known as Lewy Bodies, which are cellular inclusions that are characteristic of PD (Kouli et al., 2018). Not only that but the ubiquitin-proteasome system, which is supposed to break down aberrant proteins, is also hampered, which can cause an even higher amount of accumulation of aggregations (Gazewood et al., 2013; Kouli et al., 2018). Mitochondrial malfunction and increased oxidative stress due to reactive oxygen species may also play a role in Parkinson's disease

by inducing neuronal degeneration; in addition, there are additional proteins that play a role in the proteolytic systems and the clearing of mitochondria (Parkin, PINK1) (Ge et al., 2020; Quinn et al., 2020). It has been shown that Parkin 35 and PINK1 control mitochondrial fission, fusion, and the removal of damaged mitochondria through mitophagy (Chu, 2011). It is hypothesized that two genes, PINK1 and Parkin, have mutations linked to autosomal recessive Parkinson's disease (Ge et al., 2020).

Synucleinopathies are a set of age-related neurodegenerative illnesses connected to the pre-synaptic neuronal protein aSyn. In 1988, the tiny protein known as aSyn, which has a total of 140 amino acids, was found in the cholinergic synaptic vesicles of the electric fish *Torpedo californica* together with other homologs. The protein known as aSyn may be broken down into three distinct regions: (1) the very hydrophobic middle part (residues 61–95); (2) the highly repetitive N-terminal repeat area (residues 1–60); and (3) the acidic C-terminal repeat region (residues 96–140). Amphipathic-helical structures are formed by 11-amino acid imperfect repetitions in the N-terminal region, which contain a highly conserved hexamer motif (XKTKEGVXXX) (Fig. 6) (Mori et al., 2020). Neurodegenerative diseases, including Parkinson's disease, are characterized by the aberrant buildup of aSyn aggregates. Calcium and mitochondrial homeostasis management, poly-unsaturated fatty acid levels, and chaperone function are only a few of the many physiological processes that have been linked to aSyn (Sampaio-Marques, Guedes, et al., 2019). Numerous cellular and animal models of Parkinson's disease (PD) and other synucleinopathies have shown that cell cycle re-entry, DNA damage, activation of DNA damage responses (DDR), and cell death are also present (Camins et al., 2010).

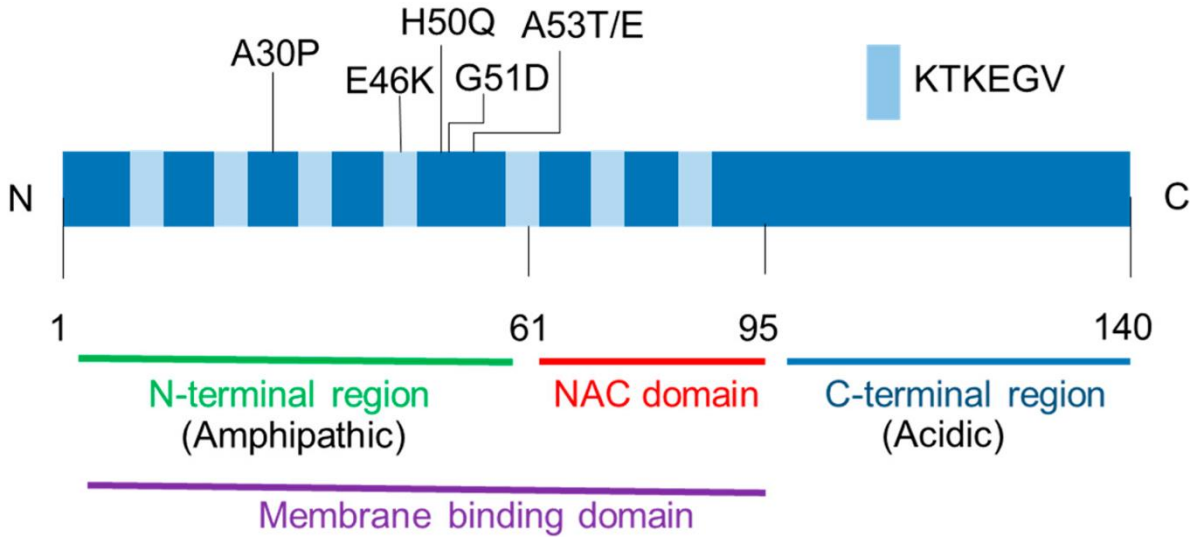

Figure 6. Model of the aSyn protein. Seven incomplete KTKEGV repeats in the N-terminal region and the non-amyloid component domain that involved and promote binding to the membrane. Picture from (Mori et al., 2020). *No permission was required due to Free worldwide access.

Recent studies have shown that aSyn has been shown to aggregate inside cells, forming poisonous inclusions made up of aSyn fibrillar form. The aSyn protein 36 inclusions in Lewy bodies are oligomeric inclusions that are amplified during stress. However, the exact mechanism by which these inclusions in protein folding contribute to their harmful effects under stress has not been elucidated. Notably, aSyn has been found in the non-phosphorylated form while the aggregated is phosphorylated inside the Lewy bodies(Mori et al., 2020; Singh & Muqit, 2020).

Surprisingly, aSyn seems to be involved in the horizontal prion-like transmission of PD between several neuronal cells. Interestingly, aSyn was found for the first time in human plasma, and it was shown that released aSyn had the potential to decrease the viability of several kinds of neuronal cells. It seems the secretion is related to the effectiveness of the proteolytic systems of the cells means the more effective the system is, the less secretion is needed (Jiang & Dickson, 2018). On the other hand, any dysfunctions in that system, like during aging, will end up with more aSyn secretion. More surprisingly, the secreted aSyn can be uptaken by other neuron cells to clear the extracellular milieu from aSyn. Based on the previous description of the exosome's role in disposing of misfolded and aggregated proteins out of the cell, it is believed that the same system is applied when it comes to aSyn secretion (Jiang & Dickson, 2018; Stefanis et al., 2019).

In several models of Parkinson's disease and other synucleinopathies, budding yeast has shown to be an invaluable component. As explained previously, the budding yeast *S. cerevisiae,* is particularly adaptable to genetic modification, the genome has been entirely sequenced, and many of the essential biological such as a lot of vital pathways like secretory autophagy and proteolytic systems and various mechanisms, are evolutionarily conserved from yeast to higher eukaryotes(Sampaio-Marques, Burhans, et al., 2019; Váchová et al., 2012). Signal transduction, membrane trafficking, and protein turnover are just a few of the many eukaryotic cellular processes that *S. cerevisiae* has helped better comprehend. Due to their striking homology, yeast is a powerful model for studying basic cellular biology processes of human neurodegenerative disorders like PD (Tenreiro et al., 2017). For instance, prions are infectious protein particles that may fold incorrectly and cluster in neurons, which can lead to neurodegeneration. The prototypical protein-folding diseases are caused by prions. Like their human counterparts, yeast prions are the heredity that spread from cell to cell through a protein-only mechanism and so affect the phenotype of the host organism (Khurana & Lindquist, 2010; Tenreiro et al., 2017). Studies demonstrated that neurodegeneration is strongly linked to mitochondrial malfunction and oxidative stress. The mitochondria is the primary organelle in yeast cells and human cells for producing reactive oxygen species (ROS). Yeast may be grown in fermentative conditions that would kill human cells, allowing for the study

of mitochondrial abnormalities that would have catastrophic effects on other organisms and play a crucial role in PD (Khurana & Lindquist, 2010). Due to the simplicity of yeast manipulation, non-yeast genes from a human can easily be heterologously expressed in yeast. For instance, regarding the synucleinopathies, under the control of a constitutive promoter, aSyn may be produced in its wild-type (WT) form either with WT *S. cerevisiae* or mutant strains (Sampaio-Marques, Guedes, et al., 2019). Notably, the effects of aSyn overexpression in yeast have been key to understanding many mechanisms, including impaired proteasome activity, accumulation of cytoplasmic lipid droplets, ER stress, mitochondrial dysfunction, and induction of autophagy and mitophagy (mediated by Sir2), among others (Menezes et al., 2015).

Hence, in this thesis, the proteotoxic model (aSyn heterologous expression) and screening for signaling molecules implicated in cell-non-autonomous causes of aging in yeast will be discussed. In addition, the importance of the effect of different promoters on the secretion of the aSyn and the survival rate of the yeast cells while chronologically aging, will be discussed in **Chapter 3, section 3.1.** Furthermore, the mechanism behind the secretion of aSyn will be evaluated in **Chapter 3, section 3.2.** Also, the details regarding the construction of split GFP plasmids for evaluating the horizontal transmission of aSyn in yeast will be discussed in **Chapter 3, section 3.3.** Protocols, materials, and procedures are all detailed in **Chapter 2.**

Finally, our goal in this thesis was to further knowledge of the underlying processes that control aging by the cell-non-autonomous mechanism by using the human aSyn protein as a proof-of-concept of secretion in yeast cells of *S. cerevisiae.*

CHAPTER 2

Materials and Methods

2.1. Strains and Plasmids

The yeast *S. cerevisiae* strains and plasmids that were used in this thesis are listed in (Table 1).

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

Yeast Strains	Genotypes	Source
W303-1A	*MATa ade2 Δ 1 trp1 Δ 1 ura3-1 leu2 Δ 3,112 his 3 Δ 11,15 can1 Δ 100*	Sampaio-Marques et al., 2018.
BY4741	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	*Euroscarf*
BY4741 *Δsnf7*	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	*Euroscarf*
BY4741 *Δvps23*	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	*Euroscarf*
BY4741 *Δgrh1*	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	*Euroscarf*
BY4741 *Δsir2*	*MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0*	*Euroscarf*
Plasmids	**Type of Plasmids**	**Source**
pYX242-SynWT-TPI1	2μ	Winderickx, J
pUG23-SynWT- EGFP-MET25	2μ	(Zabrocki et al., 2005)
pRS306 GAL-SNCAWT-GFP	Integrative	(Sampaio-Marques et al., 2012)
p426GPD-SynWT	2μ	(Tenreiro et al., 2015)
pUC23-wt_aSyn	2μ	Winderickx, J
Split GFP: pRS314-GPDp-GFP1-10	2μ	Winderickx, J
Split GFP: pRS316-GPDp-GFP1-10	2μ	Winderickx, J
Split GFP: pRS314-GPDp-GFP11	2μ	Winderickx, J

2.2. Media and Culture conditions

YEPD Medium, consisting of yeast extract (Bacto™-Belgium) (1%, w/v) peptone (Bacto™-Belgium) (1%, w/v), agar (LabChem™-Portugal) (2%, w/v), and glucose (Gibco™-USA) (2%, w/v), was used to sustain *S. cerevisiae* cell stocks. All assays were conducted in a glucose-containing synthetic complete (SC) medium with 2% glucose, as a carbon source, and 0.67% yeast nitrogen base (BD Difco™-France) without amino acids, supplemented with the amino acids and bases for which the strains were auxotrophic: 50 g/ml of histidine (Sigma™-USA), 100 g/ml of uracil (Sigma™-USA), 100 g/ml of methionine (Sigma™-USA), 300 g/ml of leucine (Sigma™-USA), and 100 g/ml of tryptophan (Sigma™-USA).

2.3. Harvesting conditioned media

Yeast cells were cultured in an SC medium at 26°C during defined time points, in which it was collected culture medium. The collected media were then centrifuged and the supernatants, designed now as conditioned media, were collected and kept at -80°C for further analysis.

2.4 Chronological lifespan (CLS)

To assess chronological lifespan (CLS), cell cultures were grown on SC medium until they reach the stationary phase, the point that was considered the time 0 of the CLS. After this time point, in which cell viability is considered 100%, cell survival was evaluated each 2-3 days, by colony-forming units (CFUs), until the observance was less than 0.1% of the viable cells. Plates of YEPD agar were used to culture serially diluted cellular samples. Prism 9, a statistical analysis program (GraphPad Software-version 9.4.1), was then used to calculate the survival rate.

2.5 Protein extraction from the pellet of cell

At the designed time points of CLS, cells were collected, and protein extraction was performed for subsequent detection of protein levels by immunoblot. For that, cells were washed one time in PBS and then spun down with centrifugation of 1 minute at 13000 rpm (Thermo™ Heraeus Biofuge-Siehe rotor), then, they were pretreated with 2 M lithium acetate at room temperature for 5 minutes. Lithium acetate is separated from the cell pellet after a brief centrifugation step with 13000 rpm (Thermo™ Heraeus Biofuge-Siehe rotor) for 1 minute. The cells were put on ice for 5 minutes after 0.4 M of NaOH was applied. After that, cells were boiled for 5 minutes in an SDS-PAGE sample loading solution before being pelleted and resuspended.

2.6 Protein extraction from conditioned culture media

Five ml of pure trichloroacetic acid (TCA) was added to 45 ml of the conditioned medium. After, samples were vigorously vortexed and incubated on ice overnight. After, the samples were centrifuged at 4500 rpm (Thermo™ Heraeus Multifuge-Siehe rotor) for 15 minutes at 4 °C. After removing the supernatant, 0.5 ml of TCA 10% was added. The mixture was mixed properly by vortex, then transferred to the microtubes to increase the yield of extraction, and then centrifuged at 13,000 rpm (Thermo™ Heraeus Multifuge-Siehe rotor) for 15 minutes at 4°C. The resultant pellet was treated with 0.5 ml of

acetone and then centrifuged for 10 minutes at 13,000 rpm (Thermo™ Heraeus Multifuge-Siehe rotor) and 4°C. The protein pellet was centrifuge-dried at high-speed using a Speed Vacuum after discarding the supernatant. In the end, the pellet was resuspended in 50 μL of SDS PAGE sample buffer.

2.7 Protein quantification and Immunoblot assay

The RC/DC protein assay (Bio-Rad) was used to determine protein concentration, and the samples were treated following the manufacturer's protocol. 20 μg of cellular pellet proteins and 30 μL of media proteins were separated on an SDS-PAGE gel at a concentration of 12% based on the results of the protein quantification. Proteins were further transferred to a nitrocellulose membrane for 12 minutes at 25 V through the Trans-Blot Turbo transfer device. After, the membrane was blocked using tris-buffered saline (TBS) with 0.1% Tween (Sigma-Aldrich™-France) 20 (TBST) and 5% bovine serum albumin (BSA) (Sigma-Aldrich™-USA). Next, membranes were incubated with primary antibodies with TBST containing 1% BSA against anti-α-syn (1:1000, Cell Signaling) and anti-PGK (1:5000; Invitrogen) overnight at 4°C. After washing the membrane with TBST, the membrane was incubated with secondary antibodies, against anti-rabbit IgG (1:5000) and anti-mouse IgG (1:5000) in 1% BSA. After incubation, the membrane was washed and visualized with a ChemiDoc XRS System (Bio-Rad) with Quantity One (Bio-Rad) software by Clarity Western ECL Substrate (Bio-Rad) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

2.8 Experimental design of Split GFP tagged with Alpha-Synuclein

2.8.1 Primers

Based on the homologous recombination strategy, the primers designed to amplify aSyn by this strategy must contain homologous arms. Table 2 are described the primers that were used for amplification of the aSyn with homologous recombination, as well as colony PCR primers.

Table 2. List of primers that were used for amplification of aSyn and colony PCR.

Primers' name	Sequence
NHF14161-10-11(FW)	CACCAGAACTTAGTTTCGACGGATGCATGGATGTATTCATGAAAGGACTT
NHR14161-10-11(RV)	CCCTCCGGATCCACTAGTTCTAGAGCGGCCGGCTTCAGGTTCGTAGTCTT
Colony PCR: CF131411-H(FW)	GTAAAACGACGGCCAGTGAATTG
Colony PCR: CR1314161110-H(RV)	CGAGGTCGACGGTATCGATAA
Colony PCR: RFS14161-10.11(FW)	GGCCGCATGGATGTATTCATGAAAGGACTT
Colony PCR: RRS141611(RV)	CTAGTGGCTTCAGGTTCGTAGTCTTG

2.8.2 Cloning strategy

Homologous recombination is the basis for plasmid construction; this process calls for an inserted gene (here *SNCA*) to have homologous arms that are complementary to the insert region in the plasmid using primers indicated in Table 2. Then split GFP plasmids (Table 1) were linearized using restriction enzymes. In this case, plasmids were digested using the *NotI* restriction enzyme. Next, competent cells were transformed with the amplified fragment and linearized plasmids so that transformed *E. coli* could construct the plasmids by re-constructing the circular plasmids with the fragment in the digested region. According to the literature, both *E. coli* and *S. cerevisiae* are capable of "gap repair" (Lyozin & Brunelli, 2020). By this strategy, the competent cells are able to insert our fragment, namely *SNCA*, before the split GFP region (Fig. 7).

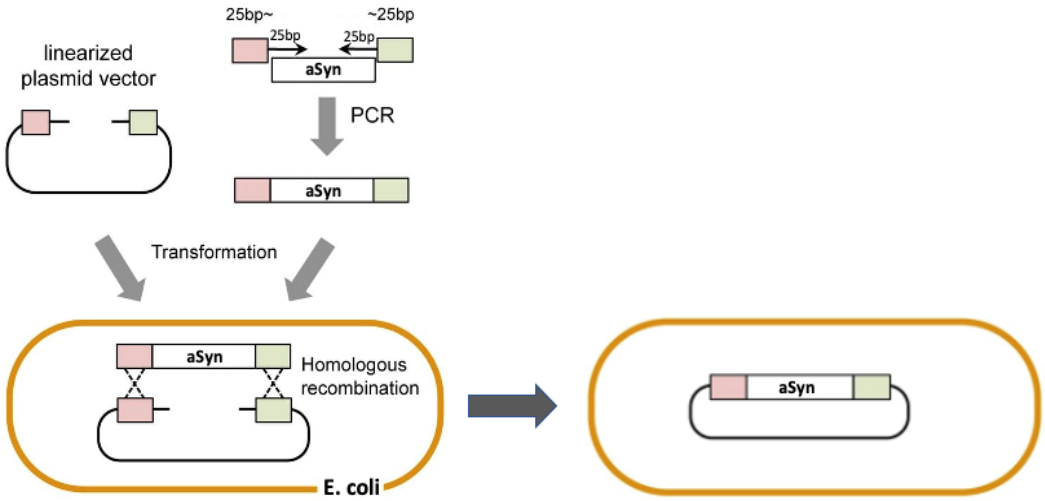

Figure 7. Schematic view of homologous recombination in *E. coli*. Adapted from (Chino et al., 2010)

2.8.3 Plasmid digestion

For linearizing our plasmids, the backbone of them (pRS314-GPDp-GFP1-10, pRS314-GPDp-GFP1-10, and pRS314-GPDp-GFP11) were digested by *NotI* restriction enzyme (Fast digest Anza - Thermo Fisher Scientific) by the mixture shown in the (Table. 3) for 15 minutes at 37°C. Then the restriction enzyme was deactivated by heat, through incubation at 80°C, for 20 min.

Table 3. Composition of the 20µL reaction mixture for plasmid digestion with *NotI*.

Components (stock concentration)	**Volume (1 reaction)**
1:10 Plasmid (100 ng/µL)	5µL
Enzyme *NotI* (Anza Thermo Fisher scientific)	1µL
Buffer 10X	2µL
Water	12µL
Final volume	20µL

2.9. Transformation of *Escherichia coli*

To perform the transformation of *E. coli*, 5µl of the digested plasmids (pRS314-GPDp-GFP1-10, pRS314-GPDp-GFP1-10, pRS314-GPDp-GFP11) and 5µl of aSyn amplification product with homologous arms were added to 90 µL of competent *E. coli* cells (*DH5-alpha*), which were then incubated on ice for 30 min. Following the incubation on ice, the cells were subjected to heat shock for 45 seconds at 42°C. Immediately, cells were incubated on ice for 10 min. For the recovery of the heat-shocked cells, 800 µL of LB-rich medium was added to each microtube and incubated at 37°C for 1 hour at 200 rpm (Thermo™ Heraeus Multifuge-Siehe rotor). Then spun down and recovered cells were resuspended with 100 µL of leftover LB media (0.5%, w/v yeast extract, 1%, w/v Tryptone, and 1%, w/v NaCl) in the microtubes and then were plated on an LB Ampicillin plate (final concentration of 100 µg/ml) overnight for growing.

2.10. Polymerase chain reaction (PCR) and Agarose gel electrophoresis for DNA

SNCA gene as our fragment was amplified with the primers of NHF14161-10-11 (Forward) and NHR14161-10-11 (Reverse) for generating the homology arms. For this purpose, high-proofreading polymerase was used to decrease the chance of mutations. Primer's Melting temperature of 57°C was used as the annealing temperature for the reaction. The PCR process is typically performed over the course of 30 cycles, with each cycle including a change of temperature by one of three predetermined increments. One or more high-temperature (>90 °C) steps are often performed before the cycle, and one hold step is typically performed thereafter for final product extension or temporary storage (Details are shown in (Table. 4)). For confirmation, the PCR product was run on the DNA gel electrophoresis using a 1 % TAE agarose gel. Notably, a higher and longer denaturation temperature (10 minutes of 98°C) was used in order to lyse the cells for the colony PCR. The primers indicated in (table. 2) were used in order to check the construction by colony PCR.

Table 4. PCR cycling parameters used to amplify aSyn

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Annealing	57°C	10 sec	30
Extension	72°C	30 sec	30
Final extension	72°C	10 min	1

2.11. Plasmid extraction from *E. coli*

Plasmids were isolated from *E. coli* cells cultured overnight at 37 °C and 200 rpm (Thermo™ Heraeus Multifuge-Siehe rotor). Cells were cultured in 5 ml of LB medium with Ampicillin (final concentration of 1000 µg/ml). The cells were spun for 2 minutes at 5000 rpm (Thermo™ Heraeus Multifuge-Siehe rotor) in an Eppendorf and the resulting supernatant was thrown away. After vigorously vortexed, the pellet was re-suspended in 250 µL of Buffer P1 (50 mM Tris-HCL pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). After that, 250 µL of Buffer P2 (200 mM NaOH, 1% SDS) was added and then inversed gently after the 5 minutes of room temperature incubation. Then 300 µL of P3 (3 M Potassium acetate-pH 5.5) was added and mixed gently. Centrifugation was performed with a maximum speed (13000 rpm-Thermo™ Heraeus Multifuge-Siehe rotor), and the supernatant was collected to the new clean Eppendorf. After that, 500 µL pure isopropanol was added and mixed gently, and then the Eppendorf was centrifuged for 5 minutes, and the resulting pellet was treated with 1ml of ethanol 70%. After another centrifugation procedure and discarding the supernatant and letting the left-over Ethanol evaporate, 50 µL of ultrapure water was added the extracted plasmid's concentration was measured by Nanodrop.

2.12. Yeast transformation

Yeast transformation via the lithium acetate (LiAc) technique was performed. For that, yeast cells were cultured in rich YEPD medium overnight and then diluted to an OD600 of 0.2 (2x10e6 cells/ml) in 100 ml of fresh YEPD the next morning. The time required to increase to an OD600 of 0.6 is, therefore, four to six hours. The cells were centrifuged in a volume of 2.5 ml, and the resulting supernatant was thrown away. After that, the cells were rinsed in deionized sterile water, centrifuged, and the supernatant was thrown away. The following were added to the individual microtube: 240 µL of a 50% Polyethylene glycol (PEG) 3500 solution (w/v), 36 µL of Lithium acetate (LiAc 1M), 50 µL of salmon sperm DNA (Boiled

for 5 minutes and cooled immediately on ice), and 20-34 µL of the plasmid. After adding the cells and transformation solution to the Eppendorf, the mixture was vortexed for 1 minute. The following stage included incubating the Eppendorf at 42 degrees for roughly one hour. Centrifuged again, and after discarding the supernatant, the resultant pellet was resuspended in 100 µL of sterile water and plated on with selective solid media (SC without tryptophan (Sigma-USA), SC without uracil (Sigma-USA)).

2.13. 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 9 statistical analysis software (GraphPad). A *p*-value of less than 0.05 was considered as a significant difference. At least three independent biological replicas were used in all the experiments with statistical analysis reported in this thesis.

CHAPTER 3

Results and Discussion

3.1. Secretion of aSyn during yeast aging

Aging is a complex and multi-factorial physiological process described as the random accumulation of damage and loss of cellular, tissue, and organ function (Sampaio-Marques, Burhans, et al., 2019). Mounting evidence revealed that aSyn propagates between different neuronal cells in the human brain in a prion-like mechanism (Choi et al., 2018). The mechanism driving the release of low molecular weight transmissible components into the extracellular environment is still not completely well-known. Therefore, herein, we aimed to study the role of cell-non-autonomous mechanisms in yeast aging modulation through the use of the yeast model heterologous expressing the human aSyn, which is a protein associated with aggregation and synucleinopathies, and its expression in yeast promotes proteotoxic stress and premature aging (Sampaio-Marques, Guedes, et al., 2019). Since aSyn toxicity is dependent on its expression level, we promoted the expression of aSyn under different levels due to the action of distinct promoters. For this aim, we first let the yeast cells age in the selective media to evaluate their survival rate during CLS. For this purpose, we used different WT backgrounds expressing aSyn by the power of different promoters to evaluate the difference between their survival rate during CLS. An immunoblotting assay was also done to evaluate the difference in the secretion of aSyn expressed by different promoters in proteins extracted from media at different time points of CLS.

To observe the effect of aSyn expression on the CLS of the cells, we began by characterizing the W303-1A cells expressing aSyn under the *GAL* promoter (Outeiro & Lindquist, 2003). The *GAL* system has been extensively used to achieve gene expression in *S. cerevisiae*, whose expression is strongly suppressed during the growth on glucose and strongly stimulated by the growth on galactose. Expectedly, a significant drop in chronological survival has been found in W303-1A cells expressing aSyn versus the control vector (CV) (Fig. 8). Also, as it is shown, yeast cells lost their viability 24 hours after aSyn expression while in comparison to the control vector, almost no cells lost their viability in 24 hours. This means that the expression of aSyn and its toxicity dramatically reduce CLS and induces premature aging.

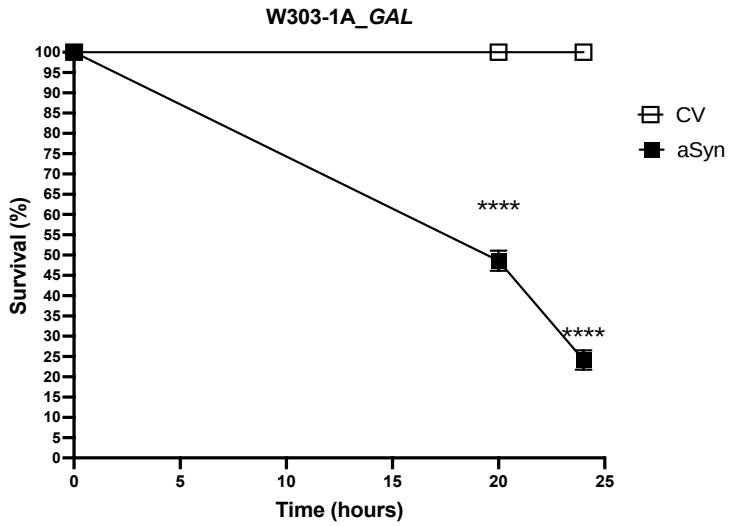

Figure 8. aSyn decreases survival of W303-1A cells during chronological lifespan. Chronological lifespan of W303 expressing WT aSyn with *GAL1* promoter in comparison with the control vector. Cell viability was measured at 2–3-day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at T0 (100%). Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (****p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM).

For evaluating the secretion of aSyn to the extracellular medium, we collected samples at 0, 20, and 24 hours of CLS, and then the protein present in the media was extracted and analyzed by western blotting against aSyn. Cell pellets were also collected, and the proteins were extracted and also analyzed by western blotting against aSyn. aSyn is strongly expressed inside the cells by galactose-induced *GAL1* promoter with the presence of galactose and the absence of glucose. As it is shown in (Fig. 9), aSyn was detected in media, suggesting that yeast cells secreted aSyn to the extracellular milieu when aSyn is expressed by the power of the *GAL1* promoter. As illustrated in (Fig. 9), immunoblot analysis revealed the presence of distinct bands. In the pellet extracts, we observed a band of 18 kDa that corresponds to the molecular weight of aSyn. Furthermore, we also detected bands with higher molecular weight, which probably corresponds to aggregated aSyn. In accordance with what was described in the literature, studies already showed, by microscope visualization, the presence of aSyn aggregates when it is expressed under the control of *GAL1* (Outeiro & Lindquist, 2003). Interestingly, in the extracts obtained from media, immunoblot analysis only showed the bands with higher molecular weight, which suggests that cells are essentially secreting the aggregated aSyn, probably as a way to get rid of the waste.

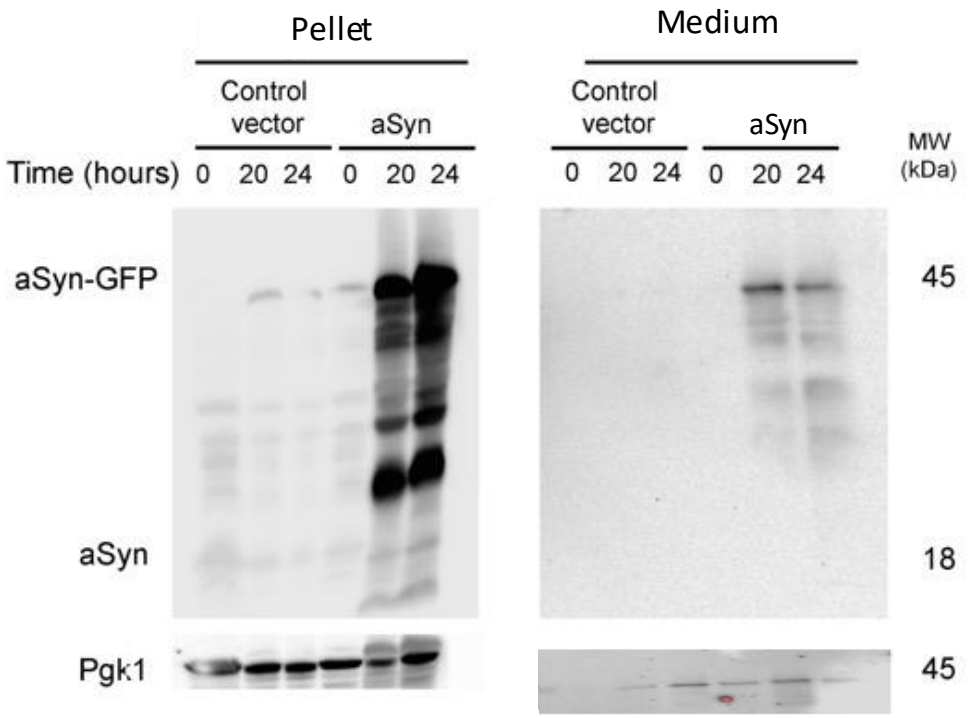

Figure 9. aSyn is secreted to the extracellular media with the aSyn expressed by the *GAL1* promoter. Western blotting analysis of the aSyn precipitated from the extracellular media of the W303-WT cells expressing WT aSyn with the Gal1 promoter and the control vector as control negative at the hours of 0, 20 and 24. At least 3 biological independent replicas were tested.

To elucidate the effect of promoter strength on the CLS and secretion of aSyn, aSyn was heterologously expressed under the *MET* promoter in BY4741 cells. As a control, the empty vector with the same plasmid containing the *MET* promoter has been used. A slight difference between the CLS of aSyn-expressing cells versus CV was observed (Fig. 10).

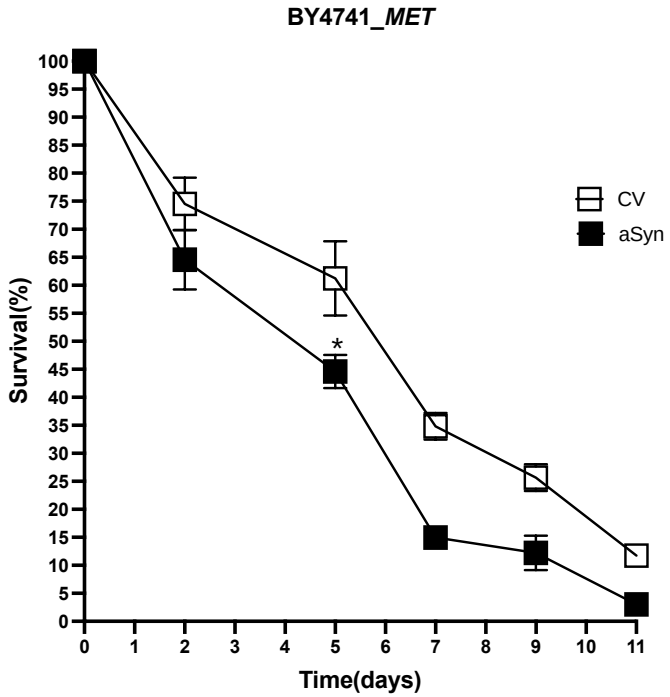

Figure 10. aSyn did not decrease the survival rate during CLS of BY4741 cells expressing aSyn under *MET* promoter. Chronological lifespan of BY4741 expressing WT aSyn in comparison with the control vector. Cell viability was measured at 2 -day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at T0 (100%). Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (*$p \leq 0.05$) between cells The error bars represent the standard error of the mean (SEM).

By the comparison of the results obtained with *GAL1* and *MET* promoters, we observed that the viability of the cells is connected to the strength of the promoter expressing aSyn (Fig. 8, Fig.10). Furthermore, the western blotting against aSyn was conducted on precipitated proteins obtained from the extracellular milieu of cells expressing aSyn and CV in order to evaluate the secretion of aSyn. The results obtained showed that aSyn is detected in the pellet fraction of BY4741 cells expressing aSyn under MET promoter, but we were not able to detect any secretion of aSyn to the extracellular milieu (Fig. 11). Based on this result, we could also hypothesize the significance of the secretion of aSyn to the extracellular medium as a way that cells have to cope with its high expression and toxicity.

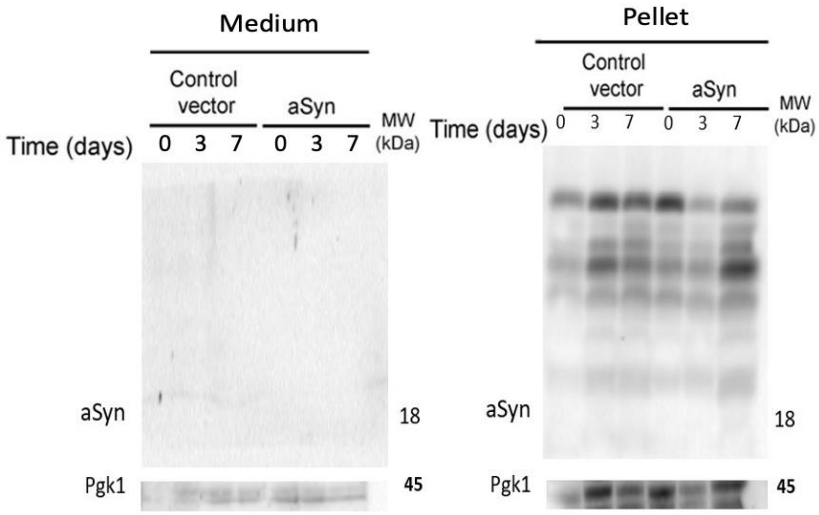

Figure 11. No aSyn secretion was detected in the extracellular media with the aSyn expressed by *MET* promoter. Western blotting analysis of the aSyn precipitated from the extracellular media of the BY4741 cells expressing WT aSyn with the *MET* promoter and the control vector as control negative at the days of 0, 3 and 7. At least three biological independent replicas were tested.

To further explore this hypothesis, we decided to use another promoter, glyceraldehyde 3-phosphate dehydrogenase *(GPD)*, to express aSyn. The *GPD* promoter is often used as a constitutive promoter for high-level gene expression, often comparable with the strength of *GAL1* (Li et al., 2008).

BY4741 yeast cells have been transformed with the aSyn-*GPD* plasmid or control vector. The lifespan of the cells expressing aSyn under the *GPD* promoter decreased overwhelmingly while cells expressing the control vector kept their viability (Fig. 12). These findings with the *GPD* promoter were almost identical to those obtained by the W303-1A-Syn-*GAL1* yeast cells (Fig. 8).

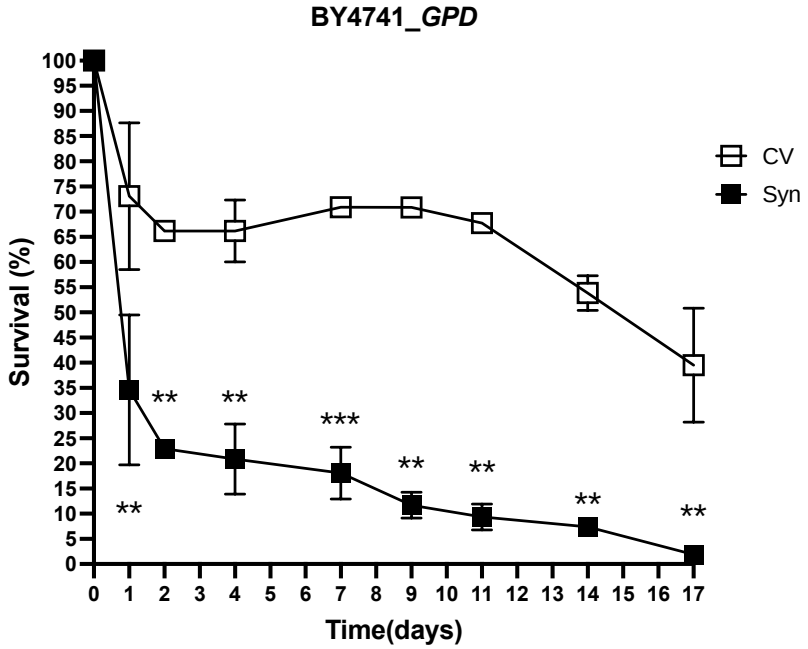

Figure 12. aSyn decreases survival during CLS of BY4741 yeast cells expressing aSyn under *GPD* promoter. Chronological lifespan of BY4741expressing WT aSyn in comparison with the control vector. Cell viability was measured at 2–3-day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at T0 (100%). Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (**p ≤0.01, ***p ≤0.001) between cells. The error bars represent the standard error of the mean (SEM).

Immunoblotting assays were carried out on aSyn to determine whether or not it was secreted under the influence of the *GPD* promoter. In this context, the medium was collected on days 0, 2, and 4 of CLS from cells expressing aSyn or the CV, and secreted proteins were extracted, and the presence of aSyn was examined. As can be seen in (Fig. 13), aSyn-expressing cells secrete the aSyn into the extracellular media during CLS. Interestingly, aSyn was detected in all the time points of a media collection. The presence of the aSyn in all of the time points may correlate with the *GPD* promoter expression level, meaning the secretion of aSyn began from day 0 of the CLS in order to discharge the accumulation of aSyn inside the cells to longer the longevity and postpone the possible cell death due to the aSyn-induced toxicity. Comparing this result with the *GAL1* promoter immunoblotting assay (Fig. 9), we observed a higher secretion of aSyn to the media by the *GPD* promoter, confirming the probable effect of aSyn expression level on the secretion of aSyn. Furthermore, as expected, aSyn was also detected in proteins isolated from pellet fraction. Notably, the higher molecular weight of aSyn may have been brought on by aSyn aggregation (Fig. 13).

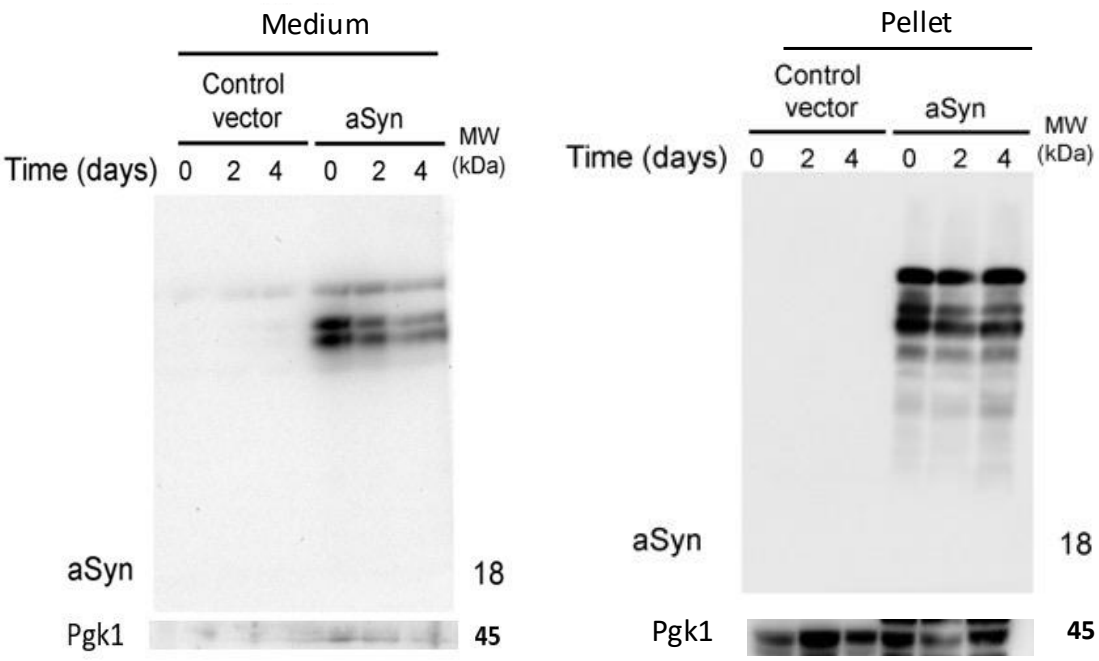

Figure 13. The presence of aSyn is detected in the extracellular medium of BY4741 cells expressing aSyn under the control of the *GPD* promoter. Western blotting analysis of the aSyn precipitated from the extracellular media of the BY4741 cells expressing WT aSyn with the *GPD* promoter and the control vector as control negative at the hours of the days 0, 2, and 4. At least three biological independent replicas were tested.

Altogether, our results showed that the level of aSyn expressed during CLS has a comprehensive correlation to the amount of aSyn that was secreted by the yeast cells. A more remarkable expression of aSyn was shown to impair cell survival. Accordingly, we deduced that the secretion of aSyn is dependent on the level of aSyn expressed during the CLS. Notably, it seems that the quantity of aSyn that is secreted into the extracellular space is proportional to the aSyn levels within the cells that are powered by the strength of the promoter. Our hypothesis suggested that the secretion may only occur if other systems are unable to cope with the proteotoxicity of aSyn, but the mechanism underlying this secretion is still not clearly understood.

3.2. Mechanisms associated with aSyn secretion during aging

We have shown that the expression levels of the aSyn correlate with the survival rate of yeast cells and with the release of aSyn to the extracellular medium during CLS. Therefore, the data above points to the travel of aSyn across cells in a yeast population. Although the mechanism behind the secretion and probable uptaken of aSyn remains poorly understood, recent research suggests that an unconventional ER-Golgi-independent mechanism may be responsible for aSyn secretion in neuron cells of the brain (Tyson et al., 2016).

Scientists have discovered that yeast cells are capable of producing vesicles (Oliveira et al., 2013), which are sent to the extracellular environment. Accumulating evidence also suggests that secretory autophagy is the first step in the cell-non-autonomous process by which these vesicles secrete low molecular weight transmissive molecules (Oliveira et al., 2010; Rodrigues et al., 2016). Hence, based on these studies, the pathways underlying the production or trafficking of these vesicles may light the mechanisms behind the section of aSyn. In this regard, the endosomal/MVB pathway may be the first candidate that needs to be analyzed. Evidence pointed to MVBs being a subset of endosomes that contain membrane-bound intraluminal vesicles. These vesicles originate from MVB lumen budding. By fusing with lysosomes, MVBs' content is destroyed; by fusing with the plasma membrane, their contents are freed into the extracellular space (Hasan et al., 2022; Oliveira et al., 2013). Vps23 and Snf7, two VPS proteins, act as a part of the ESCRT (endosomal sorting complex necessary for transport) compartment, which includes ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. Snf7 and Vps23 have been demonstrated to play unique roles in a number of activities, including MVB formation and late endosomal sorting activity (Katzmann et al., 2001; Piper & Katzmann, 2007). One of four subunits of the ESCRT-III complex; participates in the sorting of trans-membrane proteins into the MVB pathway. Activation of *SNF7* needs a substantial conformational shift to expose protein-membrane and protein-protein interfaces (Tang et al., 2015). Due to the importance of Snf7 protein in MVB's formation, we intended to evaluate the survival rate of *Δsnf7* mutant cells expressing aSyn in comparison with the cells expressing CV during CLS of the cells and to determine the secretion pattern of aSyn with these cells during CLS.

A null mutant for *SNF7* was transformed with aSyn-*TPI* plasmid to express aSyn or CV. Then we characterized cells' survival during CLS (Fig. 14-A). As already described in papers, *Δsnf7* cells presented decreased life span in comparison with the WT BY4741 cells (Marek & Korona, 2013). Regarding the lifespan of *Δsnf7*, it dropped overwhelmingly on the third day of CLS (Fig. 14-A). However, no specific differences were observed between the CLS curves of the control vector and aSyn expressing cells (Fig. 14-A), in contrast to the CLS of WTBY4741 presented in (Fig. 14-B). This data suggested that the

dysfunction of MVBs biogenesis may result in shorter longevity of the cells regardless of the expression of aSyn.

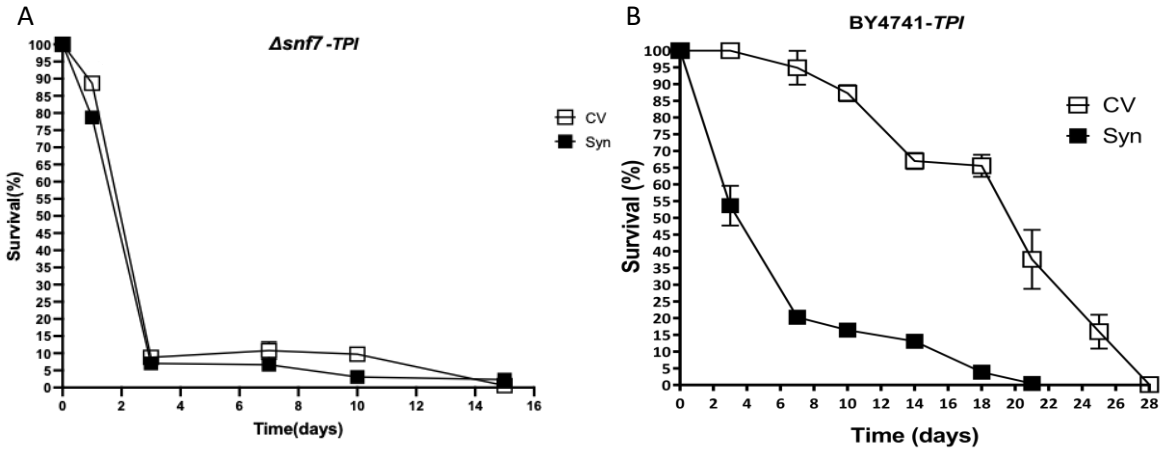

Figure 14. MVB formation dysfunction led by *SNF7* deletion decreased the lifespan of the aging cells expressing aSyn with no considerable difference with control vector (A) in contrast to the WT BY4741 expressing aSyn and CV with *TPI* promoter (B) (This same graph is used for comparison with all the mutants)**.** 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 T0 (100%). Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (*p ≤ 0,05, **p ≤ 0,01) between cells. The error bars represent the standard error of the mean (SEM).

Next, to better understand the role of Snf7 in the secretion of aSyn during CLS, we collected medium at different time points of the CLS, 0, 1, and 3 days and performed the protein precipitation. The isolated proteins were analyzed by immunoblotting assay against aSyn. As it is shown in (Fig. 15), no aSyn secretion was detected in the extracellular milieu, while the proteins isolated from the *Δsnf7* cells pellet showed the expression of aSyn. Regarding the extracts from media of the *Δsnf7* cells expressing aSyn, we were not able to detect aSyn, indicating that it is not released. These results are in accordance with the literature showing that in *Δsnf7* cells, the secretory pathway is partially inhibited (Leidal & Debnath, 2021).

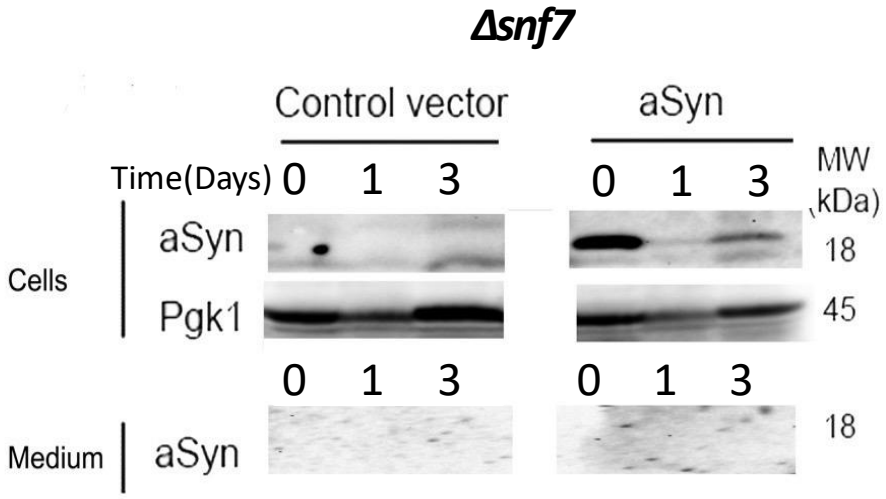

Figure 15. MVBs formation dysfunction could actively block the secretion of aSyn as no aSyn was detected in the extracellular media of *Δsnf7* cells. Western blotting analysis of the aSyn precipitated from the extracellular media of the vps23 cells expressing WT aSyn with the *TPI* promoter and the control vector as control negative at the days of 0, 1 and 3 of CLS. At least three biological independent replicas were tested.

Snf7 has a significant role in MVBs formation and exosome biogenesis, and our findings imply that it may also play a role in the secretion of aSyn, as no secretion was observed in the extracellular medium of the cells (Fig. 15). To further complement these results, we have also examined if ESCRT-based autophagy is behind the aSyn secretion during CLS. It has been postulated that *VPS23,* which is the yeast homolog of tumor susceptibility gene 101 (*TSG101*) in mammalians, functions in ubiquitin-dependent protein sorting into the endosome as a component of the ESCRT-I complex (Bishop & Woodman, 2001). Previous data also suggest that *VPS23* may be responsible for the trafficking of late endosomes (Babst et al., 2000). To further comply with the described results, we decided to use aSyn-*TPI* plasmids for the other null mutant cells. Accordingly, we obtained the null mutant of *Δvps23* and transformed it with the aSyn-*TPI* plasmid to have aSyn expressed in these mutant cells. Then we characterized the survival rate of *Δvps23* during CLS (Fig. 16-A). As it is represented, *Δvps23* cells showed longer CLS both in aSyn expressing cells and CV in comparison to the WT represented in (Fig. 16-B). These results mean that *VPS23* impairment, which leads to late endosomal trafficking dysfunction, may increase the longevity of cells during CLS. However, there is a major discrepancy between the survival rate of CV and cells expressing aSyn, despite the fact that both follow the same pattern of survival during CLS. The difference reached its peak value between days 15 and 20 when it is at its highest value.

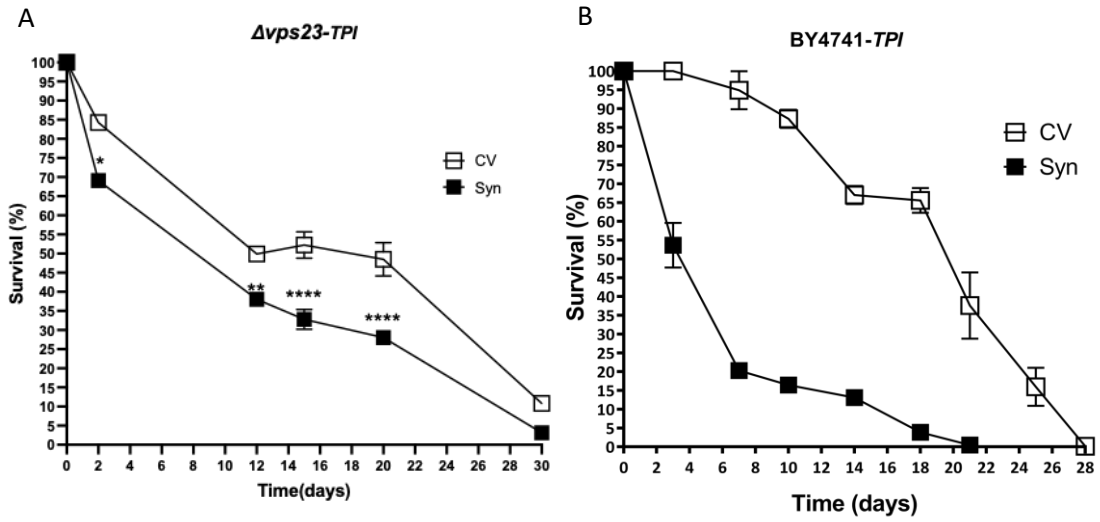

Figure 16. *Δvps23* yeast cells increased longevity of yeast cells during CLS, while aSyn expression leads to premature aging (A) in contrast to the WT BY4741 expressing aSyn and CV with *TPI* promoter (B). Cell viability was measured at 2–3-day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at T0 (100%). 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≤0.0001) between cells. The error bars represent the standard error of the mean (SEM).

To determine if the *VPS23* impairment influences the release of aSyn to the extracellular medium, we collected the conditioned media of the cells expressing aSyn on days 0, 3, and 7. Then the proteins from the extracellular medium were isolated, and an immunoblotting assay was performed against aSyn. Despite our observation of aSyn detection in cellular pellets, no secretion of aSyn was observed (Fig. 17). Notably, although aSyn secretion was not observed in the medium, we were not able to compare the results with the empty vector due to some technical problems which lead to the missing of CV immunoblotting assay (Fig 17). Based on our results, we hypothesized the ESCRT machinery might also play a crucial role in the secretion of aSyn as genetic impairment of endosomal sorting protein led to the secretion blockage of aSyn.

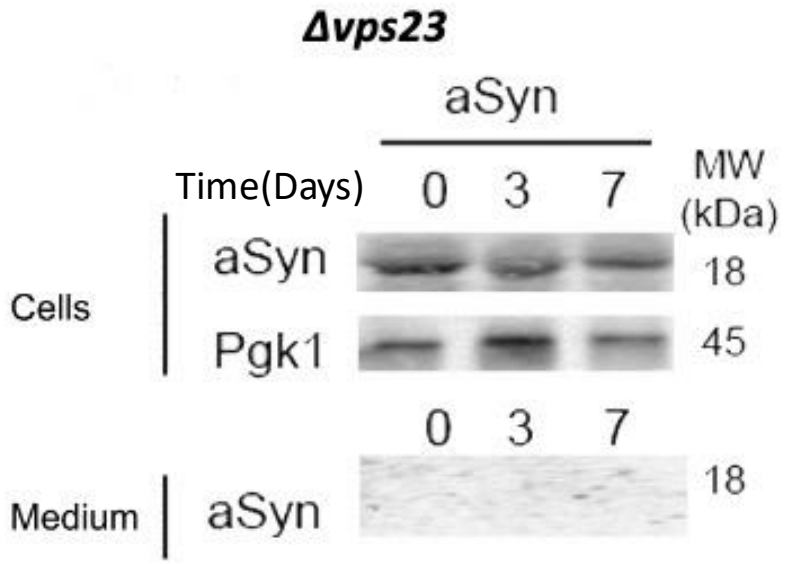

Figure 17. Late endosome sorting impairment could block the aSyn secretion during CLS of *Δvps23* cells. Western blotting analysis of the aSyn precipitated from the extracellular media of the *Δvps23* cells expressing WT aSyn with the *TPI* promoter and the control vector as control negative at the days of 0, 3 and 7 of CLS. Immunoblotting assay of CV is missed here. At least three biological independent replicas were tested.

Since the ESCRT is made up of many protein complexes, it seems that proteins from various compartments must cooperate in their efforts to control aSyn sorting, trafficking, and/or secretion. Based on the obtained results, we were capable of hypothesizing the role of MVBs formation, and sorting of the late endosomes is crucial for the secretion of aSyn as the genetic abrogation of Snf7 and Vps23 inhibits the secretion of aSyn. Though, we are still not able to note if aSyn secretion is specifically regulated by both conventional and unconventional autophagy or one of them merely.

As mentioned in the introduction, the secretion of Acb1 protein in yeast cells is an example of unconventional secretion, and it is achieved by a combination of the GRASP machinery for secretory autophagy and the specific membrane-bound compartments (CUPS) for unconventional protein secretion (Abrahamsen & Stenmark, 2010). It is noted that Grh1 is the non-essential GRASP homolog in *S. cerevisiae*. Scientists suggest Grh1's role in the organization of secretory compartments, specifically amphisomes formation, rather than its involvement at a later stage of the secretory pathway (Levi et al., 2010). Although the exact function of Grh1 is still unclear, it was shown that the secretion of Acb1 is dependent on the Grh1-mediated pathway in *S. cerevisiae* (GRASP machinery in mammalian cells), despite the fact that Acb1 secretion in yeast does not need the presence of genes involved in the endoplasmic reticulum (ER) to Golgi or Golgi to cell surface transit (Duran et al., 2010).

According to the important but unclear role of Grh1 in the organization of the secretory compartment, we intended to evaluate the effect of expressing aSyn in *Δgrh1* on CLS examination. For

this aim, we transformed the null mutant of *Δgrh1* cells with aSyn-*TPI* plasmids to express the aSyn in these cells. The control vector was transformed into the same null mutant cells. Then we characterized their survival rate during CLS. Although the immunoblotting assay has not yet been done, our data suggested a considerable increase in the longevity of these mutant cells regardless of aSyn expression (Fig. 18-A) in comparison to the WT strain of BY4741 shown in (Fig 18-B). Notably, there is also a significant drop in the CLS of the cells which express aSyn in comparison with CV (Fig. 18-A) and a drastic difference was observed between the survival rate of cells expressing aSyn and CV. Notably, on day two of CLS, the disparity between these two populations of cells widens significantly. It is noteworthy that, as *GRH1* may sort secretory compartment and amphisomes formation, genetic abrogation of this gene could significantly lengthen the lifespan of cells expressing aSyn and control vector, in comparison to the BY4741 cells shown in (Fig. 18-B). Even while aSyn-dependent toxicity may not be drastically altered, the cells lived longer as a whole as they aged chronologically. Nevertheless, the effect of the Grh1 mutant on the secretion of aSyn remained unanswered.

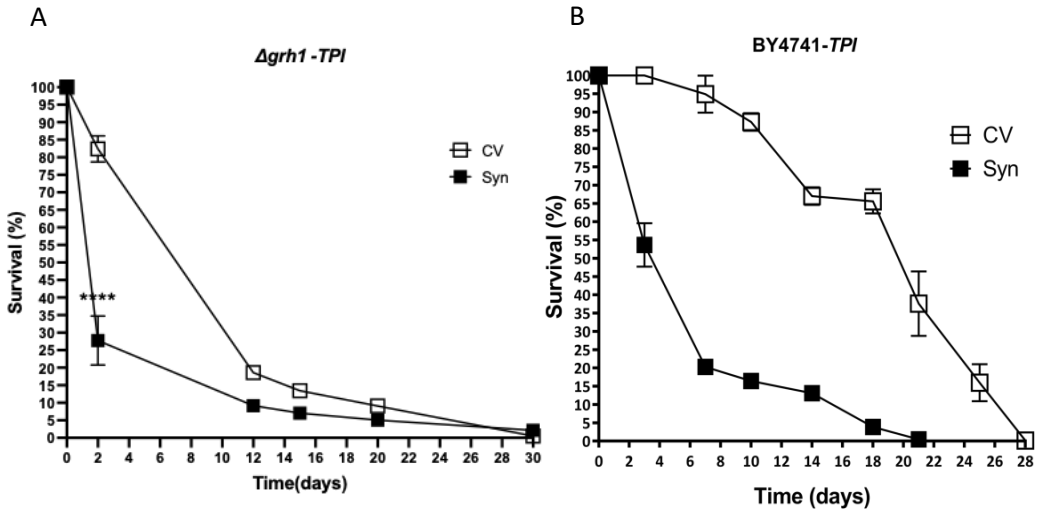

Figure 18. Impairment of secretory compartment sorting caused by *Δgrh1* increases the survival rate of both aSyn expressing cells and CV. Although aSyn expressing cells lost their viability much faster (A) in contrast to the WT BY4741 expressing aSyn and CV with *TPI* promoter (B). Cell viability was 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 T0 (100%). Data represents mean ± SEM of at least three biological independent replicas. Significance was determined by two-way ANOVA (**** p≤0.0001) between cells. The error bars represent the standard error of the mean (SEM).

According to our results, we have demonstrated that the MVBs formation and late endosome sorting impairment as a part of ESCRT machinery can block the secretion of aSyn into the extracellular milieu. The grh1-mediated pathway also seems to affect the CLS of yeast cells. It is intriguing to note that, as our results suggested that the proteins involved in aSyn secretion are also involved in

secretory autophagy pathways; therefore, the secretion of aSyn may be the result of unconventional autophagy.

As mentioned previously in the introduction, the ubiquitin-proteasome system (UPS) and autophagy have both been implicated as pathways by which aSyn influences cellular clearance and protein quality control. Mitophagy has emerged as an important new pathogenic mechanism due to recent discoveries about its central involvement in the autosomal recessive *PARK2/PARKIN* and *PINK1* genes (Chu, 2011). The function of autophagy and mitophagy in the development of PD is still up for debate. It is known that aSyn reduces mitophagy, mitochondrial fusion, and mitochondrial function, as well as autophagy efficiency (Winslow et al., 2010).

In light of this, it is essential to acquire if aSyn-induced toxicity is dependent on autophagy. *SIR2*, the yeast homolog of human *SIRT1*, selectively induces autophagy/mitophagy in aged cells, and studies have shown that this is essential for preventing aSyn toxicity (Sampaio-Marques et al., 2012). Therefore, it is crucial to examine if *Δsir2* cells expressing aSyn are capable of actively secreting the aSyn out of the cells and into the extracellular medium and whether this may influence the CLS of the cells. So, first, we initiated the evaluation of CLS by transforming *Δsir2* null mutant with aSyn-*TPI,* and after then characterized the survival rate as expected; although the lifespan of the yeast cells increased in contrast to WT (Fig. 19-B) the cells expressing aSyn showed extremely similar patterns during their CLS in comparison to the CV (Fig. 19-A). Remarkably, our results elucidated that the longevity of the cells increased as expected from (Sampaio-Marques et al., 2012) in comparison to the BY4741 with *TPI* promoter presented in (Fig. 19-B), which may confirm the toxicity tolerance of aSyn to the *Δsir2* cells.

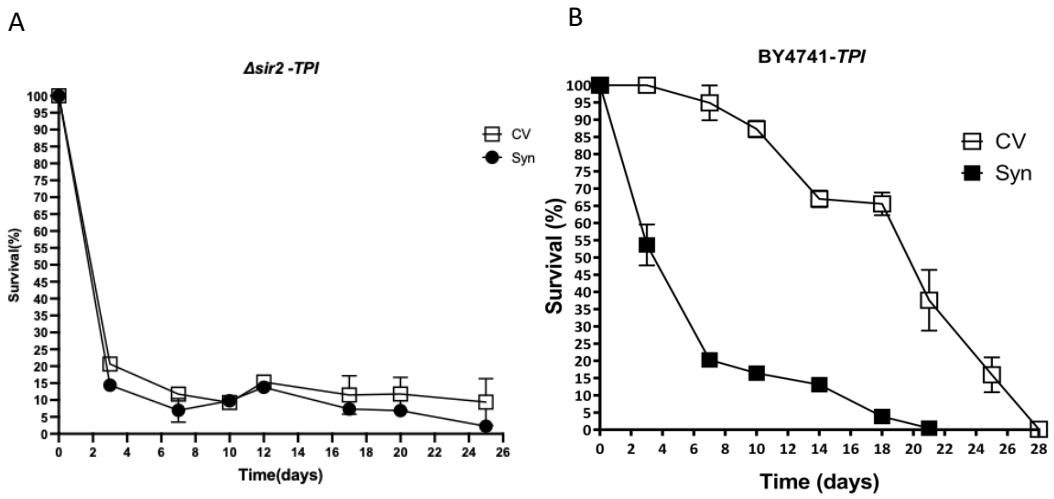

Figure 19. aSyn-induced toxicity is prevented in *Δsir2* cells (A) in contrast to the WT BY4741 expressing aSyn and CV with *TPI* promoter (B). Cell viability was measured at 2–3-day intervals beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at T0 (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 between cells. The error bars represent the standard error of the mean (SEM).

To better understand the effect of the genetic abrogation of *SIR2*, we attempted to characterize the immunoblotting assay with *Δsir2* cells. To do that, the extracellular medium was collected on days 0, 3, and 7 of the cells' CLS. Then proteins were precipitated, and the isolated proteins were analyzed by immunoblotting assay against aSyn. As shown in (Fig. 20), we could not detect the secretion of aSyn to the extracellular medium. On the other hand, our results showed the presence of aSyn in proteins isolated from the cellular pellet, meaning that although aSyn was expressed in the *Δsir2* mutant cells, they were not able to secret aSyn to the extracellular medium.

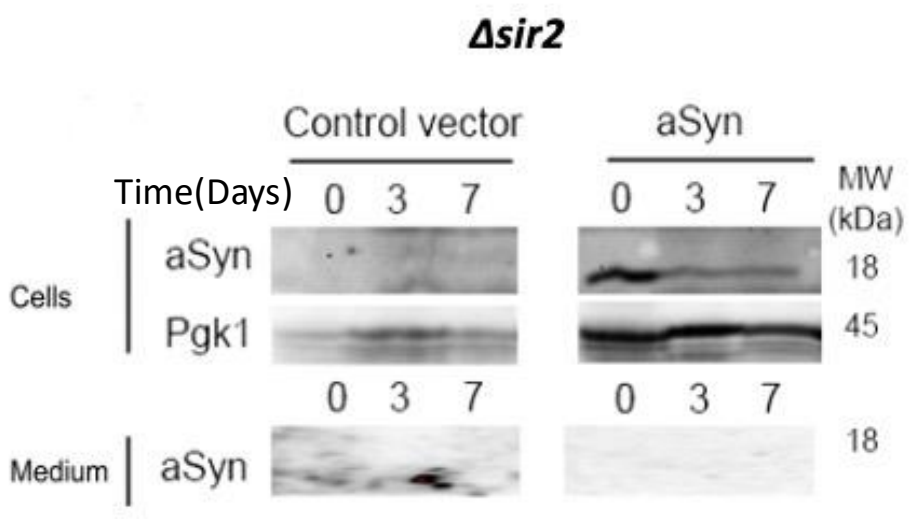

Figure 20. aSyn secretion is avoid in *Δsir2* cells expressing aSyn. Western blotting analysis of the aSyn precipitated from the extracellular media of the *Δsir2* cells expressing WT aSyn with the *TPI* promoter and the control vector as control negative at the days of 0, 3 and 7 of CLS. At least three biological independent replicas were tested.

As we could not detect aSyn in the extracellular medium of *Δsir2* cells, we can hypothesize a strong effect of Sir2 on the secretion of aSyn. Studies have previously shown that Sir2 plays a considerable role in regulating autophagy, and deficient *SIR2* can decrease autophagy (Sampaio-Marques et al., 2012). Comparing the literature with our results, we can hypothesize that decreased autophagy mechanism may alter the system clearance of the cell and therefore inhibits the secretion of aSyn to the extracellular medium of the mutant cells. According to our results of the CLS and immunoblotting assay, an alteration of the secretion system due to the tolerance of the cells to the aSyn-induced toxicity of accumulated and aggregated protein inside of the cells is suggested. Due to the variety of known roles of Sir2, from hyper-cluster formation in quiescent cells in yeast to autophagy and mitophagy regulation (Dali-Youcef et al., 2007; Sampaio-Marques et al., 2012), understanding the phenomenon behind the secretion inhibition by *Δsir2* requires further study.

3.3. Constructing split GFP plasmids for detection of synuclein secretion and uptake[1]

Endocytosis and phagocytosis remove aSyn from neuronal cell-extracellular environments (Stefanis et al., 2019). Exosomes harboring aSyn may transmit aberrant aSyn throughout neurons or be cleared by nearby cells. As we know, horizontal aSyn transmission between brain neurons may potentially contribute to Parkinson's disease with a cell-non-autonomous mechanism (Stefanis et al., 2019; Tyson et al., 2016).

Our results have indicated that yeast cells are able to secret aSyn to the extracellular medium during CLS and revealed probable important players of this secretion. Based on our results, the expression levels of aSyn are not only responsible for the presence or absence of the aSyn in the extracellular milieu but also for the regulation of longevity of aged cells.

As mentioned, tracking of aSyn through the cells and the possibility of its horizontal transmission might reveal more details regarding the underlying mechanism. In this regard, we aimed to construct split GFP plasmids with aSyn. Transgenes from the Split GFP line are created in such a way that the big GFP component (GFP1-10) will only express fluorescence in the presence of the small GFP subunit (GFP11) (Fig. 21) (Kakimoto et al., 2018). Split GFP plasmids will allow us to monitor aSyn secretion and possible endocytosis when naïve cells are in contact with aSyn medium (from hereon named conditioned medium). In this situation, the small subunit of GFP (namely GFP11) must interact with the large subunit (GFP1-10) in the cells for a fluorescent signal to be produced. Studies have revealed that the interaction with these two subunits can work vice versa, meaning the protein of interest can be tagged with the GFP1-10 subunit while it requires the interaction with GFP11 to have the fluorescent signal emerge (Kakimoto et al., 2018; Kent et al., 2008).

Figure 21. Schematic representation of split GFP plasmids interaction. Interaction between the two subunits namely GFP1-10 and GFP11 will release fluorescence signal. Adapted from (Lundqvist et al., 2019)

[1] *Part of the experiments herein described have been performed at Katholieke Universiteit Leuven/Belgium at Professor Joris Winderickx's lab during the Erasmus mobility traineeship program.*

We intended to perform the strategy of homologous recombination (gap repair) for constructing our split GFP plasmids with the fusion of aSyn gene (*SNCA*). In this strategy, the homologous sequences that are complementary to the plasmid's insert region are required. Meaning that the gene of interest (here *SNCA*) should be amplified with the primers containing homologous parts of the split GFP plasmids. Due to the homologous recombination strategy, our plasmid shall be linearized either by PCR or restriction enzyme to receive the fragment. Then, linearized plasmids, as well as amplified genes, should be transformed into *E. coli* for re-constructing the circular plasmids with the fragment inserted in the digested site. Our method ensures that the gene of interest (*SNCA*) will be placed before the split GFP subunits. A schematic view of the strategy performed to construct the split GFP fused with aSyn is shown in (Fig. 22).

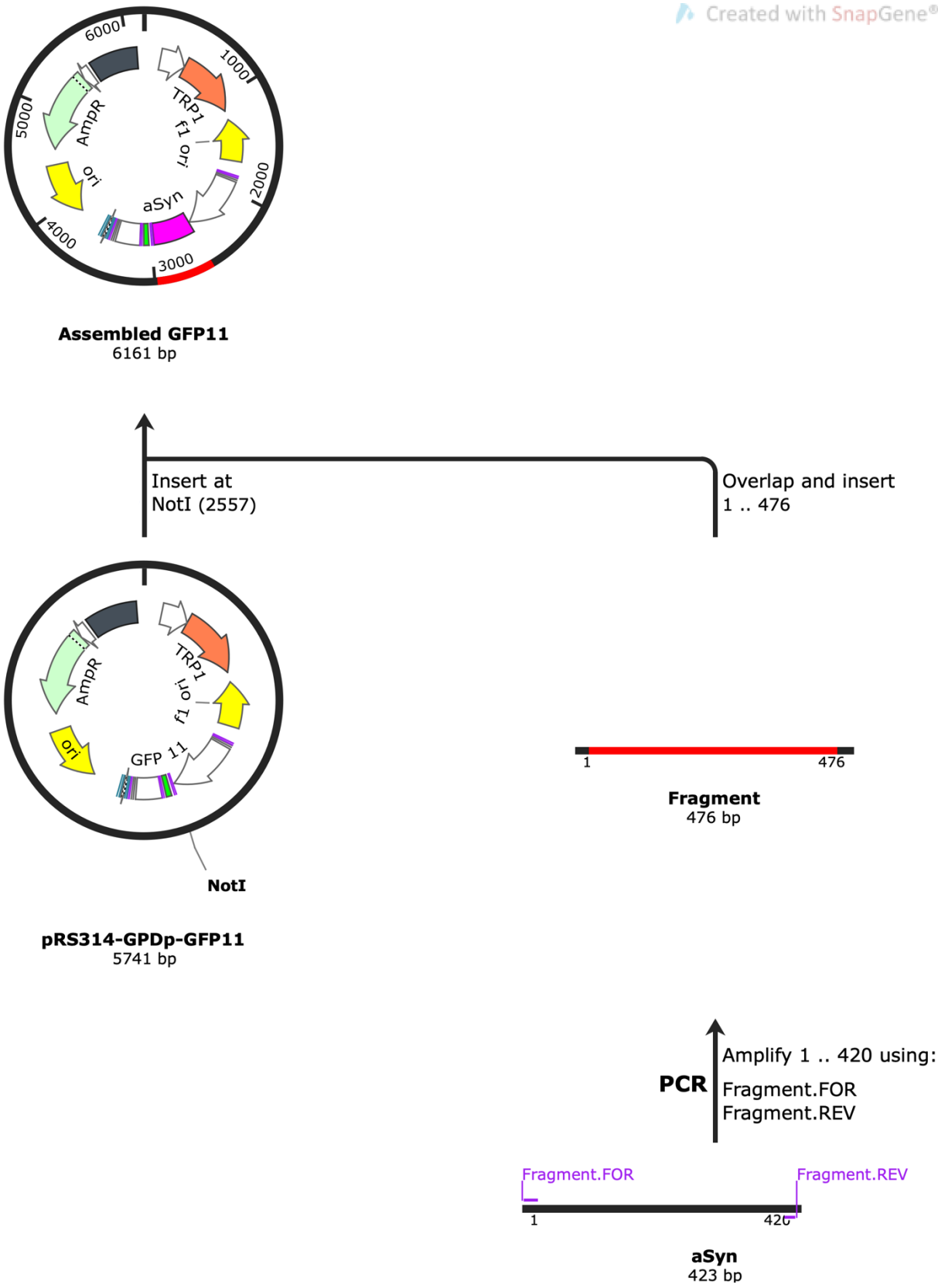

Figure 22. Schematic strategy for the construction of pRS314-GPDp-GFP11 plasmid with GFP11 subunit fused with aSyn. Based on the homologous recombination or gap repair provided by Snapgene version 6.1.1.

To have the gene that codifies aSyn tagged and fused with subunits of split GFP plasmids, we used two different plasmids: pRS316-GPDp-GFP1-10 (GFP1-10) and pRS314-GPDp-GFP11 (GFP11). In this regard, first, we amplified the *SNCA* gene from the pUC23-aSyn-WT vector by PCR with the sets of the primers (Mentioned in Chapter 2, Table 2) named NHF14161-10-11_Forward and NHR14161-10-11_Reverse. Based on these primers, the *SNCA* gene should have been amplified with a pair of homologous arms that are complementary to similar regions in both plasmids. The first step was to identify the optimal melting temperature (TM) using a PCR reaction with a gradient of temperatures from 55.0 ℃ to 65.0 ℃ in the annealing step. Data revealed that, independent of the used temperature for annealing, we were able to detect a band with 476 bp that corresponds to the length of the *SNCA* gene with homologous arms (Fig. 23). However, amplification has been done in all of the annealing temperatures, but based on (Fig. 23), an annealing temperature of 57 ℃ was chosen as smaller and thinner band corresponding to the template plasmid was observed.

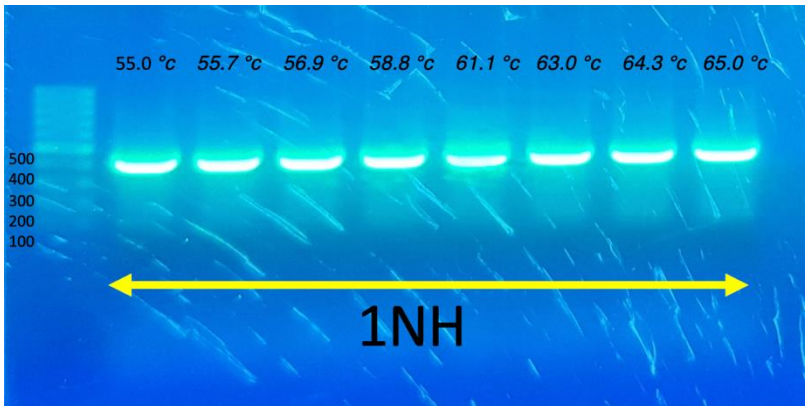

Figure 23. DNA agarose gel of the amplified aSyn with specific primers to clone *SNCA* with homologous arms (476 bp). Temperature gradient which was used is indicated on the picture. * SNCA gene amplified with homologous arms was named 1NH.

After the confirmation of the final PCR product, as the second step, due to the availability of the NotI digestion site before the subunits of GFP11 and GFP 1-10, we intended to digest out split GFP pRS316-GPDp-GFP1-10 (GFP1-10) and pRS314-GPDp-GFP11 (GFP11) by the NotI restriction enzyme. The mentioned restriction enzyme was chosen in order to linearize the plasmids before the GFP1-10 and GFP11 regions. Linearized plasmid was then checked by agarose gel electrophoresis using a 1 % TAE agarose gel. As the final step, in order to construct these plasmids by gap repair machinery of *E. coli.*, the 1NH fragment was transformed with the linearized plasmids into *E. coli* competent cells. Grown colonies were tested using colony PCR primers indicated in (Chapter 3, Table 2). As it is illustrated in Fig. 24, we could detect the corresponding bands of 1164 bp and 561 bp for the plasmids of pRS316-GPDp-

GFP1-10 and pRS314-GPDp-GFP11 fused with the *SNCA* gene, respectively. Unexpectedly, we could not re-grow the *E. coli* colony expressing *SNCA* in the pRS316-GPDp-GFP1-10 plasmid. Hence, the remaining positive colonies from the cells expressing constructed pRS314-GPDp-GFP11 fused with aSyn were undergone for plasmid extraction and then checked with other sets of colony PCR primers illustrated in (Chapter 3, Table 2). Then extracted plasmids were transformed into W303-1A auxotrophic yeast strain due to the availability of the tryptophan selective markers. In addition, the plasmids were extracted from transformed and grown yeast cells, then analyzed by colony PCR as another form of confirmation by electrophoresis, using a 1 % TAE agarose gel. As mentioned before, the complete interaction of the split GFP subunits is necessary in order to have a fluorescence signal. As a result, the empty vector plasmids were transformed into yeast with Lithium acetate/NaOH-based protocol, and then the grown colonies were isolated and analyzed by colony PCR for confirmation.

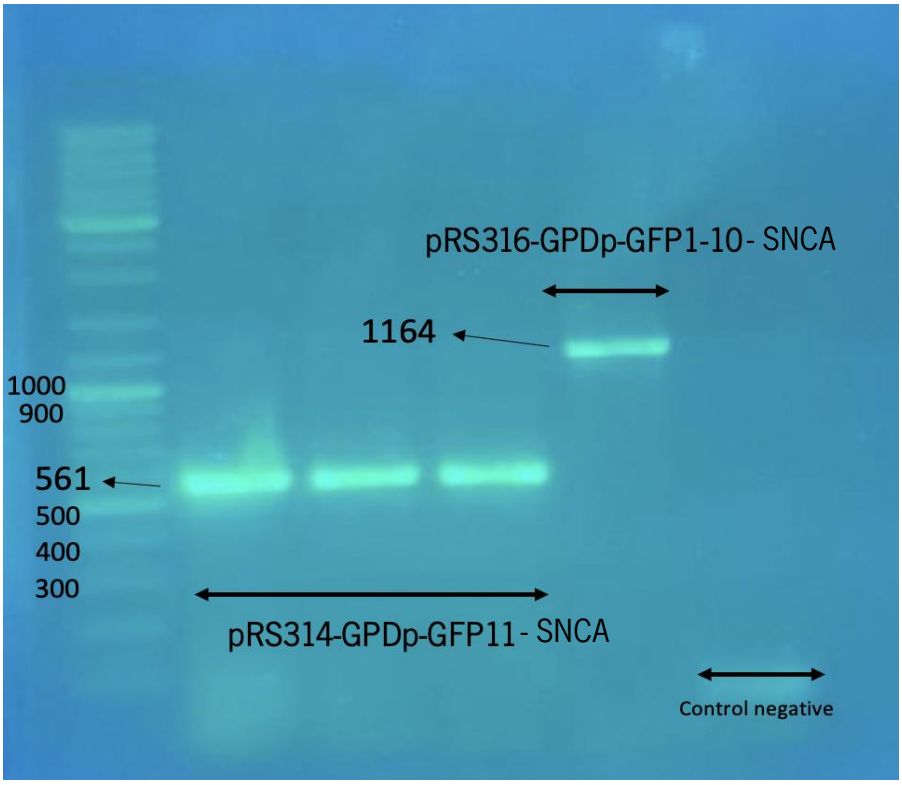

Figure 24. Colony PCR of the construction of split GFP-aSyn plasmids in *E. coli.* As shown, positive colonies with aSyn fused N-terminally with split GFP's subunits have correct band positions.

It is essential to highlight the fact that the implementation of the negative control is one of the most crucial stages in our construction progress. Studies have revealed that *E. coli* and budding yeast cells are not able to re-ligate the linearized vector without the presence of complementary regions in the amplified fragment (Jacobus & Gross, 2015). Based on that, we characterized a variety of different

negative controls. As a result, the linearized plasmid was transformed into the competent cells without the fragment, while the fragment was transformed into the competent cells without the vectors.

Plasmid construction has become a critical part of contemporary molecular biology. Plasmids are often made in the lab by using restriction enzymes to cut DNA at precise locations (restriction sites), followed by ligation to rejoin the pieces. In order to examine the structure of the produced DNA, it is often amplified in *E. coli.* The use of restriction sites limits the potential outcomes of a cloning project, whereas a second cloning approach using homologous recombination activity (commonly referred to as gap-repair cloning or GRC) provides more design freedom. In general, oligonucleotides with 5′ tails that define endonuclease recognition sites are often used in DNA cloning to amplify DNA of interest. These enable the cloning of PCR fragments into a cloning vector of one's choosing after they have been cleaved (Jacobus & Gross, 2015). Although these conventional methods of cloning are widely used, the time-consuming and tedious process of preparing reactant molecules with restriction enzymes and combining them with DNA ligase is a major drawback. Several PCR-based cloning procedures have been developed to escape these restrictions by doing away with the necessity for DNA ligase and restriction endonucleases. The PCR products used in homology-based approaches are framed on either end by sequences that are 15 to 60 bp in length and are precise complements for the beginning and end of a linear vector (Kelwick et al., 2014). Studies have revealed that both *E. coli* (Jacobus & Gross, 2015) and the budding S*. cerevisiae* are able to do gap repair (Chino et al., 2010). Based on the aforementioned facts, we intended both strategies, from restriction enzymes to homologous recombination, both in *S. cerevisiae* and *E. coli.* As a result, our several attempts for the construction with different strategies were made with failure (data not shown). However, homologous recombination has finally succeeded in *E. coli*, which was mentioned previously.

CHAPTER 4

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The accumulation of anomalies at the genomic and proteome levels is a complex and diverse aspect of aging that is impacted by hereditary, environmental, and stochastic factors (Sampaio-Marques, Burhans, et al., 2019). As a result, physiological integrity progressively declines with age, reducing functional capacity and increasing vulnerability to death. Aging may contribute to protein misfolding, which in turn contributes to protein aggregation (López-Otín et al., 2013). On the other hand, these aggregates are often considered to be the principal culprit behind neurodegenerative diseases such as Parkinson's disease (Blagosklonny, 2008). Natural proteins in the brain, such as prion, tau, beta-amyloid, and aSyn, undergo structural alterations that profoundly affect not just their functions but also their toxicity toward cells (Hansen et al., 2007). In addition, several research projects have shown that this deterioration is the primary causal agent behind the majority of the most serious diseases that may affect humans, including cancer, diabetes, cardiovascular conditions, and neurodegenerative problems (Blagosklonny, 2008).

There has been a lot of study showing that the features of aging are strikingly conserved across species (Sampaio-Marques, Burhans, et al., 2019). Hence, different model organisms have been employed to research aging since the aging system is conserved across species. (Breitenbach et al., 2012). *S. cerevisiae* is a powerful unicellular potent system for discovering constituents in longevity-regulating pathways due to the fact that many longevity-related components and processes are substantially conserved across species and the potential of investigating aging in two independent groups of Replicative aging and Chronological aging (Dilova et al., 2007; Kenyon, 2001).

Evidently, natural selection operating within an ecosystem has resulted in the evolution of both cell-autonomous and cell-non-autonomous intra-species mechanisms that govern yeast lifetime. Besides the importance of cell-autonomous mechanisms, cell non-autonomous processes, including a signaling network between numerous cells within an organism or population, have recently been discovered to contribute to modulating complicated aging biological phenomena. It has been shown that yeast cells may produce, secrete, and/or respond to such low molecular weight transmissible longevity factors when the cells are cultured (Burtner et al., 2011; Medkour et al., 2016; Mei & Brenner, 2015). It is also noted that the yeast cells are capable of secreting transmissible molecules to the extracellular milieu through the cell-non-autonomous mechanism in which these molecules can be endocytosed and taken up by the neighboring cells, and the mentioned procedure may play a vital role in regulating the lifespan of the cells (Rodrigues et al., 2016). Although the mechanism behind cells' non-autonomous activities was previously unknown, new research has demonstrated the likely importance of the leasing of extracellular vesicles, which are the lipid-bound vesicles generated by cells and released into the

extracellular environment by conventional or unconventional mechanisms (Doyle & Wang, 2019; Rodrigues et al., 2016). One of the best examples is the protein alpha-synuclein, which is ubiquitous in human neurons and whose accumulation is thought to have a role in the development of PD (Liu et al., 2016).

Here, in this thesis, we intended to demonstrate the secretion of aSyn to extracellular space over the CLS by our yeast model, *S. cerevisiae*. Our results indicated that there is a strong correlation between the expression level and secretion of aSyn to the extracellular milieu. Based on our results, we could detect the secretion of aSyn into the extracellular media during the CLS while yeast cells were heterologously expressing aSyn with *GAL1* or *GPD* promoter (Fig. 9 and Fig. 13). On the other hand, we could not detect the secretion of aSyn with the power of *MET* promoter despite the fact that aSyn was expressed weakly in the cells' pellet (Fig. 11). Comparing results obtained from heterologous expression of aSyn with *GAL1* and *GPD* promoters with *MET* promoter led us to hypothesize the strong link between the expression level powered by different promoters and the secretion of aSyn. Our hypothesis also suggests that the more powerful the promoter is, the more secretion would happen. Therefore, we could see strong secretion of aSyn from day 0 of CLS in the cells expressing aSyn with *GPD* promoter, while we could not see day 0 secretion in any other promoters. Seemingly, yeast cells were affected in a particular manner by varying levels of aSyn inside the cells, which led to more accumulation of aggregated proteins inside the cells that ended up with more secretion. We may hypothesize that this is the mechanism for cells to cope with toxicity induced by aSyn.

In addition, we showed the effect of different aSyn expression level intensities induced by different promoters during the CLS on the survival rate of the yeast cells. In this regard, we have shown that the survival rate of yeast cells expressing a higher level of aSyn with *GPD* and *GAL1* promoter (Fig 8 and Fig. 12) are dramatically shorter in comparison with results obtained with *MET* promoter (Fig. 10). Interestingly, yeast cells expressing aSyn-*MET* did not show a considerable discrepancy with the CV. We hypothesize the drastic correlation between the aSyn secretion strength and the survival rate of the cells during CLS. Seemingly, the more expression of aggregated-prone aSyn affects the survival rate of the yeast cells negatively, while the necessity of the cells' system to secrete this protein would go higher overwhelmingly. Based on this hypothesis, we might clarify the yeast cells' behavior regarding their different response to the different levels of aSyn expression while the survival rate was noticeably different from promoter to promoter.

Previous studies about protein transportation through the cells showed that most secretory proteins go along a single pathway, from the endoplasmic reticulum to the Golgi complex. The absence

of signal peptides in a number of secreted proteins has been interpreted as evidence for unconventional protein release. Although the mechanism behind the secretion of aSyn still lacks clarification, studies suggest that due to the capability of the yeast cells to secret small transmissible proteins to their external space by extracellular vesicles. Due to the prion-like behavior of the aSyn, unconventional autophagy may justify the secret behind this phenomenon (Ng & Tang, 2016; Wang et al., 2017). Therefore, *Δsnf7, Δvps23, and Δsir2* were investigated extensively to see whether they might prevent the secretion of aSyn and affect the survival rate of yeast cells as they age chronologically. We also demonstrated the effect of *Δgrh1* on the survival rate of the aSyn-expressing cells. Interestingly, the secretion of aSyn was completely blocked by the inhibition of ESCRT machinery caused by Snf7 and Vps23 deficiency. It seems that *Δsnf7* and *Δvps23* cells are deficient in the secretion of aSyn to the extracellular medium (Fig. 15 and Fig. 17). We speculated that the ESCRT mechanism and the formation of MVBs are both crucial for the secretion of aggregated proteins into the surrounding environment. Our results also illustrated the shortened lifespan of *Δsnf7* cells (Fig. 14-A) in comparison to the wild type (Fig 14-B). In contrast, *Δvps23* cells had an exceptionally higher CLS (Fig. 16-A). In spite of the fact that both groups of cells (aSyn and CV) had the same overall survival rate distribution, we were able to demonstrate a significant difference between them. We hypothesize that a deficiency of MVBs biogenesis and late endosomal trafficking can inhibit the secretion of aSyn during CLS, while the survival rate was affected differently.

On the other hand, although *Δsir2* cells were unable to secret aSyn to the medium (Fig. 20), there is not any considerable difference between the survival rate of aSyn expressing and CV cells during chronological aging (Fig. 19-A). We hypothesized that decreased autophagy system caused by generic abrogation of *SIR2* inhibits the secretion of aSyn to the extracellular medium. Considerably, deficiency in the *SNF7* gene has shortened the lifespan of the cells regardless of the expression of aSyn, while the lifespan of *SIR2-deficient* cells increased the lifespan considerably due to the tolerance of the cells to aSyn-induced toxicity and aSyn-expressing cells could survive almost as long as the empty vector. Importantly, on Grh1-deficient cells, the survival rate is drastically different when expressing aSyn compared to the empty vector. Our observation suggests that the dysfunction of the Grh1-mediated pathway caused by abrogation of *GRH1* can increase the lifespan during CLS (Fig. 18-A) in comparison to the WT illustrated in (Fig. 18-B) despite the fact that cells affected by aSyn-induced toxicity lost their viability distinctly in comparison to CV. However, the effect of the Grh1-mediated pathway on the secretion of aSyn is not yet understood.

Furthermore, for a better understanding of the secretion of aSyn in yeast cells during CLS and probable uptake of aSyn from the extracellular medium, we constructed split GFP plasmids based on GFP1-10 and GFP11 subunits of GFP, which only show fluorescence signal if they interact. Based on the prion-like behavior of aSyn in the neurons, we could design a system for checking not only the secretion of aSyn but also the endocytosis of aSyn by the neighboring cell. To achieve this goal, split GFP plasmids were constructed while the big or small compartments of the GFP, namely GFP1-10 and GFP11, were fused N-terminally with the aSyn. In this case, pRS316-GPDp-GFP1-10 and pRS314-GPDp-GFP11 plasmids were constructed in order to screen for the secretion and uptaken of the aSyn by the neighboring cells which explains the details about the cell-non-autonomous mechanism. With this system, we will be able to go further into the secretion and endocytosis mechanism of horizontal aSyn transmission.

This thesis relies mainly on information gleaned from the human aSyn expression system in yeast as a model for studying cell-non-autonomous processes that regulate longevity. Our findings highlight the significance of aSyn expression level to the cells' biological behavior and determine the probable players behind this secreting route during CLS.

4.1. Future perspectives

There is evidence in the articles published to support the hypothesis that neurons are capable of both secreting and uptaking aSyn (Gousset et al., 2009). Due to the ample examples of the conserved mechanism between humans and yeast, investigating horizontal transmission of aSyn in yeast should provide light on many hitherto unanswered questions. As a result, we designed a system that we believe is able to open a new world by revealing the mechanism behind the horizontal transmission of aSyn between the cells with more accuracy. In our work plan, shown below (Fig 25), we developed a screening approach that can be performed in the future.

Based on the system we designed, the horizontal aSyn transmission could be confirmed. This will be performed in yeast wild-type cells from distinct backgrounds. Therefore, the plasmids will be transformed in BY4741 and W303 yeast cells due to different selective markers. First, we will perform growth curve assays to check if the plasmids have any impact on cell growth. Next, we will evaluate the transformed cells with the split GFP (subunit 11 or 1-10) fused with aSyn during CLS. The main aim is to study the aSyn horizontal transmission between cells during CLS. Afterward, yeast cells transformed with empty vectors of split GFP (receptor cells) with different subunits will chronologically age in the extracellular medium from aSyn aged expressing cells. During the CLS of the receptor cells, a microscope fluorescence screening should be performed to find cells expressing GFP. The presence of GFP

fluorescence is an indication that aSyn is transmitted between cells. Only when receptor-naïve cells uptake aSyn from a conditioned medium does the appearance of a fluorescence signal indicate the interaction between two subunits of split GFP.

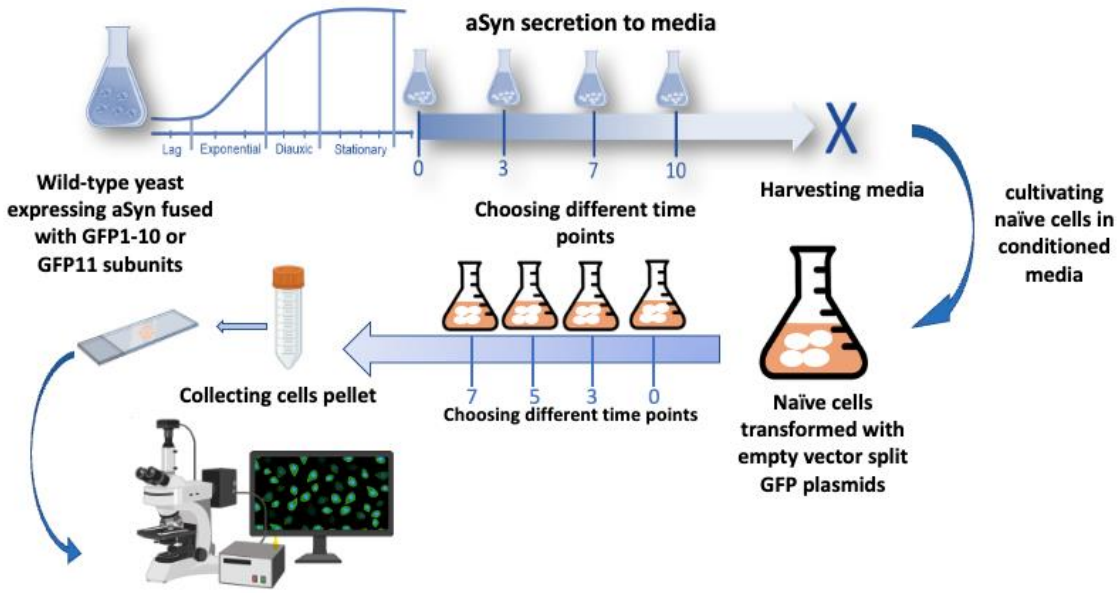

Figure 25. Schematic view of the future perspective workplan of evaluating horizontal aSyn transmission in yeast by split GFP.

Finally, this thesis, in the end, is a sincere effort to a unique and intriguing study subject that will never stop in which fresh information emerges every second throughout the globe.

CHAPTER 5

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