Cristina Martins

Amino acids homeostasis meets aging: studies in the yeast model

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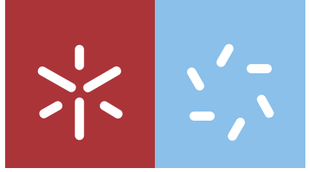

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e da
Professora Doutora Sandra Cristina Almeida Paiva

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A homeostase de aminoácidos é um modelador do envelhecimento: estudos no modelo de levedura

RESUMO

O envelhecimento é reconhecido como a uma acumulação progressiva de danos ao longo do tempo. Este processo é um dos principais fatores de risco para a maioria das doenças humanas. Atualmente, devido ao aumento do número de idosos, o conhecimento dos fenótipos que favorecem o envelhecimento saudável está entre os principais desafios da medicina e da biologia. Os aminoácidos são essenciais para a sobrevivência celular e, garantir sua homeostase é essencial para aumentar a longevidade. Estudos anteriores demonstraram que a suplementação de aminoácidos de cadeia lateral ramificada (BCAA) regula o envelhecimento, aumentado a longevidade de várias espécies. Com base nestes resultados, o objetivo principal deste trabalho foi o de estudar o impacto da suplementação com aminoácidos de cadeia lateral carregada (CSCAAs) na regulação da longevidade cronológica (CLS). Para cumprir o objetivo, estudamos o tempo a CLS e alguns mecanismos autónomos das células, como ciclo celular, acumulação de espécies reativas de oxigênio, autofagia e modulação do pH extracelular. Os dados demonstraram que a suplementação com Arginina e a MIX de aminoácidos, prolongam a CLS, retardando o processo de envelhecimento. No entanto, a suplementação com lisina resulta em envelhecimento prematuro. Os CSCAAs também afetam o ciclo celular, a acumulação de espécies reativas de oxigénio e o pH extracelular. Também estudamos as vias de sinalização envolvidas na modulação da CLS promovida pelos CSCAAs. Observamos que a quinase TOR1 não está envolvida na modulação da longevidade da levedura por esta classe de aminoácidos. No entanto, a resposta da célula à suplementação de lisina e arginina ou MIX de aminoácidos está sob o controle de Sch9 e Gcn2, mas aparentemente com efeitos distintos. Um dos principais responsáveis pela manutenção da homeostase dos aminoácidos é a família de transportadores AVT. A deleção dos genes *AVT3* ou *AVT4* anula a extensão da longevidade promovida pela suplementação com arginina ou a MIX. Isso significa que os transportadores de AVT também desempenham um papel importante na modelagem da CLS nestas condições de suplementação. Em geral, os resultados demonstram que a suplementação com arginina e a MIX promove fosforilação de Sch9 que controla a atividade de Gcn2, Avt3 e Avt4, assegurando a homeostase de aminoácidos, e o aumento da longevidade. Em contraste, a suplementação com lisina induz a ativação de Gcn2 que, consequentemente, reduz a longevidade das células.

PALAVRAS-CHAVE: Envelhecimento; aminoácidos; vida cronológica; ciclo de célula; espécies que reagem ao oxigênio

Amino acids homeostasis meets aging: studies in the yeast model

ABSTRACT

Aging is the progressive accumulation of damage over time. This process is the main risk factor for most human diseases. Currently, due to the increase in the number of elderly people, increasing knowledge of the phenotypes that favor healthy aging, is among the main challenges of medicine and biology. Amino acids are recognized as essential for cell survival, and ensuring their homeostasis is essential to increase yeast longevity. Previous studies have demonstrated that branched side-chain amino acids (BCAA) supplementation regulates aging, increasing the longevity of several species. Based on these results, the main goal of this work was to study the impact of charged side chain amino acids (CSCAAs) supplementation on lifespan regulation. To accomplish the objective, we studied the chronological lifespan and some cell-autonomous mechanisms, as cell cycle, reactive oxygen species accumulation, autophagy, and extracellular pH modulation. The data demonstrated that Arginine and amino acids MIX supplementation, significantly extend chronological lifespan, delaying the aging process. However, lysine supplementation leads to premature aging. CSCAAs supplementation also impacts on cell cycle, reactive oxygen species accumulation, and extracellular pH modulation. We also studied the signaling pathways involved in the modulation of CLS promoted by CSCAAs. We observed that TOR1 kinase is not involved in the yeast longevity modulation by this class of amino acids. However, the cell's response to lysine and arginine or amino acid MIX supplementation is under the control of Sch9 and Gcn2, but apparently with distinct effects. One of the main responsible for maintaining amino acids homeostasis is the AVT family of transporters. Deletion of *AVT3* or *AVT4* genes abrogates the longevity extension promoted by arginine or amino acids MIX supplementation. This means that AVT transporters probably also play an important role in CLS modeling under these CSCAAs supplementation conditions. Overall, the results demonstrate that Arginine and amino acids MIX supplementation leads to phosphorylation of Sch9 that controls the activity of Gcn2, Avt3, and Avt4 promoting amino acid homeostasis and longevity. In contrast, Lysine supplementation induces the activation of Gcn2 that consequently reduces cells longevity.

KEYWORDS: Aging; amino acids; chronological lifespan; cell cycle; reactive oxygen species

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LIST OF ABBREVIATIONS AND ACRONYMS

AMP	5' AMP-activated protein kinase
ALP	Autophagy-lysosome
ATF4	Activating transcription factor 4
AVT	Vacuolar amino acid transport
BCAAs	Branched side-chain amino acids
BSA	Bovine serum albumin
CR	Caloric restriction
CFUs	Colony-forming units
CMA	Chaperone-mediated autophagy
CLS	Chronological lifespan
CSCAAs	Charged side-chain amino acids
Cvt	Cytoplasm-to-vacuole targeting
DHE	Dihydroethidium
DHR	Dihydrorhodamine
DMSO	Dimethyl sulfoxide
EGOC	EGO complex
ERCs	rDNA circles
ESR	Environmental stress response
FeS	Iron-sulfur
FOXO	Forkhead box class O
FRTA	Free radical theory of aging
GR	Growth Hormone
HisRS	Histidyl tRNA synthetase domain
IGF-1	Insulin-like growth factor 1
ISR	Integrated Stress Response
LeuRS	Leucyl-tRNA synthase
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mtNOS	NO mitochondrial synthase
OXPHOS	oxidative phosphorylation system

PBS	Phosphate-buffered saline
P-eIF2α	Phosphorylation of the α-subunit of eIF2
PKA	Protein Kinase A
PKR	Protein kinase double-stranded RNA-dependent
PHS	Phytosphingosine
RLS	Replicative lifespan
ROS	Reactive oxygen species
RNC	Raptor-like N-terminal-Conserved
RNR	Ribonucleotide reductase
SEAC	Seh1-associated complex
SEACAT	SEAC activating TORC1
SEACIT	SEAC inhibiting TORC1
SDS-PAGE	Sodium dodecyl sulphate–Polyacrylamide gel electrophoresis
SOD	Superoxide dismutases
Sir2	Sirtuin 2
TBS	Tris-buffered saline
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
tRNAs	Transfer RNAs
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome systems
V-ATPase	Vacuolar ATPase
YNB	Yeast Nitrogen Base

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CHAPTER 1

INTRODUCTION

1.1.Aging

Throughout history, aging phenomenon has always been the subject of interest and curiosity of scientists and philosophers. The first attempts to unravel this phenomenon arose in the religious milieu, since aging affected humans but not the gods, they thought that this decay process could be avoided by attaining immortality.[1] However, it was only in 1939 that research in the aging field was revolutionized. It was found that caloric restriction (CR) increases life expectancy in rats, in addition to reducing the development of age-related diseases, later the study was repeated with comparable results in several organism aging models, demonstrating the phylogenetic conservation of longevity pathways.[2]

Initially, gerontologists sought to develop a single theory to explain the aging phenomenon, such as a single gene or the decline of the immune system. However, they found that several interacting processes are in the basis of aging, which is a complex and multifactorial process.[3] In this sense, in recent decades, several genetic studies have been performed, and several theories have been developed, but none has been fully accepted by the scientific community.[2,4,5,6] In 1968, Bjorksten developed one of the main theories, which relates aging to free radical-induced damage, after which many other researchers developed theories about aging as changes in immune functions, telomere shortening and the presence of senescence genes in DNA . More recently, Rattan has developed a theory that encompasses genes, performance of genetic maintenance and repair systems, highlighting the need for a systematic and integrative analysis of the aging process.[3]

Nowadays, we define aging as a complex and multifactorial physiological phenomenon. It can be described as a progressive accumulation of damage over time, which reduces the body's capacity to maintain homeostasis. This process is also responsible for the increased susceptibility to age-related diseases such as cancer, cardiovascular and neurodegenerative diseases.[2,3,7,8,9,10] Longevity and risk for some diseases differ both between individuals of different species and the same species, with differences that can rate from days to decades, so biologists have long known that longevity has also a genetic basis.[2,4,7,11,12] Despite advances in understanding the molecular bases of aging, we still know little about the causal mechanisms, both molecular and environmental, involved in the aging process, as well as underlying diseases associated with age and longevity.[12,13]

Currently, due to the increase in the number of elderly people, increasing knowledge about the fundamentals of human longevity, as well as the knowledge of the phenotypes that favor healthy aging, are among the main challenges of medicine and biology.[2,10,14,15,16]

1.2 Aging hallmarks

Aging is a common process for all cells and tissues and can be caused by the combination of several intracellular and extracellular factors.[17] Although there are hundreds of theories explored and developed, our understanding of the aging process remains limited.[18,19] Despite this, in 2013, López-Otin and colleagues categorized, for the first time, the main cellular hallmarks of aging.[9,16,19] These hallmarks are intertwined and contribute to the aging process and together determine the aging phenotype.[9] Ideally, each hallmark should follow three criteria, it should manifest itself during normal aging, its experimental decline should accelerate aging, and its experimental improvement should delay the normal aging process and therefore increase healthy life expectancy.[9,16,18]

The defined nine hallmarks (Fig. 1) are divided in three main categories: primary, antagonistic and integrative hallmarks.[9] The primary hallmarks, which includes DNA damage, epigenetic drift, and defective proteostasis, are all unequivocally negative events that cause damage that accumulates progressively with time. The antagonistic hallmarks, senescence, ROS production and nutrient sensing, have opposite effects depending on their intensity. At low levels, they mediate beneficial effects, but at high levels, they become deleterious. The progressive changed effects of these antagonistic hallmarks, from positive to negative function, are in part promoted or accelerated by the primary hallmarks. Finally, the integrative characteristics that affect homeostasis and tissue function are stem cell exhaustion and altered intercellular communication.[9,18,20,21]

The definition of aging hallmarks contributed to the study of the molecular mechanisms of aging, as well as to design interventions to improve human health. However, there are still numerous challenges regarding the understanding of this complex biological process.[9]

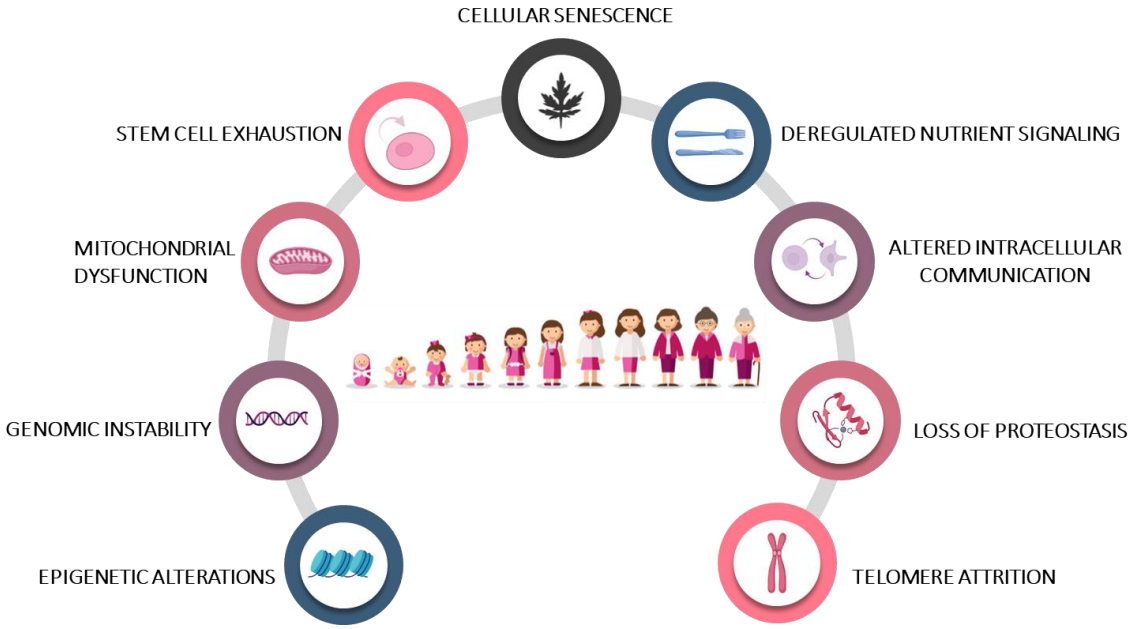

Fig. 1 –Graphical representation of the nine hallmarks of aging (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and, altered intercellular communication) described by López-Otín and colleagues in 2013.

Genomic instability, one of the first hallmarks, refers to the accumulation of genetic damage that occurs in the cell over time. This hallmark is an important facator in the aging process in all eukaryotic organisms.[22,23] DNA is constantly exposed to many extrinsic (chemicals, radiation) and intrinsic (ROS, replication errors, spontaneous hydrolytic reactions) agents that damage it. DNA damage consists in deviations from the normal chemical structure of DNA and changes in information content.[24] Every day, cells are subjected to thousands of lesions, like backbone breaks (single and double stranded), base deletions, and DNA modifications. If DNA damage is not properly repaired, these lesions can result in permanent alterations, including mutations (deletions, additions, and substitutions) and chromosomal rearrangements. Furthermore, DNA damage is reversible since it can be restored by the DNA repair systems, while mutations are not discovered by this system and thus become permanent changes in the genome.[25]

DNA damage affects the genome at random, but some chromosomal regions are more susceptible to modification. This is the case of the terminal sections of chromosomes where the telomeres are located. During aging, as most somatic cells lack the enzyme telomerase, which is the only DNA polymerase that allows the complete replication of the terminal ends of linear DNA molecules, progressive loss of

telomeres occurs in many organisms.[23,26] Therefore, several studies verified an age dependent accumulation of mutations in organisms ranging from yeast to humans.[24]

DNA replication stress play also a role in the modulation of chronological aging. It was described a strong correlation between longevity extension and reduction in the frequency by which cells fail to arrest in G0/G1 in stationary phase, under a variety of experimental conditions. Replication stress can be influenced by several factors, including the glucose concentration. In yeast, it was demonstrated that the frequency of budded cell death is augmented in medium containing elevated concentrations of glucose.[27] In addition, it was also observed that the loss of reproductive capacity observed in high glucose medium is further accelerated in the *mec1-21* strain which does not encode a protein (*mec1*) required for cellular responses to both replication stress and DNA damage. Furthermore, additional evidence that replication stress modulates aging is provided by the observation that ribonucleotide reductase (RNR) activity is limiting in wild-type cells during transitions to stationary phase, even in the absence of exposure to hydroxyurea. This is indicated by suppression of apoptosis in stationary phase cells ectopically expressing the RNR subunit Rnr1p.[28]

Another hallmark of aging is related with epigenetic alterations. This hallmark corresponds to changes on patterns of DNA methylation, post-translational modification of histones and chromatin remodelling, which modify genetic expression without changing the DNA sequence.[29] The basis of this mechanism involves the methylation of cytosine in C-G nucleotide pairs, histone modifications and non-coding RNAs, which will influence how accessible a certain genome locus is to be transcribed.[29] Sirtuins, a family histone deacetylases, are the main players involved in transcription regulation. Mounting evidence suggests a role for these proteins in aging progression. In yeast, the model organism where sirtuin 2 (Sir2) was first described, overexpression of Sir2 is implicated in a lifespan extension in a mitotic (replicative) lifespan[9,30,31,32], whereas in a post-mitotic (chronological) lifespan, Sir2 is implicated in aggravated protein toxicity.[31] Contrasting to DNA mutations, epigenetic changes are theoretically reversible, suggesting that manipulation of the epigenome can be used to prevent age-related pathologies and prolong a healthy life.[9,33]

Aging and age-related diseases are associated with loss of protein homeostasis (proteostasis), other aging hallmark.[9] Proteins are responsible for the central cellular processes and the maintenance of the proteome, protein homeostasis or proteostasis integrity crucial for cell function.[34,35,36,37] The presence of damaged proteins and protein aggregates suggests that proteostasis is compromised.[9,36,38] In fact, numerous aging-related neurodegenerative pathologies such as Alzheimer's diseases, Huntington's

disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis are associated with the aggregation of certain proteins.[36,37] To struggle with this proteotoxicity, eukaryotic cells activate the proteostasis network, in an attempt to maintain protein folding at homeostatic levels. This network comprises 1) the chaperone-mediated protein folding, mediated by several chaperones as those from the heat-shock family of proteins; 2) the unfolded protein response (UPR), which is activated upon ER stress; and 3) the proteolytic systems, including ubiquitin-proteasome systems (UPS) and autophagy-lysosome (ALP) (vacuoles in yeast). Furthermore, the proteostasis network have also a spatial protein quality control that limits the toxicity of damaged and misfolded/aggregated proteins and assurances that the damage is not inherited to other generations.[39]

During aging, it is observed an increment of increased mutations, accumulation of reactive oxygen species (ROS) and changing in lysosomal/vacuolar pH, which could consequently affect protein stability and lead to the formation of aggregates.[24,40,41] Furthermore, it is also established that the proteolytic systems capacity decline with age,[42,43] which culminate in the accumulation of protein aggregates and also in the consequent disruption of the proteostasis network.[44]

The proteolytic systems have an important role in the prevention of proteotoxicity resultant from a variety of stresses. As above described, the proteolytic systems include the UPS and ALP. UPS is the main system responsible for the ATP-dependent degradation of short-lived, misfolded and truncated proteins. It requires the coordinate action of a large number of proteins, which are involved in the recognition and conjugation of multiple ubiquitin molecules to the substrate, to produce the poly-ubiquitin chains, which will be further degraded by the proteolytic machinery, namely by the 26 proteasome.[45]

Evidence proposes that UPS can interact with the other major proteolytic system, ALP, nevertheless, the exact mechanisms of crosstalk is still undisclosed.[46] Recent findings suggest that there is an overlap of target proteins between both proteolytic systems, as ubiquitin-tagged proteins can also be degraded in the cytosol by ALP. Furthermore, it is also described that proteasomal inhibition with various compounds (bortezomib, MG132, lactacystin, etc.) or by genetic abrogation modulate ALP.[46,47,48]

The ALP, pathway is the other proteolytic system responsible for the proteostasis network, namely by the degradation of cargos such as long-lived proteins and whole organelles. Autophagy is considered a membrane trafficking process that translocate its cargo to the lysosome/vacuole for degradation. In higher eukaryotes, three different mechanisms are described: microautophagy, which is responsible for the direct invagination of the lysosome/vacuole membrane to envelope small portions of cytoplasm[49]; macroautophagy, herein called autophagy, which involves the formation of a double membrane vesicle -

the autophagosome - that later fuse with the lysosome or vacuole (in yeast) where degradation occurs; and the Chaperone-mediated autophagy (CMA) that degrades soluble intracellular proteins by direct engulfment by lysosomes. This particular type of autophagy (CMA) does not exist in yeast. In the yeast *Saccharomyces cerevisiae*, autophagy occurs mainly by two mechanisms: macroautophagy and microautophagy. Other pathways in yeast include cytoplasm-to-vacuole targeting (Cvt)[50] and secretory autophagy.[51]

Autophagy is an important mechanism involved in the maintenance of protein homeostasis, particularly in post-mitotic cells, that do not benefit from the dilutions of cellular damage that comes with cell division.[52] It was also described to be required for G1/G0 cell cycle arrest and quiescence in yeast cells.[53] However, autophagic activity also decreases during aging, as demonstrated in several model organisms such as flies, worms, yeast and mammals.[52,54] This loss of proteostasis could also be aggravated by aging-associated diseases linked to proteotoxic stress promoted by protein misfolding and aggregation.[52,55] On the other hand, overexpression of autophagy related genes and pharmacological or environmental factors, such as caloric restriction (CR), that enhance autophagy resulted in protein homeostasis and consequent lifespan extension.[48,52,56,57] Nevertheless, it is also described that late-life exacerbate levels of autophagy could result in deleterious effects to cells, leading to premature aging. Indeed, studies on yeast point towards shortening of lifespan and increased cell death associated with sustained activation of autophagy at later stages of chronological lifespan (CLS).[48,57] Together, this data suggests that autophagy activity should be tightly balanced to extend lifespan. Overall, data above described showed that loss of proteostasis is associated with aging. There is mounting evidence across several models that inducing proteotoxicity with the expression of proteins associated with age-related pathologies promote premature aging, while genetic and non-genetic interventions that enhances proteostasis improve lifespan.[9,55,58]

The last two hallmarks, deregulated nutrient sensing, and mitochondrial dysfunction, are the most important for the development of this project and will be detailed in the following sections.

1.2.1. Mitochondrial dysfunction

Mitochondria are important cellular organelles, considered essential for the eukaryotic cell's life.[59,60] This organelle is mainly known for its biochemical functions, such as pyruvate oxidation, citric acid cycle, electron transport, oxidative phosphorylation, and ATP generation, being considered the largest

generator of energy in the cell.[61,62] Mitochondria are also responsible for lipid and amino acid biosynthesis, cytosolic calcium buffering, iron-sulfur (FeS) complex formation, and some stages of heme biosynthesis, and the urea cycle.[59,61,62] This organelle is also integrated into central metabolic pathways, such as autophagy, regulated cell death, steroid synthesis, and hormonal signaling, regulating communication between cells and tissues.[59,60,62,63]

Mitochondria have their own genome, which is a small circular DNA molecule (mtDNA), replicated independently of the host genome. [59,60] Its replication is not related to the cell cycle, which can be performed numerous times, causing several mutations.[59] As mtDNA encodes proteins related to the oxidative phosphorylation system (OXPHOS), mutations in mtDNA mostly lead to OXPHOS dysfunction.[59,60,64,65]

Mitochondria are responsible for generating ATP for cells through the functioning of eletron respiratory chain and OXPHOS. A byproduct of electron respiratory chain is reactive oxygen species (ROS).[60,65] ROS consists of radical and non-radical oxygen species and is a natural subproduct of normal oxygen metabolism.[45,67,68] The nitric oxide synthase reaction is a major mitochondrial sources of ROS.[66] Together with electron respiratory chain ROS derived from mtROS include singlet oxygen (O_2), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radical ($OH^\cdot$) and, hydroxyl ion (OH^-).[59] Initially, oxygen is reduced to O_2^-, which is the precursor to other ROS. The superoxide anion can be converted to H_2O_2 by the anti-free radical enzyme superoxide dismutases (SOD). In turn, H_2O_2 can be converted to OH or water.[59,69,70,71] Particularly in yeast, the production of $NO^\cdot$ by mitochondria remains an open question, but it is speculated that it may be produced by NO mitochondrial synthase (mtNOS).[59]

ROS regulates several physiological functions, such as signal transduction, expression, and gene proliferation.[68,72] However, high levels of ROS overload the cellular antioxidant defense systems causing oxidative stress, which can occur by increasing ROS levels or decreasing the cellular antioxidant capacity.[45,67,68] Oxidative stress can damage cellular macromolecules such as DNA and proteins, playing an important role in several chronic diseases related to aging.[64,66] Some diseases related to oxidative stress are carcinogenesis, neurodegeneration, atherosclerosis, and diabetes.[67] Several pieces of evidence, also suggest that mitochondrial ROS may influence homeostatic signaling pathways, controlling cell proliferation and differentiation, and contributing to adaptive stress signaling pathways such as hypoxia.[63]

ROS can act in different levels. For example, $OH^\cdot$ is very reactive and removes electrons from other molecules, turning them into new radicals, making the ROS formation a chain reaction. The H_2O_2 is less reactive, which permits their diffusion in the cytoplasm and may eventually come to the nucleus and

interact with DNA damaging it.[69] ROS have the property of being or generating free radicals. In 1956, Harman explained the impact of aging on biological systems, established a link between the ROS production and the aging process and created the free radical theory of aging (FRTA). According to him, aging is a response to the accumulation of damage caused by free radicals, produced by mitochondria as byproducts of normal metabolism, being responsible for the functional decline characteristic of aging.[59,71,73,74,75,76]

In response to oxidative stress, organisms developed antioxidant defenses and repair systems. These defenses include the enzymes of SOD, catalase, and glutathione system that detoxify ROS and reactive nitrogen species. Moreover, non-enzymatic mechanisms can protect cells and eliminate free radicals, such as ascorbic acid, α-tocopherol, and other substances, such as cysteine, uric acid, and glutamine. However, it is hypothesized that the efficiency of protective systems decreases with age, which is also a potential contributor to the aging process.[77]

Through the analysis of the free radical theory of aging, we can conclude that the decrease in the production of ROS is accompanied by decreased oxidative damage and increased CLS.[70,71] However, recent studies in model organisms have called into question the FRTA veracity,[71,78] and a new theory evolved. Some studies showed that CR and other experimental manipulations enhanced longevity through the induction of hormesis effects in association with increased ROS.[78,79] Furthermore, through other studies, it has been observed that increased antioxidant defenses have contradictory effects in invertebrate aging models.[71]

1.2.2. Deregulated nutrient sensing

Metabolic signaling pathways coordinate environmental factors such as nutrient availability and cell status to ensure metabolic homeostasis.[80] More than 80 years ago, McCay *et al* demonstrated, for the first time, the relevance of nutrients in the aging process.[16] Cell signaling pathways detect nutrient's availability as well as the energy state of cells and, in conjunction with hormones and growth factors regulate metabolic homeostasis throughout the body.[80]

Aging results in the gradual deterioration of metabolic regulation, including the sensors and molecular targets of nutrients involved in homeostatic regulation.[17,81] However, there is a bidirectional relationship between aging and metabolic regulation. While aging affects the functioning of the main metabolic signaling pathways, metabolic dysregulation accelerates the aging process.[80]

CR is one of the best-characterized strategies that favors the life expectancy extension of several species, reducing the accumulation of ROS.[14,65,80,82] CR is characterized by the reduction of calorie intake

throughout life without causing malnutrition.[14,21] It is well established that organisms subjected to CR live longer, a phenomenon that is sustained by the delayed onset of age-related deterioration and pathologies. Indeed, CR has been associated with a several health benefits, such as reduced risk of diseases, including cancers, neurodegenerative disorders, autoimmune diseases, cardiovascular complications, and diabetes.[58,83] These beneficial effects are associated with increased stress resistance, enhanced proteostasis, and reduced inflammation.[80] Although not fully understood, CR is responsible for repressing Growth Hormone(GH) /Insulin-like growth factor 1 (IGF-1), insulin/hosphoinositide 3-kinases (PI3K), and mammalian target of rapamycin (mTOR) pathways and activating the 5' AMP-activated protein kinase (AMPK) pathway.[80,84] Under CR conditions, plasma glucose levels decrease, decreasing insulin release. As insulin levels decrease, IGF-1 will also decrease, preventing its binding to the receptor, so IGF-1 remains unphosphorylated and is unable to activate PI3K. AKT is not phosphorylated by PI3K, so mTOR remains inactive. The inactivation of mTOR causes longevity extension and forkhead box class O (FOXO) is activated. FOXO is a subfamily of transcription factors involved in main longevity pathways, such as stress response, cell proliferation, apoptosis, and autophagy. [84] Decreased glucose availability increases the AMP/ATP ratio, and consequently activates AMPK,[17,84] which has several beneficial metabolic effects, promotes glucose uptake, and fatty acid oxidation.[80] AMPK activation is also responsible for mTOR inactivation and increases the NAD+/NADH ratio.[17,84] In association with NAD^+, high levels of AMP activate sirtuins, responsible for the insulin signaling pathway and regulation of longevity.[17,85]

Sirtuins, which are a family of NAD+-dependent deacetylases,[23,85,86] have been also reported as downstream targets of CR in several organisms supporting a role for Sirtuins as mediators of longevity.[82,87,88] Sirtuins play a role in chromatin formation and are also involved in several other cellular functions, including maintaining genome stability and promotion of asymmetric inheritance of oxidatively damaged proteins during cytokinesis.[89]

1.3. Aging models

As life expectancy increases, it becomes increasingly important to understand the mechanisms involved in aging. During the last decades, several model organisms have been used to understand this phenomenon as mice, *Caenorhabditis elegans*, *Drosophila melanogaster* and the yeast *S. cerevisiae*.[90] The short lifespan of these organisms is a key advantage, as well as reduced cost and simpler ethical

concerns than human models. Several studies have also revealed that several pathways associated with aging are conserved among these species.[13]

1.3.1. Aging yeast models

The budding yeast *S. cerevisiae* reflects a prototype of a eukaryotic cell. Like all model organisms, yeast is not a perfect model, nevertheless, the yeast *S. cerevisiae* has been widely used as a cellular aging model. Yeast cells exhibit some essential characteristics to be used as a good model organism in aging studies, such as the short generation time and the cheap manutention, the availability of a collection of precise deletion mutants of every gene in the genome, the existence of databases of yeast protein–protein interactions and gene expression patterns.[10,91,92] Additionally, the yeast *S. cerevisiae* genome was the first eukaryote genome sequenced in 1996. Afterwards, several studies using this microorganism allowed a better understanding of the signaling pathways, the identification of molecular actors of aging, and the screening of new potential interventions anti-aging.[93,94] Several relevant aging pathways are conserved in yeast, such as nutrient signaling, cell cycle regulation, DNA repair mechanisms, mitochondrial homeostasis, stress response, and regulated cell death. Several studies have shown that about half of the essential yeast proteins can be replaced by their human orthologs.[93]

Two different models of aging are established in *S. cerevisiae*, the replicative lifespan (RLS) and the chronological lifespan (CLS).(Fig. 2)[92,93,95,96] RLS represents the number of times a mother cell can divide before dying, this model is used for proliferating cells, such as stem cells and represents a model for studying aging of mitotically active cells.[90,92,93,95,97] Whereas CLS represents the length of time that a mother cell can survive in stationary phase, and it is proposed to work as a model for aging of post-mitotic cells. In this thesis, we will focus on the chronological ageing paradigm.

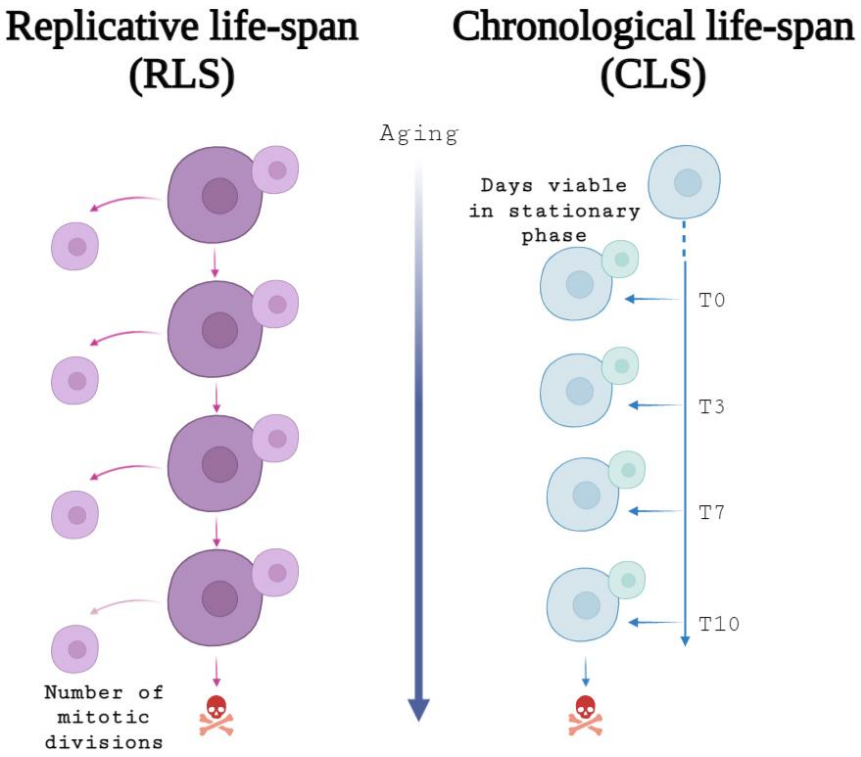

Fig. 2 – Yeast aging models. Graphical representation of chronological and replicative lifespan. Adapted from: Kaeberlein, M., Burtner, C. R., and Kennedy, B. K. in PLoS genetics (2007).

1.4 Nutrient signaling pathways that regulate aging in yeast

The master longevity regulator molecules are part of evolutionary conserved nutrient and energy signaling pathways, many of which were first described in the yeast *S. cerevisiae*. The TOR /serine-threonine-protein kinase (Sch9) (homolog of human S6K) pathway, the Ras/Protein Kinase A (PKA) pathway and sirtuins are major aging regulators described in yeast.[30]

TOR1 complex (TORC1) is composed by four main components: the catalytic subunit,[98] which has two homologous proteins, Tor1 and Tor2, responsible for yeast resistance to the growth inhibitory properties of rapamycin,[99,100,101] and three other subunits Kog1, Lst8, and Tco.[98] TORC1 activation is modulated by nutrients, growth factors and stress condition, being responsible for the regulation of cell growth by targeting both protein synthesis and protein degradation.

The Ras/PKA pathway regulates cellular processes such as cell growth, metabolism, and stress responses, and it is modulated through alteration in levels of glucose availability. In yeast, PKA is a heterotetramer composed by a regulatory subunit, Bcy1, and three redundant catalytic subunits, Tpk1, Tpk2 and Tpk3. In response to glucose availability, Ras proteins (Ras1, Ras2) are activated and

consequently stimulate adenylate cyclase, Cyr1 to produce cAMP. After, cAMP binds to the regulatory subunit of PKA, Bcy1, allowing its dissociation from the PKA catalytic subunits, and the consequent activation of PKA.[82,102,103,104] PKA activation results in the inhibition of the Msn2/Msn4 transcription factors, and consequently aging. Under low glucose levels, this cascade of protein interactions does not occur, which results in the expression of the Msn2/Msn4 transcription factors, and increased longevity.[91,92,102,] Furthermore genetic abrogation of PKA activity, through deletion of one of its subunits, results in extended longevity and this extension is not further increased by limiting glucose availability.[105]

Sirtuins have less pronounced anti-aging roles during CLS.[30] In yeast, Sir2, the NAD-dependent deacetylase, has a major role RLS.[92] It is well described that Sir2 suppresses homologous recombination in ribosomal DNA, which is one of the main defects leading to premature ageing on RLS.[10] Thus, the *SIR2* deletion causes premature aging in replicative cells, and its overexpression prolongs RLS by 40%, reducing rDNA recombination and extrachromosomal rDNA circles (ERCs) accumulation.[82,85,89,106,107] Sir2 also plays an important role in reducing epigenetic changes in histones, for example, the increase in H4K16 acetylation happens in parallel with the decrease in Sir2 levels during aging.[23]

During CLS, Sir2 appears to have a different role. Under non-CR conditions, deletion or overexpression of *SIR2* does not promote any effects on the CLS. However, it is also described that the CLS extension promoted by extreme CR is dependent on the role of Sir2. Therefore, these findings suggest that in yeast, Sir2 deacetylases can promote both pro- and anti-aging roles, dependending on the contexts.[89]

1.4.1 The impact of carbon sources and stress in TORC1 activity

TORC1 is a crucial regulator of metabolism, stress response, autophagy and protein synthesis. The two main TORC1 targets in yeast are Sch9 and Tap42/PP2A branches, in combination with several other signaling routes.[98,99,108] Sch9 belongs to the AGC protein kinase family and it is an ortholog of the mammalian S6 protein kinase 1 (S6K1).[109,110] Sch9 is a highly conserved kinase that has a multitude of functions.[109,111,112] Sch9 is phosphorylated directly by TORC1 at different sites at its C terminus and coordinates nutrient availability with cell cycle progression, cell size, autophagy, stress resistance, sphingolipid metabolism, and protein synthesis.[108] Once phosphorylated by TORC1, Sch9 consequently phosphorylates Rim15, which is a kinase responsible for the induction of cell cycle arrest at G_0 and longevity extension through the activation of Msn2/4 transcription factors. Thus, Sch9 phosphorylation

of Rim15 is able to inhibit longevity. Sch9 is also crucial for the activity of Gis1 transcription factor, independently of Rim15 and TORC1. Furthermore, in response to both glucose and amino acid availability, Sch9 inhibits Sod1/2, the mitochondrial superoxide dismutase enzyme, involved in the stress response to ROS.[10,91,113] In addition, although mostly described as a substrate for Tor1 phosphorylation, there are at least two more kinases, the sphingolipids activated kinases Pkh1/2 and the energy sensing regulator Snf1 kinase, capable of phosphorylating Sch9 at different residues, in response to different stimuli.[108,111,114] Therefore, the activity of Sch9 is also involved in the metabolism of sphingolipids, since phytosphingosine (PHS), an intermediary of this metabolism, regulates the activity of PKh1/2, which phosphorylates Sch9.[114,115]

The other TORC1 target, Tap42-2A phosphatase is responsible for the transcription of genes involved in nitrogen and amino acid metabolism, the environmental stress response (ESR) and autophagy in response to adverse conditions.[98,99,108] Under normal conditions, the Tap42-2A phosphatase is associated with the localization of TORC1 in the vacuolar membrane, but it is released in response to rapamycin treatment and stress conditions. Once released, the Tap42-2A phosphatase dephosphorylates several factors downstream of the TORC1 pathway, including Gcn2.[98] TORC1 can also directly regulate autophagy by the interaction with the Atg proteins, particularly the Atg1 kinase complex by the Atg13 phosphorylation state.[116,117,25]

Several evidences also suggest that TORC1 may be the mediator of the exchange between growth, and maintenance and life extension due to CR.[2,118] The studies show that deletion of *TOR1*, in yeast, like exposure to rapamycin, triggers a stress response similar to the nutrient starvation phenotype.[2,101] This evidence show that TORC1 signaling is dependent on the availability of nutrients.[101,119] The amino acid limitation increases CLS by activating Gcn2 which inhibits TORC1-Sch9 pathway The inhibition of TORC1-Sch9 activates several transcription factors involved in the regulation of CLS. Among them are Msn2/4, Gis1 and Hsf1, and these players activate the environmental stress response (ESR) activating up-regulation of enzymes involved in ROS detoxification. Gis1 together with Rph1 regulates the metabolism of glycerol and acetate, furthermore it also represses the transcription of the Rev1 subunit of DNA polymers, preventing DNA damage.[108]

Inhibition of TORC1-Sch9 also activates transcription factors Hcm1 and Hap4 ensuring the transcription of respiratory genes and increased mitochondrial respiration. It is also described that TORC1-Sch9 represses the expression of the ceramidase genes YDC1 and YPC1, however, this branch is not well disclosed. The downregulation of TORC1-Sch9 also promotes the catabolism of pro-aging carbon

sources and the up-regulation of glycerol biosynthesis. All these mechanisms contribute to the extension of the CLS.[108]

1.4.2 The impact of amino acids in TORC1 activity

All organisms are occasionally exposed to stress, putting the organism's cellular integrity at risk. Likewise, organisms activate different signaling pathways to deal with specific stresses.[120] One of the stress that can affect the homeostasis of cells is the deprivation of amino acids, in these conditions the general protein synthesis is reduced, and proteins needed for stress conditions begin to be expressed.[98]

Amino acids are fundamental nutrients used by cells as building blocks. They are metabolic intermediates, that contribute to many cellular functions, such as protein synthesis, tissues homeostasis, and as substrates essential for low molecular weight synthesis (for example, polyamines, glutathione, creatine, carnitine, carnosine).[121,122,123,124,125,126] The term "amino acids" refers to any organic compound that contains amine (-NH_2) and carboxyl (-COOH) functional groups.[123,127] Amino acids can be classified as essential when obtained from the diet, and non-essential when cells synthesize them from metabolic intermediates.[121,122,127,128] Sometimes, in special situations and disease states, non-essential amino acids are considered essential, since they are obtained from the diet and are called conditionally essential.[126] Some non-essential amino acids play other roles such as blood flow, nutrient transport, intestinal microbial growth, antioxidant responses, and innate immune responses.[122] Amino acids are also a source of energy-producing carbohydrate-like compounds, providing intermediaries for the TCA cycle and gluconeogenesis.[121]

When cells age, cellular processes are activated to degrade old components and replace them with new ones. The key that coordinates these processes is amino acid homeostasis, which consists of five primary processes: absorption, synthesis, utilization, recycling, and catabolism.[128] To maintain amino acid homeostasis, the concentration of free amino acids in the cytoplasm must be strictly controlled, depending on nutritional conditions, to ensure efficient protein synthesis.[129] When amino acids are scarce, protein synthesis is turned off and catabolic processes are activated, the proteins can be degraded by the protease or by autophagy, the amino acids are recycled, and new proteins are synthesized to restore cell function.[123,124,129] Studying the impact of amino acids on aging has aroused the interest of researchers because, although the shortage of amino acids normally induces autophagy in eukaryotes, numerous studies have shown that high concentrations of certain essential amino acids extend the CLS.[128]

According to the TORC1 response, the nitrogen sources have been divided into two classes. One of the classes, referred as the less preferred nitrogen sources, which includes serine, the branched chain amino acids, the negatively charged amino acids and the hydrophobic amino acids, only induce a short initial TORC1 activation. Whereas the preferred nitrogen source, including arginine, asparagine, glutamine and NH_4^+, promote a sustained TORC1 response.[130]

One of the mechanisms by which amino acids regulates TORC1 activity comprises the EGO complex (EGOC), which is a vacuolar/endosomal anchored complex. EGOC is composed by the scaffolding proteins Ego1-3 and the Rag-family GTPases Gtr1 and Gtr2.[131] Activation of Gtr1 and Gtr2 leads to the binding of them to the TORC1-specific subunit Kog1, enhancing TORC1-activity.[132,133] Different studies suggested that EGOC is only required for the transient initial activation of TORC1.[133,134]

A wide range of amino acids are known to induce the TORC1 response, but only the mechanisms by which leucine and methionine signal to TORC1 are well studied. Thus, it is described that leucine stimulates the interaction between the leucyl-tRNA synthase (LeuRS) Cdc60 and Gtr1.[135] By other side, it is described that methionine, in turns activate the Seh1-associated complex (SEAC). SEAC comprise two subcomplexes SEAC inhibiting TORC1 (SEACIT) and SEAC activating TORC1 (SEACAT). High cytosolic methionine levels lead to the phosphorylation of SEACIT by a yet unknown protein kinase.[136,137] In addition, apparently, leucine starvation also indices interaction between SEACIT and Gtr1, which consequently results in the downregulation of TORC1 activity.[133]

Apparently, EGO system also has additional amino acid-dependent response via Lst4 and Lst7. Stimulation of amino acid leads to the interaction between Lst4-Lst7 with inactive Gtr2, thereby triggering the activation of TORC1. It is defined that glutamine, aspartate, asparagine, methionine and cysteine have the capacity to activate this mechanism.[130,138]

1.4.3 The role of Gcn2 in response to amino acids during aging

Gcn2 is part of the Integrated Stress Response (ISR) signaling pathway.[16] This adaptive and pro-survival pathway, conserved in eukaryotes, allows cells to recognize loss homeostasis and respond by activating protein kinases that change the cell's translation and transcription programs. ISR detects stress, downregulates global protein synthesis, and increases biosynthetic and autophagic pathways.[139] Gcn2 was first described in budding yeast as a regulator of eIF2α phosphorylation in response to amino acid deprivation.[16,140,141] The function of this protein kinase is highly conserved from yeast to human cells at

structural and functional levels.[140,141,142,143] Gcn2 regulates mRNA translation initiation rates according to amino acid availability, allowing cells to adapt to nutrient deprivation.[143] CR promotes the accumulation of deacylated transfer RNAs (tRNAs) close to the ribosome, favoring its binding to Gcn2 through the histidyl tRNA synthetase domain (HisRS).[140,141,142,144,145] To ensure the transfer of uncharged tRNA to Gcn2, the cofactor Gcn1 must bind to the ribosome.[140,141,144,145] The binding of tRNAs to Gcn2 promotes a conformational change of Gcn2, allowing its autophosphorylation at a threonine residue and its activation, leading to the phosphorylation of its substrate, eIF2α.[142] Phosphorylation of eIF2α at Ser51 inhibits the general translation initiation and favors the selective translation of some mRNAs that induce the expression of genes that help cells adapt to amino acid deprivation.[143,146] One of the transcribed mRNAs encodes the transcriptional activator Gcn4, which is translated using an alternative and functional open reading frame, this gene promotes cell survival and adaptation during amino acids insufficiency.[16,141,139] Since translation is a process that involves expending about 50% of the cell's energy, overall inhibition of translation and promotion of selective translation saves cell energy, which can be used to restore and maintain the requirements of the cell. Therefore, selective translation ensures that homeostasis is better preserved.[16]

1.4.4 Relationship between the general amino acid control pathway and TORC1

During amino acid deficiency, discharged tRNAs accumulate in the cell and bind to the Gcn2 kinase, the central regulator of general amino acid control (GAAC), initiating its autophosphorylation due to a conformational change, and the simultaneous phosphorylation of the translation initiation factor, eIF2α at serine 51. This phosphorylation decreases the overall initiation of protein translation, but favors transcription of the transcription factor GCN4, which promotes transcription of genes involved in amino acid synthesis, homeostasis and cell survival.[147,148,149]

In *S. cerevisiae* the activation of Gcn2 is dependent on TORC1, as it prevents the dephosphorylation of Gcn2 through the inhibition of one or more phosphatases. Amino acid deprivation inhibits TOR signaling which leads to the removal of inhibitory phosphorylation of Gcn2, activating it and allowing phosphorylation of eIF2α. Thus, in budding yeast, after amino acid deprivation, activation of Gcn2 occurs due to the accumulation of uncharged tRNAs and the release of an inhibitory effect of TORC1.[120,150] Furthermore, it has been also proposed that in *S. cerevisiae*, in response to leucine or histidine starvation,

Gcn2 can phosphorylate the RNC (Raptor-like N-terminal-Conserved) domain of Kog1, thereby inhibiting the TORC1 activity.[98]

1.5. The function of vacuolar amino acid transporters during CLS

pH homeostasis is the maintenance of the proper pH in closed cellular compartments by membranes,[151] and it is crucial for several molecular operations and physiological processes.[152,153] The pH of each organelle influences the biochemical reactions that occur along the endocytic and secretory pathways.[151,153] In eukaryotic cells, intracellular pH affects enzyme activity, and protein folding, is important for vesicle trafficking and affects organelle function and integrity.[153] In humans, changes in intracellular pH are related to different pathologies, such as diabetes mellitus, obesity, epilepsy, myocardial arrhythmia, and glaucoma.[152]

Maintaining pH homeostasis is one of the main challenges for cell survival.[151] The yeast pH regulation is performed by the yeast vacuolar ATPase (V-ATPase) and Pma1. The V-ATPase is an ATPase, with a complex structure, with two main domains, peripheral V1 and V0 linked to the vacuolar membrane,[154] which mediates the protons translocation.[154] V-ATPase pumps protons from the cytosol to the vacuole,[152,153] endosomes, and Golgi compartments.[154] Pma1 is a P-type ATPase, which pumps protons across the plasma membrane to acidify the extracellular space.[153,154] Pma1 is the most abundant protein in the plasma membrane of yeast cells, consumes at least 20% of total cellular ATP, its activity is strictly regulated by metabolic activity and the physiological conditions of the cells, for example, in response to a decrease in intracellular pH or increased potassium uptake.[154] To ensure pH homeostasis, V-ATPase and Pma1 work in close harmony. The loss of V-ATPase activity causes the incorrect localization of Pma1 to the vacuole and other compartments, reflecting a compensatory mechanism. In addition, to V-ATPase and Pma1, there are several other proton pumps and exchangers in yeast, which probably adjust the pH control of the cytoplasm and each organelles.[153]

Vacuoles are highly complex organelles, occupy about 25% of the cell volume of *S. cerevisiae*.[155,156,157,158] In this organelle 60% of cellular amino acids are stored. The remaining amino acids are in another pool located in the cytoplasm that is involved in metabolism and protein synthesis. Amino acids are unevenly distributed throughout organelles,[159] most basic amino acids and glutamine are located in the vacuole, while acidic amino acids are located in the cytosol.[155,156,158,159] The concentration of cytosolic free amino acids is strictly regulated as a function of nutritional conditions to ensure efficient protein

synthesis. In starvation conditions, proteins are degraded, to compensate, amino acids from the vacuole are transported to the cytoplasm where they are recycled and reused in the synthesis of new proteins.[129] Therefore, to ensure the efficient synthesis of proteins, it is necessary to maintain the concentration of amino acids in the cytoplasm, changes in the level of amino acids can result in damage to cell metabolism and loss of survival capacity.[129,159]

Ensuring the maintenance of amino acid levels implies some active and specific transport mechanisms in the vacuolar membrane.[129,158] Kinetic experiments for the vacuolar transport activities suggested seven independent transport systems in the vacuolar membrane of *S. cerevisiae*: arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and isoleucine-leucine.[157,159] In yeast, there are three main transporter families of amino acids across the vacuolar membrane: the VBA-, AVT- and PQ-loop family transporters. The VBA-family (Vba1-4) and PQ-loop family (Ypq1-3) proteins, in addition to Btn1 and Vsb1, are classified as basic amino acid importers,[156,160,161] suggesting that they can be linked to the preferential storage of basic amino acids in the vacuolar lumen. In contrast, the AVT transporters are recognized as a more diverse family of transporters, since they are related with both import and export of many different types of amino acids. Avt1 imports several neutral and basic amino acids,[162] while Avt3 and Avt4 are essentially responsible for the export and recycling of neutral amino acids.[157,163] Avt6 mediates the efflux of acidic amino acids, glutamate and aspartate.[123,129,156,159] Avt7 exports glutamine, and proline.[123,129] The activity of Avt2 and Avt5 remains unknown. Avt3 and Avt4 appear to have a redundantly function under normal growth conditions, as single deletion mutants lacking either *AVT3* or *AVT4* do not display obvious changes in vacuolar amino acids, while the double deletion mutant displays a remarkable increase of several amino acids in the vacuole.[163] The activity and vacuolar abundance of the referred transporters is dependent of the nitrogen availability.[160,163]

Recently, it was showed that vacuolar acidity has been linked to the maintenance of mitochondrial integrity, which reveals an important function in lifespan regulation.[111,40] Even though, their mode-of-action is undisclosed, it seems that *TORC1*, *Sch9* and *PKA* deleted cells revealed a maintenance of vacuolar acidity and mitochondrial integrity during RLS,[40] as well as CLS.[153] Furthermore, overexpression of *AVT1* resulted in the accumulation of many amino acids in the vacuole, which improved mitochondrial fitness and prolong the RLS.[40]

CHAPTER 2

AIMS AND SCOPE OF THESIS

The overall objective of this master's thesis is to investigate if medium supplementation with charged side chain amino acids (CSCAAs) impacts on aging, resulting in a longevity extension. Therefore, yeast cells were supplemented with CSCAAs and the effects on aging and in aging-associated pathways were explored. Finally, this thesis was also focused on the function of the vacuolar amino acid's transporters and their role on aging regulation. To achieve these objectives, two main specific aims were defined.

Aim 1. To elucidate how differential charged side chain amino acids (CSCAAs) supplementation impacts on yeast chronological lifespan. This aims allows to understand the impact of medium supplementation with CSCAAs, namely arginine, histidine, lysine, aspartic acid and glutamic acid, on aging modulations, as well as the cellular mechanisms elicited by these supplementations.

Aim 2. To determine the role of vacuolar amino acids transporters on yeast CLS. A balance in the cytosolic and vacuolar amino acid concentrations must be maintained since loss of this maintenance results in damage to cellular metabolism and viability. Therefore, with this aim we will study the involvement of vacuolar transports in the regulation of longevity under CSCAAs supplementations.

CHAPTER 3

MATERIAL AND METHODS

3.1. Yeast stains, and growth conditions

All yeast and plasmids are listed in Table 1 and table 2 were inoculated into regular complete medium containing 0.67% (w/v) Yeast Nitrogen Base (YNB) (Difco Laboratories, Detroit, MI, USA) and 2% (w/v) glucose as a carbon source, supplemented with the appropriate amino acids for which the strains were auxotrophic: 100 mg / L uracil; 300 mg/L of leucine, 50 mg/L of histidine and 50 mg/L of lysine. Cultures were grown at 26°C, with shaking at 150 rpm.

Table 1 - Yeast strains used in this work.

Yeast strain	Pertinent genotype	Complete genotype
BY4741	Will type	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ*
TOR1	*tor1Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, torΔ::KanMX*
SCH9	*sch9Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, sch9Δ::KanMX*
GCN2	*gcn2Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, gcn2Δ::KanMX*
AVT3	*avt3Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, avt3Δ::KanMX*
AVT4	*avt4Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, avt4Δ::KanMX*
AVT3 AVT4	*avt3Δ avt4Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, avt3Δ::KanMX avt4 Δ::KanMX*
AVT3 SCH9	*avt3Δ sch9Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, avt3Δ::KanMX sch9 Δ::KanMX*
AVT4 SCH9	*avt4Δ sch9Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, avt4Δ::KanMX sch9 Δ::KanMX*
AVT3 AVT4 SCH9	*avt3Δ avt4Δ sch9Δ*	*MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ, avt3Δ::KanMX avt4Δ::KanMX sch9Δ::KanMX*

Table 2 - Plasmids used in this work.

Plasmids	Type of Plasmid	Source
pRS416-GFPAtg8	2μ	Guedes. A *et al* (2016)
pRS416-SCH9	2μ	Urban. J *et al* (2007)
pRS416-SCH9-2D3E	2μ	Urban. J *et al* (2007)
pRS416-SCH9-5A	2μ	Urban. J *et al* (2007)

3.2. Chronological lifespan (CLS) assays

To analyze the CLS cells were grown for two days in regular medium until the stationary phase, this is considered CLS day zero. On this day, cultures were supplemented with 10Mm CSCAAs - 10 mM

of arginine; 10 mM of lysine or amino acids MIX (10 mM of arginine, 10 mM aspartate and 10 mM of glutamate). To determine cell survival, cultures were serially diluted, and plated on plates containing YEPD agar medium, consisting in 0,5% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar. Survival was assessed by colony-forming units (CFUs) method, the method was started on day 0 when survival is considered 100%, and repeated every 2-3 days, until only 0.1% of the cells remain viable.

3.3. Assessment of intracellular superoxide anion and hydrogen peroxide accumulation

Accumulation of reactive oxygen species, particularly hydrogen peroxide and superoxide anion, was measured using the two probes, dihydroethidium (DHE) (Molecular Probes, Eugene, OR, USA) and dihydrorhodamine 123 (DHR) (Molecular Probes, Eugene, OR , USA), respectively. The cells were collected on days 0, 3, 7, and 10, and then incubated with the two probes. DHE was added to a final concentration of 5μM in dimethyl sulfoxide (DMSO), followed of incubation in dark for 10min at 30°C. DHR was added at 15mg/ml of cell culture from in ethanol, followed of incubation for 2h at 30°C in dark. The DHE and DHR signals were detected using FACSCaliber2 flow 6 cytometer (BD-Biosciences, Franklin Lakes, NJ, USA) with a 488nm excitation laser. Signals from 30,000 cells/sample were captured in FL3 (> 670 nm) at a flow rate of 1,000 cells/s. Data collected with the FACSCaliber2 flow cytometer were processed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

3.4. Cell cycle analysis

Cell samples (1mL) for cell cycle analysis were collected on days 0, 3, 7, and 10, and fixed with 70% ethanol for 30 min at 4°C, followed by sonication, treatment with RNAse for 1h at 50°C in sodium citrate buffer (50mM sodium citrate, pH 7.5), and subsequent incubation with proteinase K (0.02 per mg 107 cells). Cell DNA was then stained overnight with SYBR Green 10 000X (Molecular Probes, Eugene, OR, USA), diluted 10-fold in Tris-EDTA (pH 8.0), and incubated overnight at 4°C. Before cytometric analysis, samples were diluted 1:4 in sodium citrate buffer. Determination of cells in each phase of the cell cycle was performed offline with ModFit LT 3.2 software (Verity Software House Inc., Topsham, ME, USA).

3.5. Assessment of medium pH

For evaluation of medium pH, 5mL of yeast cells were collected, at days 0, 3, 7 and 10, vortexed and measured by the Five Easy Plus pH/mV Bench Meter (Mettler Toledo, Columbus, OH, USA).

3.6. Protein extraction and quantifications

The cells were harvested in a centrifuge and washed once with PBS 1X. The cells were pre-treated with 500μl of lithium acetate (2M), incubated at room temperature for 5 minutes, and then centrifugated. The supernatant was discarded, and the pellet resuspend in 500μl of NaOH (0.4M), incubated for 5 minutes on ice, and then centrifugated again. The supernatant was discarded, and the pellet resuspend in 100μl of Laemmli Sample Buffer (BioRad), boiled for 5 minutes, and centrifuged at maximum speed for 30 minutes at 4°C. Finally, the protein-enriched supernatant was collected to new tubes.

Total protein quantification was performed with the RC DC protein assay (Bio-Rad) and processed according to the manufacturer's instructions.

3.7. Immunoblot analysis

Western blots of protein extracts were carried out by resolving 20 μg of the total protein 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After protein were transferred to a nitrocellulose membrane for 10 minutes in the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 hour at room temperature in tris-buffered saline (TBS) with 0.1% tween 20 (TBS-T) containing 5% BSA and afterwards incubated overnight at 4°C with the different polyclonal primary antibodies: rabbit anti-eIF2α (1:1000; Cell Signaling), rabbit anti-phospho-eIF2α (1:1000; Cell Signaling), goat anti-GFP (1:5000; Abcam), mouse anti-Pgk1 (1:5000; Invitrogen) in TBST containing 1% BSA and primary antibody. Then membranes were washed with TBST, and incubated one hour with the correspondent secondary antibody: HRP-conjugated anti-rabbit IgG, anti-mouse IgG or anti-goat IgG at a dilution of 1:5000 in TBST containing 1% BSA. Protein bands were detected with ChemiDoc XRS+ system (Bio-Rad) after incubation with Clarity Western ECL Substrate (Bio-Rad) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Digital imagens were obtained in the ChemiDoc XRS+ System (Bio-Rad) with the Quantity One Software (Bio-Rad) and, subsequently, the densitometric

analysis was performed using the Image Lab software. At least, tow independent biological replicates were performed.

3.8. Monitoring autophagy by GFP-Atg8 assay

To monitor GFP-Atg8 processing, the delivery of Atg8 to the vacuole was followed by N-terminally tagged with GFP to Atg8. Thus, cells were transformed with the plasmid pRS416-GFPAtg8, in which, Atg8 is under the control of its endogenous promoter. As GFP is more resistant to the vacuolar degradation than Atg8, bulk autophagy results in the accumulation of free GFP in the vacuole (Cheong & Klionsky, 2008; Klionsky et al., 2016). Therefore, after protein extraction and western blot assay (described above), GFP-Atg8 and free GFP were detected using a GFP-specific antibody (described in the antibodies section). Immunoblot bands were quantified by densitometric analysis using the Quantity One software. Graphical representation of densitometric analysis refers the ratio between the free GFP and total GFP.

3.9. Statistical analysis

Results shown are mean values and standard error of the mean (SEM) of at least three independent biological replicates. Statistical analyzes were determined using two-way ANOVA or t-test (GraphPad Software, San Diego, CA, USA). A value of $p \leq 0.05$ will be considered statistically different.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Medium supplementation with amino acids from the class of the charged side chain amino acids (CSCAAs) impacts on yeast chronological lifespan and cell-autonomous mechanisms

Numerous proteins involved in a wide range of cellular processes regulate aging. These proteins are conserved across species and belong to nutrient and energy detection signaling pathways.[165] To extend longevity, researchers have begun to develop experiments that interfere with these signaling pathways regulation. Several authors have described the CR as a robust and reproducible method to extend lifespan in various organisms. However, the scientific community continues to look for other approaches.[166]

Amino acids are essential for yeast cells growth and survival, since they can support cells with sources of carbon and nitrogen.[167] Previous studies have tested the supplementation impact of branched side-chain amino acids (BCAAs: isoleucine, leucine, and valine) in cells longevity. As results, these studies demonstrated that BCAAs supplementation regulates aging, leading to increased longevity in yeast,[168] mice,[169,170] and *C. elegans*.[171] In contrast, decreasing BCAAs supplementation increases *Drosophila* longevity[172]. In addition, numerous reports link BCAAs to diseases such as sarcopenia, obesity, diabetes mellitus, and cardiometabolic risk factors.[173] Based on these results, we decided to focus our study in the impact of side-chain charged amino acids (CSCAAs: lysine, arginine, histidine, aspartic acid, glutamic acid) supplementation on lifespan regulation.

We started to study the impact of CSCAAs supplementation on yeast CLS. For that, at beginning of CLS, 48 hours of growth, we supplemented the medium without (non-supplemented, as control) or with 10 mM of arginine, 10 mM of lysine or a MIX of amino acids (10 mM arginine, 10 mM glutamate and 10 mM aspartate). Data showed that supplementation with arginine or the amino acids MIX promoted a strong increase on CLS of wild type cells (BY4741 background) in comparison with the CLS presented by non-supplemented cells (Fig. 3A). Interestingly, in contrast, lysine supplementation led to a shorter CLS compared to the CLS of cultures without supplementation and supplementation with the other amino acids (Fig. 3A).

Together, these results demonstrate that CSCAAs, like BCAAs, are associated with longevity modulation. It is described that during aging, oxidative damage could promote a random modification of the side-chain charge of proteins, which can lead to loss of protein stability. Thus,

supplementing the cells cultures with "native" loaded side-chain amino acids can restore protein stability and promote longevity.[174]

To further understanding how CSCAAs supplementation impacts on the yeast longevity modulation, we tested whether these amino acid supplementation conditions modulate cell-autonomous mechanisms, particularly cell cycle, accumulation of reactive oxygen species (ROS) and autophagy.

Yeast CLS is associated with cell cycle regulation. Some authors have already identified a relationship between the CR beneficial effects on yeast lifespan and the cell cycle, which is defined by an efficient cell cycle arrest at G0/G1 phase.[175] Using flow cytometry, we analyze the impact of amino acids supplementation on the cell cycle profile of the cells. Results showed that cells supplemented with arginine or the amino acids MIX presented an efficient cell cycle arrest at G0/G1 phases (Fig. 3B), in comparison with the cell cycle profile revealed by non-supplemented cells. In this last condition, as already describe,[176] cells undergo a cell cycle arrest at S phase (Fig. 3B), Indeed, short-lived cells tend to arrest in a budded state, creating a subpopulation stopped prematurely in the S-phase.[177] Lysine supplementation that shortened the cells CLS triggered a cell cycle arrest at S-phase, typically followed by cell death (Fig. 3B). These results reinforce the results previously obtained with the CLS evaluation (Fig. 3A).

Oxidative stress is the imbalance between ROS formation and antioxidant defense mechanisms. Due to the biological effects caused by ROS accumulation, numerous studies have been developed focused on the oxidative stress role as a regulator of aging.[63] Therefore, we proceed to the evaluation of ROS accumulation under the studied conditions. For this, we use two dyes dihydrorhodamine (DHR) and dihydroethidium (DHE), which label the hydrogen peroxide and superoxide anion species, respectively. DHR is mostly oxidized by hydrogen peroxide (H_2O_2), accumulates in mitochondria, and emits fluorescence, whereas DHE is oxidized by superoxide anion ($O_2^{\cdot 2}$), accumulates in the nucleus, and emits fluorescence. Regarding the impact of amino acids supplementation on the accumulation of H_2O_2, data revealed that supplementation with arginine and amino acids MIX increased the accumulation of H_2O_2 at time 0 of CLS, with subsequent reduction during CLS (Fig. 3C and D) in comparison with the H_2O_2 levels presented by non-supplemented cells. Probably, this cell's response is associated with an already disclosed hermetic effect.[84] On the other hand, lysine supplementation does not decrease ROS accumulation (Fig. 3C and D), and we detected an exponential accumulation of H_2O_2 during time (Fig. 3C).

In relation with the levels of O_2^{2-}, data revealed that during time, arginine or the amino acids MIX supplementation significantly reduces the accumulation of this ROS specie, while lysine supplementation present similar levels compared with the levels showed by non-supplemented cells. (Fig. 3D)

Together, results suggest that arginine and amino acids MIX supplementation lead to an improvement in mitochondrial function and a decrease in mitochondrial superoxide accumulation and oxidative damage, preventing protons leakage, thus maintaining cellular ATP levels.

Autophagy is a highly conserved catabolic process, which contributes to the maintenance of energy homeostasis. Autophagy also protects cells from stress, by facilitating lysosomal/vacuolar degradation and recycling of intracellular macromolecules and dysfunctional organelles. Studies in several model organisms have demonstrated the needed of the autophagy machinery in the regulation of lifespan.[178] In this sense, our next questions was related with the effects of amino acids supplementation in this central mechanism. Therefore, we analyzed autophagy through the immunoblot analysis of GFP-Atg8 processing assay. For that, we transformed cells with a plasmid containing the Atg8 protein bound to GFP, and we monitored the vacuolar delivery of GFP-Atg8 by western blotting. Under pro-autophagy conditions, the GFP-Atg8 reporter is transported to the vacuole and processed by hydrolases, as GFP is more resistant to vacuolar degradation than Atg8, when autophagy occurs free GFP accumulates in the vacuole.[179] We evaluated autophagy at different time points, during the cells CLS. Data revealed that ratio of free GFP and total GFP levels was similar independently of the supplementation with the different amino acids. Thus, apparently CSCAAs supplementation did not have a major impact on autophagy flow (Fig. 4A and B).

Until here, it was clear that arginine and amino acids MIX supplementation extends yeast longevity, associated with a cell cycle arrest at G0/G1 phase and reduction of ROS accumulation, while lysine supplementation decreases longevity, with a cell cycle arrest at S-phase and ROS

accumulation, in comparison with non-supplemented cells. Next, we tried to understand which signaling pathways modulate the effects of CSCAAs supplementation on yeast lifespan.

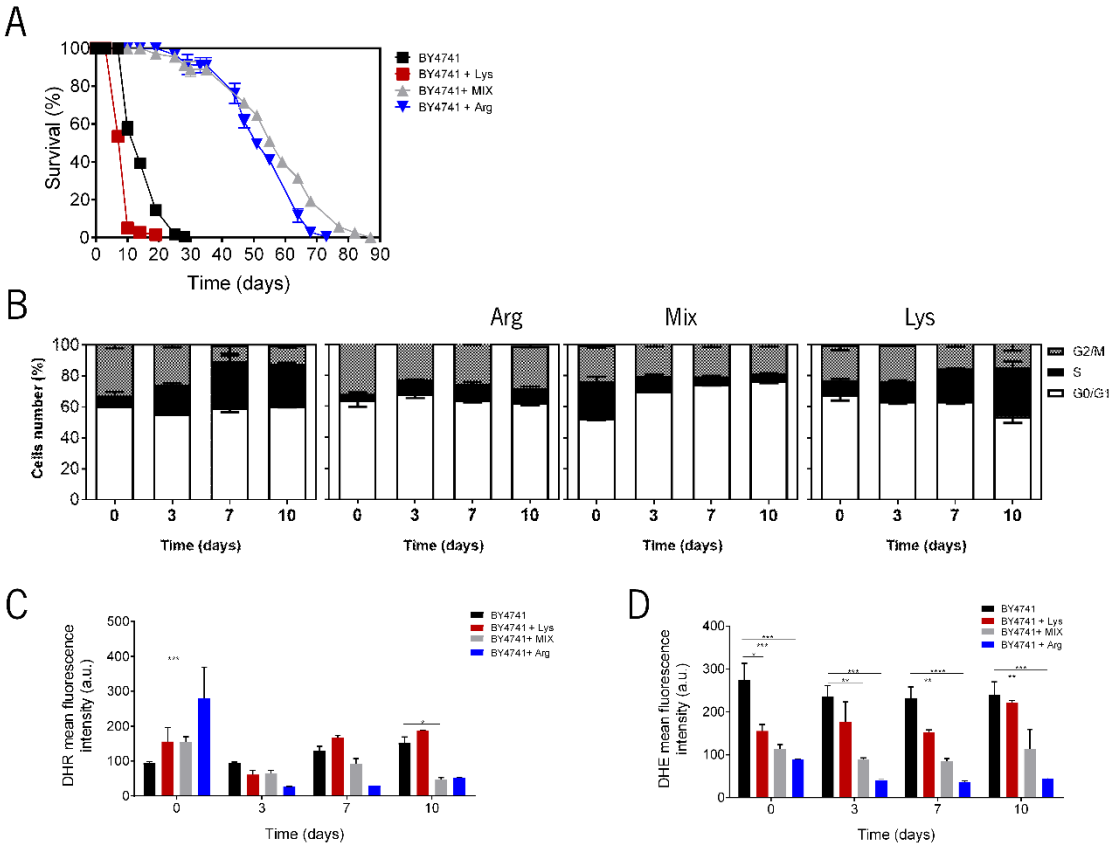

Fig.3 Global effects of CSCAAs supplementation: The supplementation of wild-type cells (BY4741) with CSCAAs modulate **(A)** the yeast Chronological lifespan (CLS). Cell viability was measured at 2-3 days interval beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The supplementation with CSCAAs also modulate **(B)** the cell cycle, and accumulation **(C)** of hydrogen peroxide **(D)** and superoxide, two different species of ROS. The cell cycle was evaluated by the measurement of DNA content by flow cytometry, and ROS accumulation was evaluated with FACS measurements of superoxide anions and hydrogen peroxide using the superoxide-specific probe DHE and using the peroxides-specific probe DHR. FACS measurements (20,000 cells per sample in three independent experiments) of DHE-positive cells and DHR mean fluorescence. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA (*p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001).

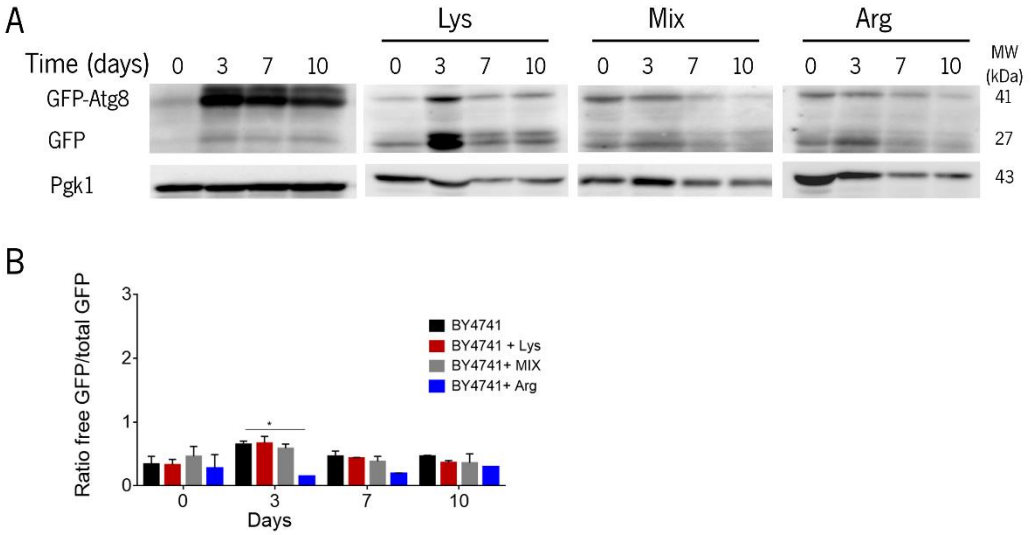

Fig.4 CSCAAs supplementation does not significantly impact autophagy flow: Monitoring of autophagy levels by ratio between GFP bound to ATG8 and free GFP in the vacuole of wild-type yeast cells (BY4741).by **(A)** western blotting. **(B)** To better analyze the autophagy flow, we also did the Graphical representation of the ratio between GFP/GFP+ATG8 protein expression levels obtained by densitometric analysis. PJK was used as a standard control. Densitometric analysis was performed using the ImageLab4.1TM software. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA (*p<0.05).

4.2. Signaling pathways involved in modulation of CLS promoted by the charged side chain amino acids (CSCAAs)

All living organisms have mechanisms that detect the scarcity or abundance of extracellular and intracellular amino acids in the microenvironment. Furthermore, it is already disclosed that these pathways modulate CLS.

Eukaryotic cells have nutrient sensors, which sense the fluctuating of several nutrients levels, including amino acids, and program the cellular machinery to mount an appropriate response.[148] Amino acid restriction increases the uncharged tRNAs, which activate the Gcn2 pathway, promoting eif2α phosphorylation and reducing activation of the TOR pathway, which results in decreased protein synthesis. When the availability of amino acids increases, the accumulation of uncharged tRNAs reduces, preventing GCN2 activation and, consequently, eif2α phosphorylation. Furthermore, when the availability of amino acids increases, Tor1 is sequestered into the vacuole, leading to increased protein synthesis.[148] Sch9 is the main target of TORC1 phosphorylation and has also been shown to be an important player in CLS modulation in response to amino acid availability. Therefore, we ask whether any of these kinases are involved in CLS modulation by

CSCAAs supplementation. To answer this question, we started to evaluate the impact of CSCAAs supplementation on the longevity of *sch9Δ*, *tor1Δ*, and *gcn2Δ* cells. For that and using the same approaches above described, we supplemented or not the culture medium of *sch9Δ*, *tor1Δ*, and *gcn2Δ* cells with 10 mM of arginine, 10 nm lysine or a MIX of amino acids (10 mM arginine, 10 mM glutamate and 10 mM aspartate), and evaluate their CLS, cell cycle and ROS accumulation.

Concerning *tor1Δ* cells, we observed, that like wild-type cells, the medium supplementation with arginine or amino acids MIX increases CLS, in comparison with the CLS presented by non-supplemented mutant cells (Fig. 5A). Furthermore, this augmented CLS was accompanied with reduced accumulation of ROS, both H_2O_2 (Fig. 6A) and O_2^{2-} (Fig. 6B) and a cell cycle arrest at G0/G1 cell cycle phase (Fig. 7A). In the opposite way, in comparison with non-supplemented condition, the medium supplementation with lysine resulted in shorter the CLS of *tor1Δ* cells (Fig. 5A), which was accompanied with enhancement of oxidative stress, through the accumulation of, both O_2^{2-} (Fig. 6A) and H_2O_2 (Fig. 6B). Concerning the cells cycle profile, the supplementation with lysine also resulted in the cell cycle arrest at G0/G1 phase (Fig. 7A), which suggests that CLS modulation promoted by lysine supplementation of *tor1Δ* does not occur via cell cycle regulation.

Sch9 is the main target of TORC1 phosphorylation, it is part of the nutrient-induced signaling network, regulates several cellular functions, such as protein synthesis and cellular homeostasis. Some studies performed with *sch9Δ* cells have already revealed that the deletion of this gene extends CLS. As described in the literature, *sch9Δ* cells are long-liver cells,[109,112] which we also confirmed (Fig. 5B). However, medium supplementation with arginine or the MIX of amino acids led to a reduction of the CLS. Furthermore, as observed to wild type cells, medium supplementation with lysine also resulted in a shorter CLS (Fig. 5B).

Supplementation of *sch9Δ* cells with amino acids MIX and lysine did not reduce the ROS accumulation, whereas arginine supplementation is also associated with a reduction of ROS accumulation (Fig. 6C and D). It is described that arginine could be related to the improvement of mitochondrial function,[180] therefore, this data suggests that arginine is still able to ameliorate the accumulation of ROS. Furthermore, the medium manipulation with the different formulation of amino acids drive cells to arrest at S-phase cells cycle phase (Fig. 7B).

Some studies have shown that amino acid deprivation activates Gcn2 kinase, by decreasing protein synthesis and consequently the reduction of amino acids consumption, which might

prolong the yeast lifespan.[118] Abrogation of *GCN2* by itself is associated with a shorter lifespan, in comparison with the CLS presented by wild-type cells (Fig. 5C). This same shorter CLS was also observed when *gcn2Δ* cells were cultivated in medium supplemented with arginine or the amino acids MIX (Fig. 5C). Surprisingly, supplementation with lysine resulted in the extension of CLS in comparison with the CLS presented by non-supplemented *gcn2Δ* cells (Fig. 5C).

gcn2Δ cells supplementation with arginine, lysine or the amino acids MIX alleviates oxidative stress, since, in all these conditions we observed a decrease in the accumulation of both H_2O_2 (Fig. 4E) and O_2^{2-} (Fig. 6F) levels, in comparison with the non-supplemented mutant cells. Furthermore, the medium manipulation with the different formulation of amino acids also drives cells to arrest at G0/G1 cells cycle phase. This arrest was more efficiently observed for the mutant cells supplemented with lysine (Fig. 7C).

Together results suggest that Tor1 kinase is not involved in the yeast longevity modulation by amino acids. In contrast, we observed that both Sch9 and Gcn2 are key regulators of CSCAAs supplementation, although they have a distinct role under supplementation. Sch9 kinase appears to a role in the beneficial effects promoted by Arginine and amino acids MIX supplementation, while Gcn2 look to have a dual role, pro-survival and anti-survival, depending on the supplemented amino acids, suggesting that it may activate different pathways in response to conditions of CSCAAs supplementation.

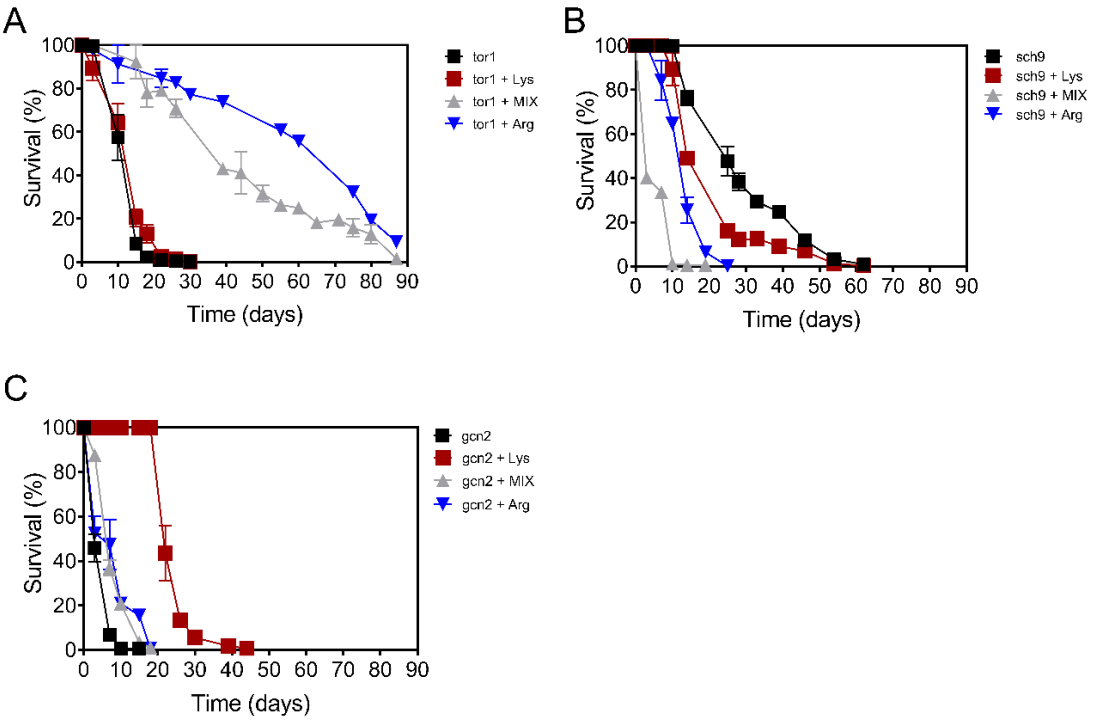

Fig.5 The signaling pathways modulate CLS under these CSCAAs supplementation conditions: Modulation of chronological lifespan of mutants **(A)** *tor1Δ* **(B)** *sch9Δ* **(C)** *gcn2Δ* non-supplemented, supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid). CLS of cells in non-supplemented medium were used as control. Cell viability was measured at 2-3 days interval beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA.

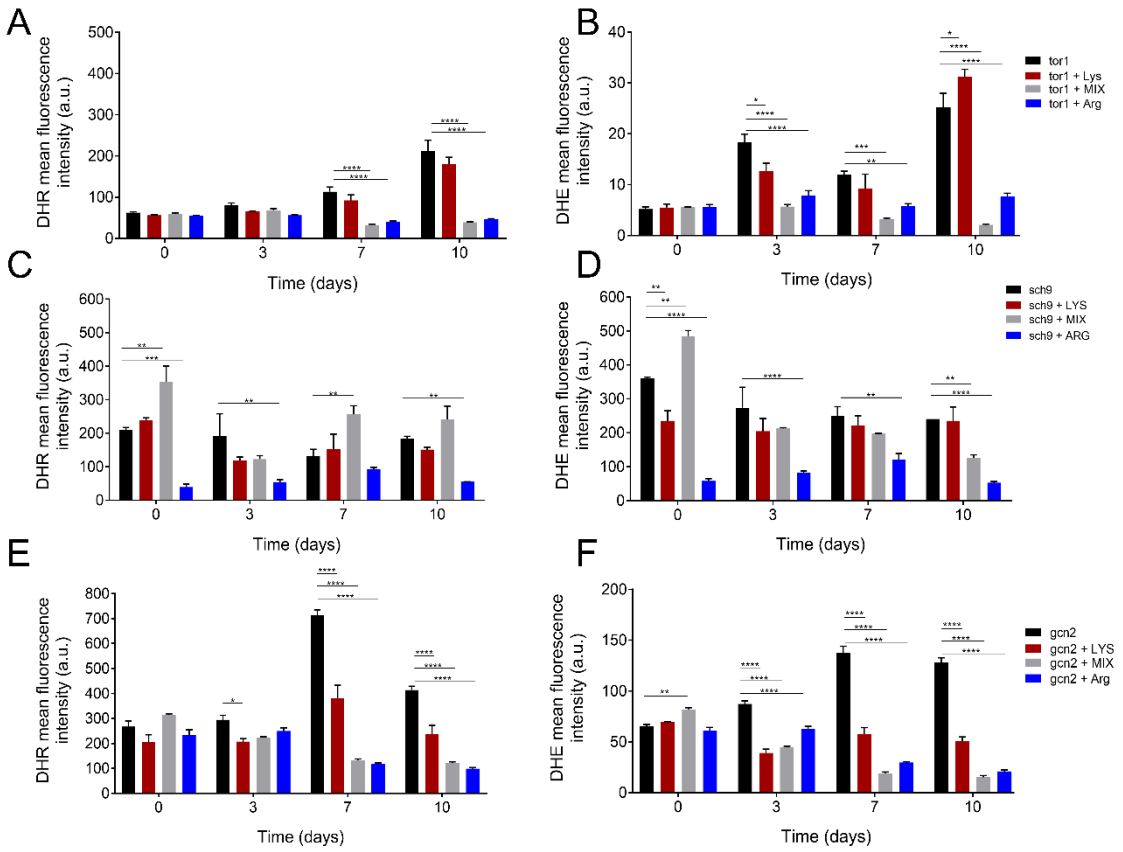

Fig.6 The signaling pathways modulates ROS accumulation under these CSCAAs supplementation conditions: We analyzed the Ros accumulation of the mutants non-supplemented, supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid) with two difrent dyes. We label **(A)** *tor1Δ* with DHR, **(B)** *tor1Δ* with DHE, **(C)** *sch9Δ* with DHR **(D)** *sch9Δ* with DHE **(E)***gcn2Δ* with DHR **(F)** *gcn2Δ* with DHE, FACS measurements of superoxide anions and hydrogen peroxide using the superoxide-specific probe DHE and using the peroxides-specific probe DHR.FACS measurements (20,000 cells per sample in three independent experiments) of DHE-positive cells and DHR mean fluorescence. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA (*p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001).

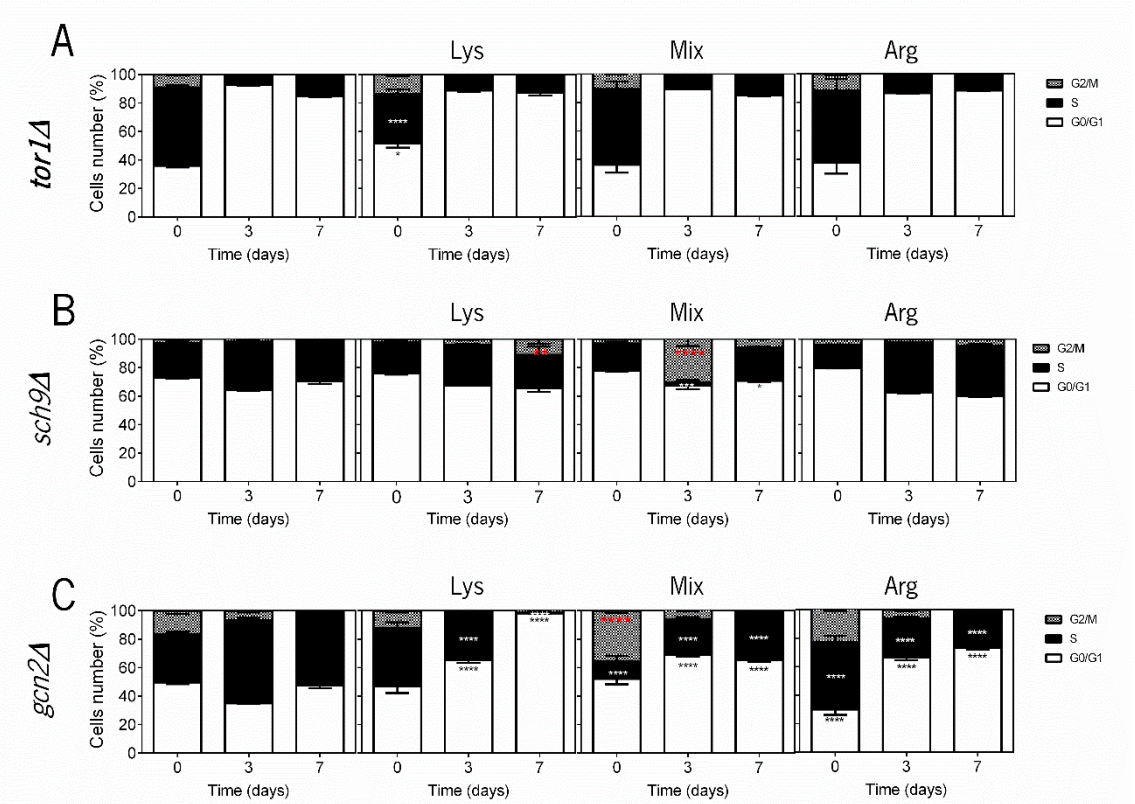

Fig.7 The signaling pathways modulates cell cycle under these CSCAAs supplementation conditions: Cell cycle analysis evaluated by the measurement of DNA content by flow cytometry of mutants **(A)** *tor1Δ* **(B)** *sch9Δ* **(C)** *gcn2Δ* non-supplemented with supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid). The data represents mean ± SEM of three independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p≤0.05; **p≤0.01; ****p≤0.0001), being related to Non-sup control graph.

4.3. Regulation of Sch9 activity under CSCAAs supplementation conditions

According to the literature, several pathways can regulate the Sch9 kinase activity, but the main responsible for its phosphorylation is TORC1.[111] As we observed earlier, Tor1 does not appears to participate in the CLS extension promoted by CSCAAs supplementation. Still, to rule out the hypothesis that Tor1 is involved in this pathway as a Sch9 regulator, we designed a new strategy, by using cells harboring a mutant version of Sch9, in which its TORC1 phosphorylation sites are mutated. TORC1 can phosphorylate Sch9 at five different sites, the used vectors had all these sites modified.[181] Herein, we tested the requirement of TORC1-dependent Sch9 phosphorylation in wild-type and sch9Δ mutant strains complemented with either wild-type Sch9, the $Sch9^{5A}$ or the phosphomimic $Sch9^{2D3E}$ alleles.[111] In $Sch9^{5A}$ vector all serine/ threonine residues located at the

phosphorylation site were replaced by alanine. This amino acid exchange mimics the absence of Sch9. In Sch9^{2D3E} vector, all serine /threonine residues were replaced by glutamate / aspartate, causing the loss of TORC1 activity.[182]

Following the same approaches already described concerning the medium supplementation with the distinct amino acids formulas, as expected expression of wild type Sch9 allele in the *sch9Δ* cells resulted in a profile similar to the one showed by wild type cells (Fig. 3A) for the distinct supplementations (Fig. 8A). In this assay the transformation of sch9Δ cells with a vector harboring the full *SCH9* gene is equivalent to wild type cells (Fig. 3A). In *sch9Δ* expressing Sch9^{2D3E}, which cannot be phosphorylated by TORC1 but can be activated by other key players, supplementation with arginine or the amino acids MIX increased the CLS in comparison with the same cells without supplementation (Fig. 8B). On the other hand, expression of Sch9^{5A}, which cannot be activated by any means, has completely lost the arginine and amino acids MIX supplementation beneficial effects (Fig. 8C).

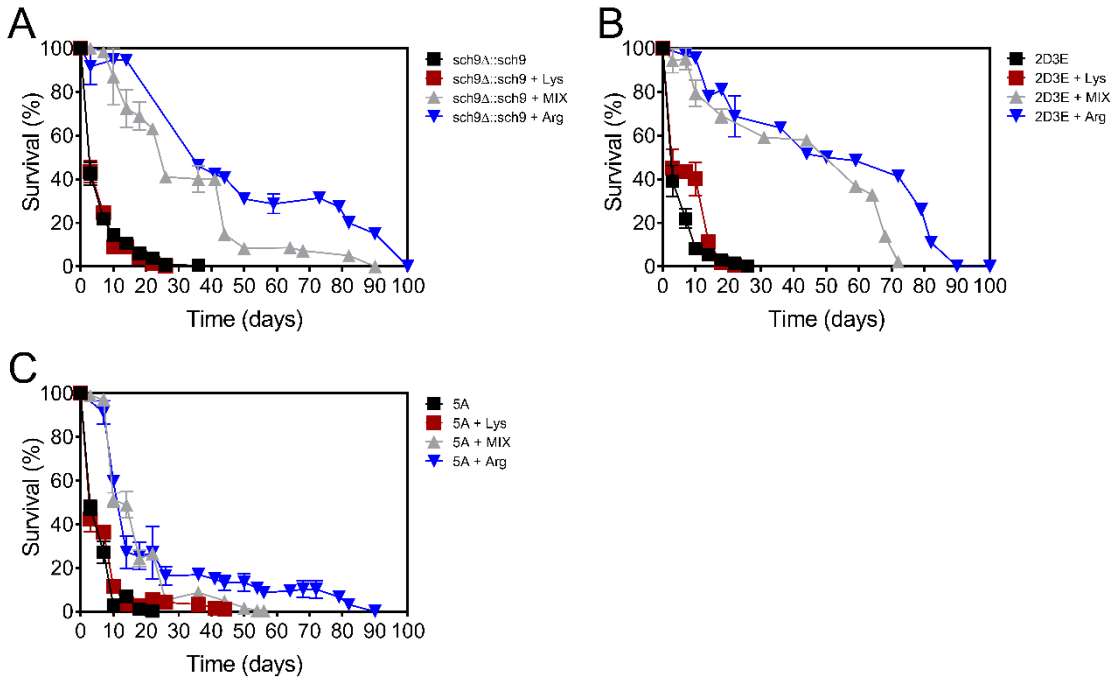

Fig.8 Activation of Sch9 in CSCAAs supplementation conditions: Chronological lifespan of vectors **(A)** *Sch9Δ::sch9* **(B)** *SCH9*^{2D3E} **(C)** *SCH9*^{5A} non-supplemented, supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid). CLS of cells in non-supplemented medium were used as control. Cell viability was measured at 2-3 days interval beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%).

Our data establishes that the role of Sch9 kinase in the CLS extension promoted by arginine and amino acids MIX supplementation is independent of TORC1, suggesting the involvement of other regulators in this mechanism.

4.4. The relationship between the two key regulators of CSCAAs supplementation, Sch9 and Gcn2

Gcn2 and eIF-2α belong to the main nutrient detection pathway in all eukaryotes called "General Control of Amino Acids" in yeasts. Amino acid deficiency activates Gcn2, increasing eIF-2α phosphorylation, causing inhibition of translation initiation and reduced overall protein synthesis. Phosphorylation of eIF-2α, on the other hand, favors the translation of mRNA encoding the transcription factor (TF) Gcn4 which induces the expression of many genes involved in biosynthesis and amino acid uptake and autophagy.[149]

Until here, we showed that Gcn2 and Sch9 are key regulators in conditions of CSCAAs supplementation. So, we want to understand if there is any crosstalk between Gcn2 and Sch9 in our working conditions, for this, we evaluate the phosphorylation levels of eIF-2α.

First, we evaluated the phosphorylation levels of eIF-2α in wild type cells, and we observed that supplementation with amino acids MIX and arginine promotes the phosphorylation of eIF-2α. However, supplementation with lysine decreases the phosphorylation of eIF-2α (Fig.9A and B). This result suggests that supplementation with MIX and arginine activates Gcn2.

Next, we evaluated the phosphorylation levels of eIF-2α in *sch9Δ* cells, and we observed that abrogation of *SCH9* gene abolishes the phosphorylation of eIF-2α promoted by supplementation with amino acids MIX and Arginine. However, abrogation of *SCH9* gene promotes the phosphorylation of eIF-2α in cells supplemented with lysine (Fig. 10A and B).

Lastly and as a negative control, we evaluated the phosphorylation levels of eIF-2α in *gcn2Δ* cells, and we observed that as expected, deletion of *GCN2* gene abrogates the phosphorylation of eIF-2α, independently of the supplementation conditions (Fig.11A and B).

Overall, the results suggests that Sch9 and Gcn2 collaborate in the same pathway to promote the CLS extension under condition of medium supplementation with amino acids MIX and Arginine.

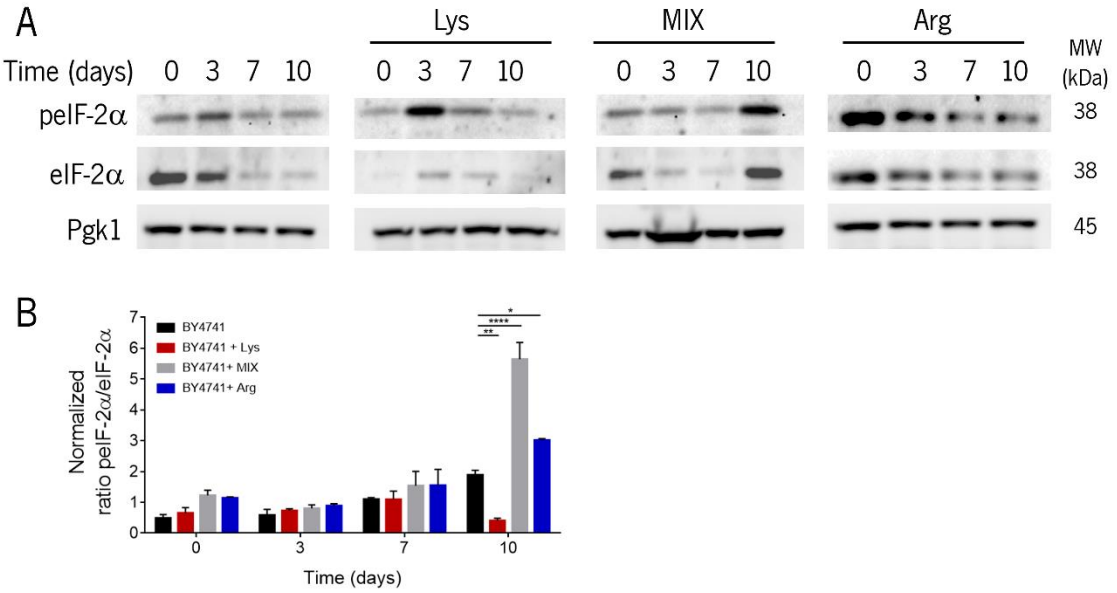

Fig.9 Supplementation with MIX and arginine increase eIF-2α phosphorylation levels in wild type cells: Western blotting of **(A)** phosphorylation levels of peIF-2α, eIF-2α and PJK, and **(B)** graphical representation of the ratio between peIF-2α/ eIF-2α protein expression levels obtained by densitometric analysis. PJK was used as a standard control. Densitometric analysis was performed using the ImageLab4.1TM software. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA (*p<0.05,**p≤0.01; ****p≤0.0001).

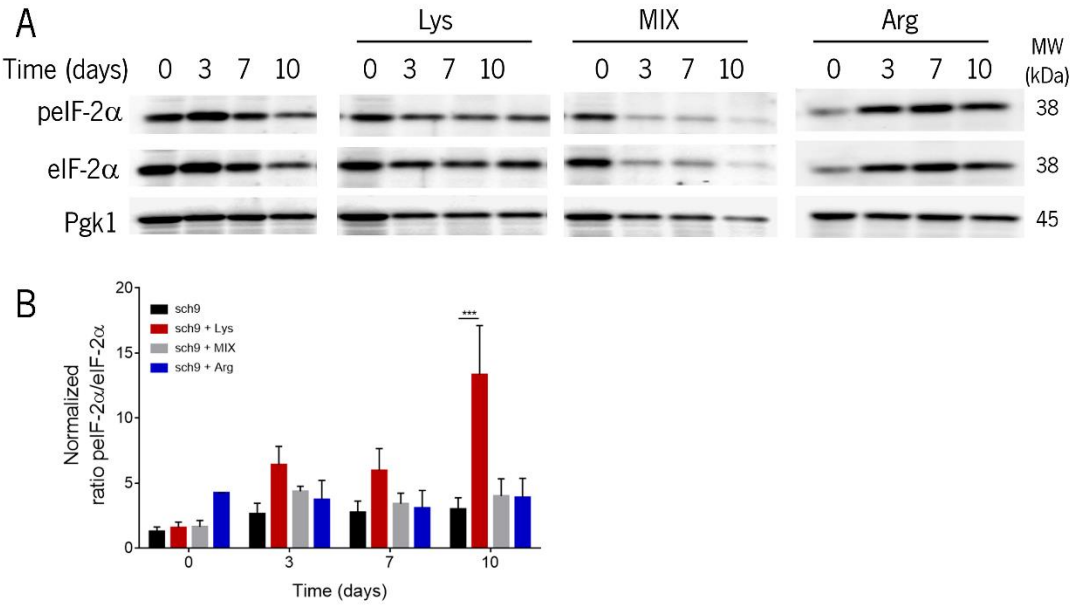

Fig.10 Supplementation with lysine increase eIF-2α phosphorylation levels in *sch9Δ* cells: Western blotting of **(A)** phosphorylation levels of peIF-2α, eIF-2α and PJK, and **(B)** graphical representation of the ratio between peIF-2α/ eIF-2α protein expression levels obtained by densitometric analysis. PJK was used as a standard control. Densitometric analysis was performed using the ImageLab4.1TM software. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA (p***≤0.001).

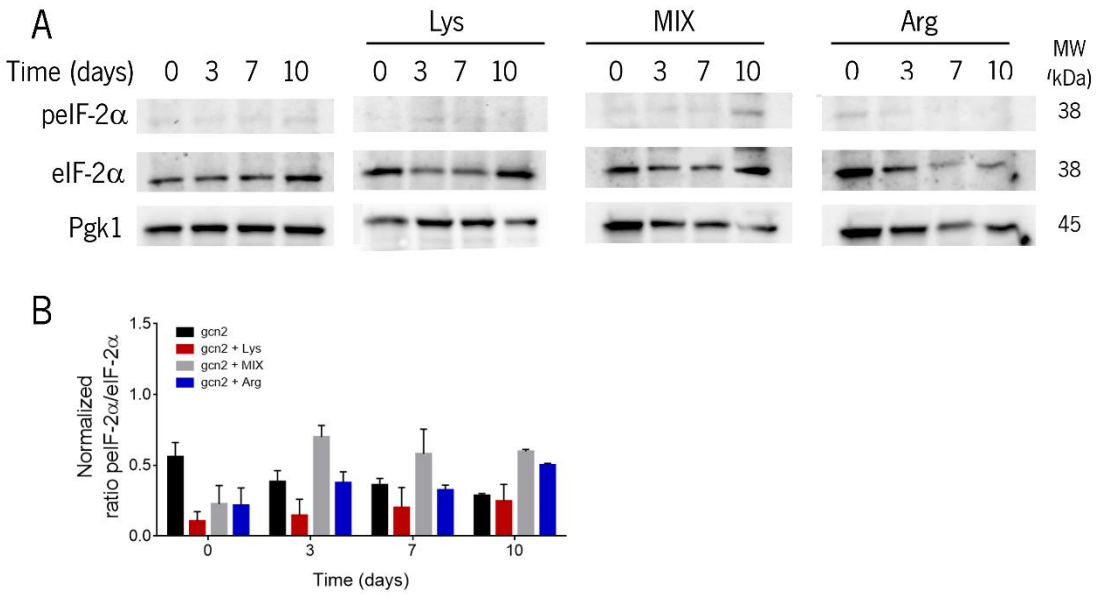

Fig.11 *GCN2* deletion abrogates eIF-2α phosphorylation independently of the supplementation: Western blotting of **(A)** phosphorylation levels of peIF-2α, eIF-2α and PJK, and (B) graphical representation of the ratio between peIF-2α/ eIF-2α protein expression levels obtained by densitometric analysis. PJK was used as a standard control. Densitometric analysis was performed using the ImageLab4.1TM software. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA.

4.5. Medium alkalization under CSCAAs supplementation is not associated with the enhancement of longevity

It has been reported by several authors, that the medium pH influences yeast aging.[183,184] According to the literature, medium alkalinization promotes cell survival, as low pH values, caused by yeast metabolism and increased oxidative stress, accelerate aging.[174] Based on this, we wondered if the effect caused by supplementation with CSCAAs on aging is related to medium pH changes.

In wild type cells, supplementation of cultures with lysine did not cause significant changes in medium pH compared to non-supplemented cultures. However, cultures supplemented with arginine and amino acids MIX increased medium pH (Fig. 12A), suggesting that the pro-survival effects of these supplementations may be related to medium alkalinization. This effect is similar to CR, which also promotes medium pH increase, some authors attribute this effect to the accumulation of acetic acid reduction, which occurs with aging and is toxic to cells.[184]

After verifying that CSCAAs supplementation influences the medium pH of wild type cells, we tested whether supplementation has the same effect on *tor1Δ, sch9Δ,* and *gcn2Δ* cells. Arginine and amino acids MIX supplementation also promote medium alkalization (Fig. 12B, C and D), just as happens in wild type cells.

Herein, results suggests that pH is not related to pro-survival effects caused by CSCAAs supplementation because some supplementations that increase mutants CLS does not increase the pH, while the supplementation of these mutants decreased the CLS and increased pH.

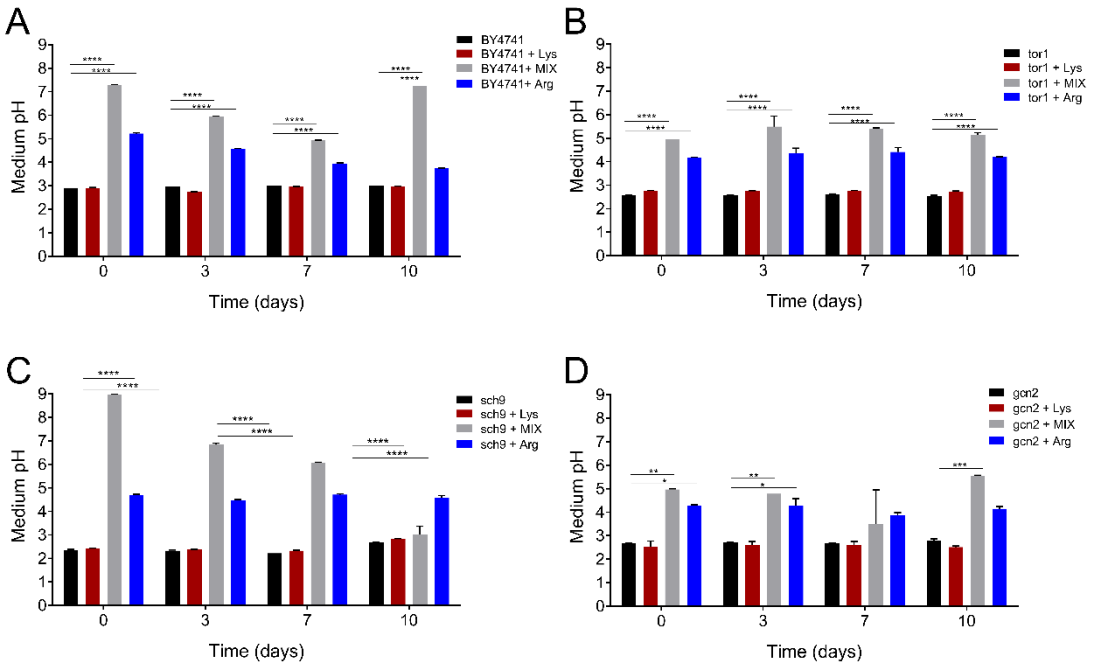

Fig.12 CSCAAs supplementation increase extracellular pH: Medium pH of **(A)** wild-type yeast cells (BY4741), **(B)** *tor1Δ* **(C)** *sch9Δ* **(D)** *gcn2Δ,*non-supplemented, supplemented with arginine, lysine, and MIX. Values indicate mean ± SEM from three independent experiments. Significance of the values between control and supplemented mediums was determined by two-way ANOVA (*p≤0.05; ****p≤0.0001).

4.6. The contribution of vacuolar amino acid transporters for the longevity regulation in conditions of CSCAAs supplementation

The vacuole is an important yeast organelle, analogous to the mammalian lysosome, playing several roles in the cellular stress response.[185] This organelle is also the major storage compartment for amino acids in the cell, and there is a correlation between vacuolar and amino acids homeostasis. [129,158] Several observations suggest that pH controls V-ATPase activity and consequently regulates the transport of protons to the vacuole, altering vacuolar pH.[186]

Several membrane transporters use V-ATPase as an electrochemical proton pump to ensure the necessary gradient for substrate transport.[155] These transporters can mediate the gradient of amino acids inside the cells, they are actively transported from the cytoplasm to the vacuole and mitochondria or vice versa. In this sense, next we tested the role of the vacuolar transporters in the modulation of yeast longevity under amino acids supplementation. As in this work, we manipulated the concentration of neutral (glutamate and aspartic acid) and basic (lysine and arginine) amino acids, we chose to study the transporters AVT3 and AVT4, because AVT3 exports neutral amino acids, and AVT4 exports neutral and basic amino acids.

To understand whether the vacuolar amino acid transporters contribute to the CLS regulation in these conditions of CSCAAs supplementation, we used *avt3Δ*, *avt4Δ,* and *avt3Δavt4Δ*, under the same supplementation conditions, and started by evaluating their CLS. We observed that the *AVT3* deletion alters CSCAAs supplementation effects. In wild type cells, amino acids MIX and arginine supplementation significantly increased the yeast CLS, however in *avt3Δ* cells, amino acids MIX and arginine supplementation loses the capacity to increment longevity (Fig. 13A). On the other hand, lysine supplementation did not promote premature aging in *avt3Δ* cells (Fig. 13A) as observed in wild type cells*,* since the CLS of mutant cells were similar under lysine supplementation and non-supplementation conditions. In *avt4Δ*, the effects of supplementation with the different amino acids are like the data observed for *avt3Δ* cells. Deletion of *AVT4* abrogates the CLS extension promoted by arginine or amino acids MIX supplementation, while lysine supplementation slightly increases the CLS of these mutant cells (Fig. 13B). Deletion of both *avt3Δ* and *avt4Δ* also abrogate the beneficial effects enhanced by arginine and amino acids MIX supplementation observed in wild type cells. In addition, supplementation with lysine did not result in a shorter CLS (Fig. 13C).

So far, we have found that Sch9 has a key role in the modulation of CLS by CSCAAs supplementation. Furthermore, we also found that supplementation impacts pH homeostasis, which interferes with AVT transporters activity. Therefore, we asked about the relationship between AVT transporters and Sch9 under CSCAAs supplementation conditions. For this, we used the *sch9Δavt3Δ*, *Δsch9Δavt4*, and *Δsch9Δavt3Δavt4* cells, and once again, we started cultures without supplementation or supplemented with arginine, lysine or the amino acids MIX, and we started by analyzing the CLS of these mutant cells. Like to effects promoted by arginine and amino acids MIX supplementation in *sch9Δ* cells, these interventions, also reduced the CLS of

sch9Δavt3Δ, *Δsch9Δavt4*, and *Δsch9Δavt3Δavt4* in comparison with non-supplemented conditions (Fig. 13D, E and F). Furthermore, the absence of *SCH9* gene in *avt3Δ, avt4Δ* or *avt3Δavt4* cells resulted in an even more pronounced CLS reduction in comparison with the *avt3Δ, avt4Δ* or *avt3Δavt4* cells (Fig. 13). Concerning lysine supplementation, like to observed for *sch9Δ* cells, this intervention is responsible for an enhancement of CLS comparing the CLS of non-supplemented cells. This effect was even more evident in *Δsch9Δavt4* cells (Fig. 13E).

Together, results suggests that besides Sch9, also Avt3 and Avt4 modulate the cells response to amino acids supplementation. Furthermore, abrogation of Sch9 exacerbates even more the effects promoted by abrogation of *AVT3* or *AVT4* genes. Data suggests that Sch9 is an upstream regulator of the transporters. Furthermore, the cells response to lysine and arginine or MIX supplementation appears to be completely distinct.

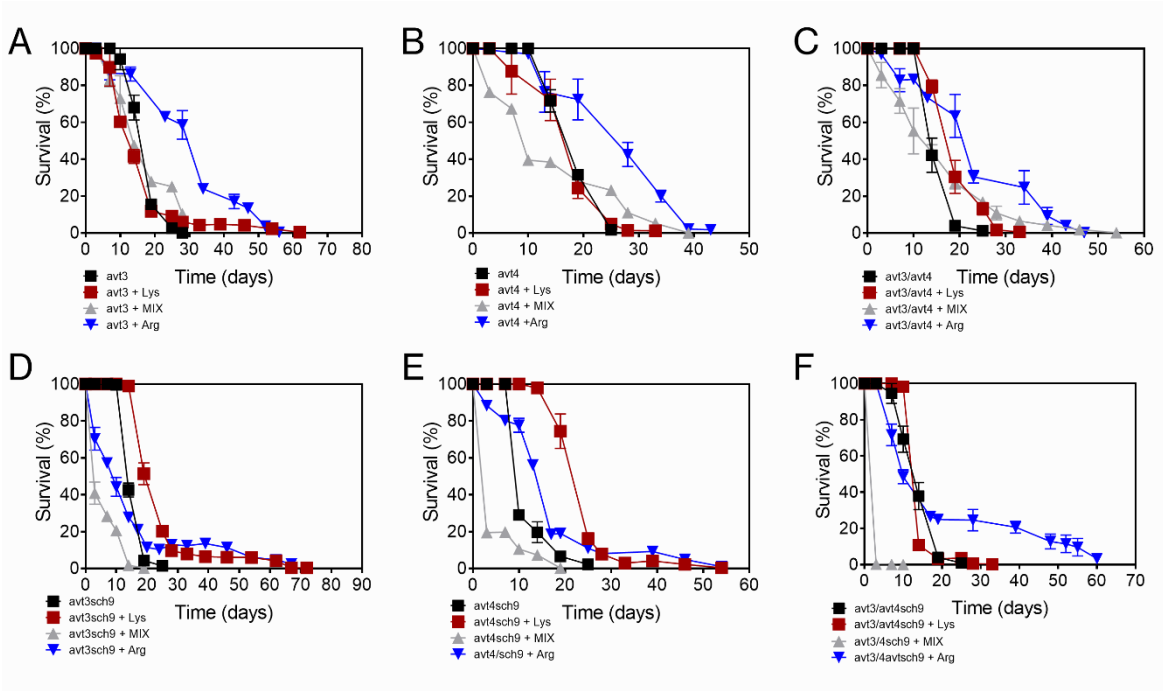

Fig. 13 Supplementation with CSCAAs modulates the CLS of AVT transporters: Chronological lifespan of mutants **(A)** *avt3Δ* **(B)** *avt4Δ* **(C)** *avt3Δavt4Δ* **(D)** *sch9Δavt3Δ* **(E)** *sch9Δavt4Δ* **(F)** *sch9Δavt3Δavt4Δ* non-supplemented, supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid). CLS of cells in non-supplemented medium (Non-sup) were used as control. Cell viability was measured at 2-3 days interval beginning at the day that cultures achieved stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%).

4.7. Contribution of vacuolar amino acid transporters in the autonomous mechanism's regulation in conditions of CSCAAs supplementation.

To conclude this study, we also evaluate whether these CSCAAs supplementation conditions modulate cell-autonomous mechanisms of vacuolar transporter mutant cells. As we have already discarded the affected of CSCAAs supplementation on autophagy, herein we just evaluate the cell cycle and ROS accumulation.

Concerning the single and the double transporter mutants, *avt3Δ* , *avt4Δ avt3Δavt4Δ*, respectively, supplementation with arginine or amino acids MIX present a more evident cell cycle arrest at S-phase in comparison with the cell cycle profile showed by non-supplemented cells. In contrast, the profile presented by lysine supplemented cells is similar to non-supplemented cells (Fig.14A, B and C). Abrogation of *SCH9* in these mutant cells abrogate the S-phase cell cycle arrest and induced an arrest at G0/G1 cell cycle phase (Fig.14D, E and F). The only exception is related with the supplementation of the amino acids MIX in *sch9Δavt3Δavt4Δ* cells, in which an arrest at S-phase is still observed (Fig.14F).

Next, we assessed the levels of endogenous accumulation of ROS, H_2O_2 and O_2^{2}, during CLS of the transporter mutants. We found that for all the used transporter mutants, supplementation with arginine or amino acids MIX decreases the accumulation of ROS in comparison with the non-supplemented cells and observed to the wild type cells. (Fig.15) In relation with lysine supplementation, accumulation of ROS has a similar profile as the non-supplemented cells (Fig.15). Thus, apparently ROS is not one responses mounted by these mutant cells to amino acids supplementation.

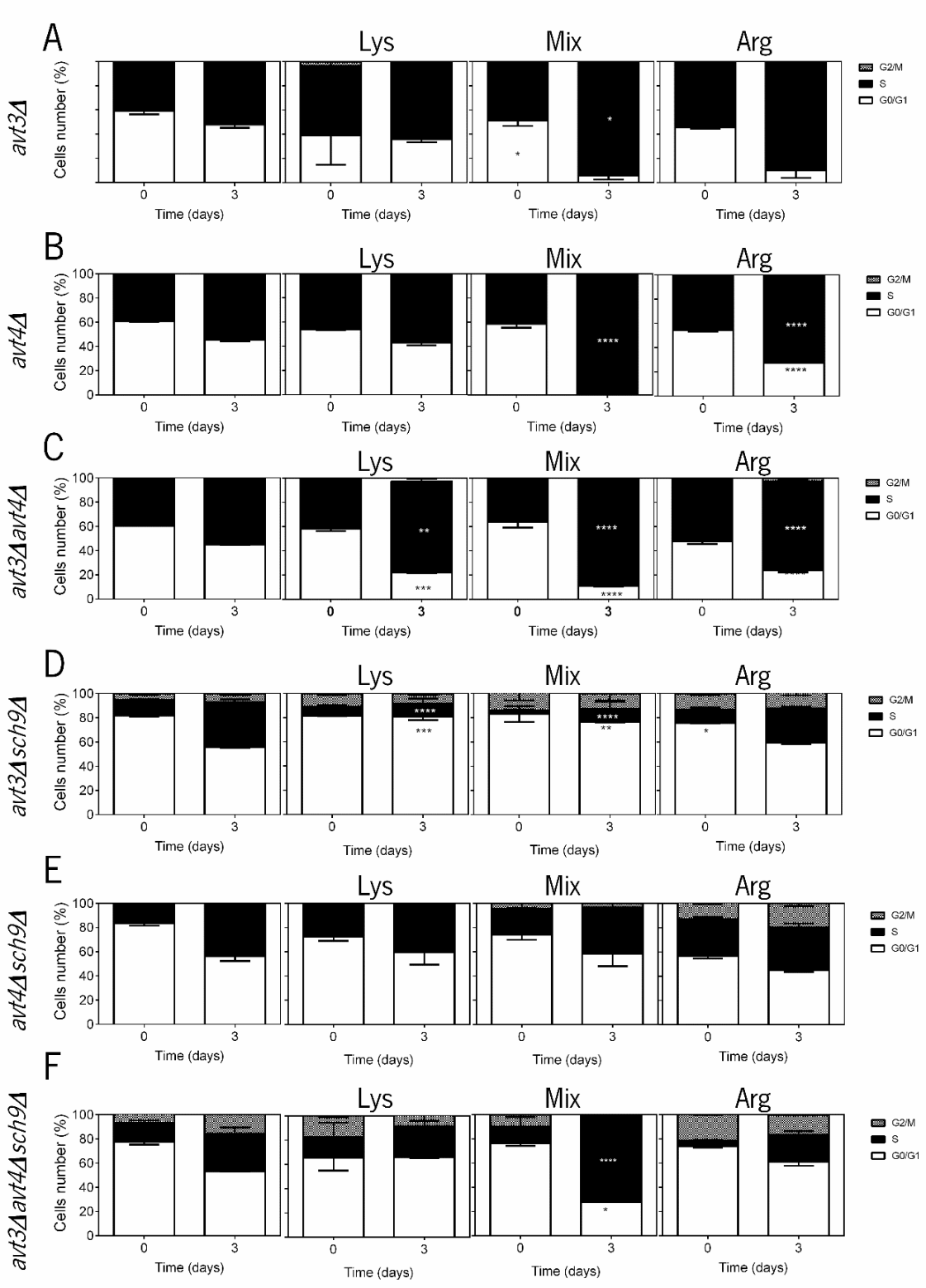

Fig. 14 Supplementation with CSCAAs modulates the cell cycle of AVT transporters: Cell cycle analysis evaluated by the measurement of DNA content by flow cytometry of mutants **A)** *avt3Δ* **(B)** *avt4Δ* **(C)** *avt3Δavt4Δ* **(D)** *sch9Δavt3Δ* **(E)** *sch9Δavt4Δ* **(F)** *sch9Δavt3Δavt4Δ* non-supplemented with supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid). The data represents mean ± SEM of three independent replicas. The

error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA (*p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001), being related to non-sup control graph.

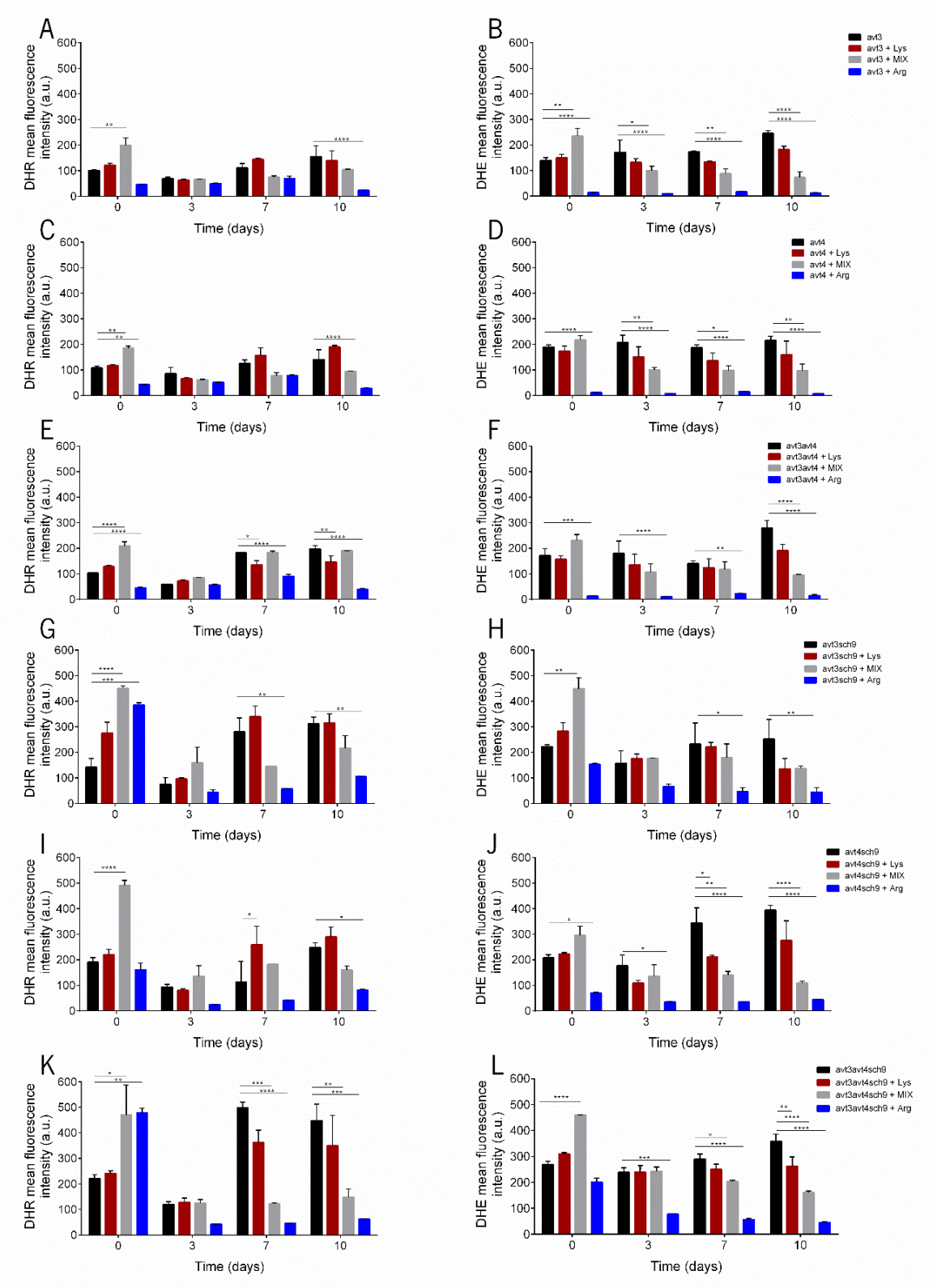

Fig.15 Supplementation with CSCAAs modulates the ROS accumulation of AVT transporters: FACS measurements of superoxide anions and hydrogen peroxide using the superoxide-specific probe DHE and using the

peroxides-specific probe DHR. We observed **(A)** *avt3Δ* label with DHR **(B)** *avt3Δ* label with DHE **(C)** *avt4Δ* label with DHR **(D)** *avt4Δ* label with DHE **(E)** *avt3Δ avt4Δ* label with DHR **(F)** *avt3Δ avt4Δ* label with DHE **(G)** *sch9Δ avt3Δ* label with DHR **(H)** *sch9Δ avt3Δ* label with DHE **(I)** *sch9Δ avt4Δ* label with DHR **(J)** *sch9Δ avt4Δ* label with DHE **(K)** *sch9Δ avt3Δ avt4Δ* label with DHR **(L)** *sch9Δ avt3Δ avt4Δ* label with DHE, non-supplemented, supplemented with arginine, lysine, and MIX (Arginine, Glutamic acid and Aspartic acid). FACS measurements (20,000 cells per sample in three independent experiments) of DHE-positive cells and DHR mean fluorescence. Values indicate mean ± SEM from three independent experiments. Significance of the values was determined by two-way ANOVA (*p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001).

CHAPTER 5

CONCLUSION

Amino acids are recognized as essential for cell survival, but with age, there is an overall decrease in amino acids.[187] Some researchers have previously showed that a specific class of amino acids called branched side-chain amino acids (BCAAs) can increase the lifespan of mice,[169,170] *C. elegans*,[171] and yeast.[168] In the same line, studies in budding yeast demonstrated that isoleucine, threonine, and valine increase the CLS.[187] However, the players that contribute to these beneficial effects are still poorly explored. In this project, we explored the impact of another specific group of amino acids, the CSCAAs, on yeast CLS and the signaling pathways elicited by their supplementation.

Our data demonstrate that supplementation with CSCAAs, particularly with Arginine, Lysine, and a MIX of amino acids (Arginine, aspartic acid, glutamate), significantly interferes with the yeast CLS. However, the effect of supplementation with these amino acids is different. While arginine and amino acids MIX supplementations increase the yeast CLS, lysine supplementation decreases CLS. These results suggest that different amino acids may activate distinct mechanisms underlying the modulation of longevity. Furthermore, our data also indicate that the pro-longevity effects of amino acid supplementation depend on the modulation of cell-autonomous mechanisms, such as the cell cycle and the accumulation of reactive oxygen species, to reduce cell damage and promote homeostasis.

Cellular aging is characterized as a gradual decline in proliferative capacity, and a progressive accumulation of cellular and molecular damage, that results in genomic instability and loss of proteostasis.[2] Some researchers have already shown that cell cycle arrest in the G0/G1 phase is associated with increased longevity, as it prevents cells in the stationary phase from entering the cell cycle again.[27] Our results demonstrated that arginine and amino acids MIX supplementation, which were the most efficient in increasing CLS, promote an effective arrest in G0/G1 phase. These data suggest that supplementation with these amino acids decreases the genomic instability characteristic of aging. The same researchers also described that G0/G1 arrest is accompanied by decreased levels of superoxide anion (O_2^{2-}).[27] As revealed, medium supplementation with arginine and amino acids MIX, which increased the arrest in G0/G1, decreased the accumulation of O_2^{2-}. Furthermore, our data show that in addition to lowering O_2^{2-} levels, supplementation with these amino acids also lowers H_2O_2 levels. Therefore, our results suggest that CSCAAs supplementation reduces ROS accumulation.

Aging is a complex and multi-factorial process that leads to a cumulative development of molecular alterations that disrupt the cellular functions.[188] Recently, it was suggested that the nutritional balance, particularly the ratio between key nutrients in the growth medium, instead merely CR might enhance longevity. For example amino acids and glucose independently and additively affect longevity by the activation of distinct evolutionarily conserved protein kinases.[108] In particularly, it is described that amino acids affect longevity through, Gcn2 as well as through activation of the upstream regulators of Sch9, such as TORC1.[108] In fact, in this study, we showed that enhancement of longevity promoted by medium supplementation with CSCAAs is related with the activation of Sch9 and Gcn2 kinases. Furthermore, it is described that TORC1 is the major regulator of Sch9, a pathway that coordinate nutrient availability with cell cycle progression, cell size, autophagy, stress resistance, sphingolipid metabolism and protein synthesis.[108,188] Nevertheless, herein, we showed that the role of Sch9 in aging modulation by CSCAAs supplementation is independent of TORC1, thus discarding a possible TORC1 function in this growing conditions. It is described that Sch9 is activated by at least three kinases, TORC1, Pkh1/2 kinases and Snf1, which phosphorylate Sch9 on different residues.[188] We hypothesized that in these conditions of CSCAAs supplementation, activation of Sch9 could be under the control of one of the above kinases. Therefore, in the future, we intend to understand the crosstalk between Sch9 and Pkh1/2 kinases or Sch9 and Snf1, to further complement this intricate puzzle.

Importantly, we also disclose that the cells response to lysine and arginine or amino acid MIX supplementation is under the control of Sch9 and Gcn2, but apparently with distinct effects. Therefore, data suggests that medium supplementation with arginine or amino acid MIX results in the activation of Sch9 and Gcn2 to enhance cells longevity. Furthermore, data also indicates that Sch9 and Gcn2 are coordinate in the same pathway, and in fact Gcn2 is a downstream target of Sch9, since deletion of SCH9 gene abrogates eIF-2α phosphorylation. In contrast, in conditions of medium supplementation with lysine, Gcn2 appears to be the major regulator, with a minor contribution of Sch9. Moreover, in these conditions, Gcn2 activation results in premature aging. Further studies needed to be performed to better corroborate the obtained data.

Ensuring amino acid homeostasis is essential to increasing yeast longevity.[128] One of the main responsible for maintaining homeostasis is the AVT family of transporters that ensure the transport of amino acids from the vacuole, the main storage site of amino acids, to the cytoplasm and mitochondria.[123] Based on this, we also studied the importance of AVT transporters in

modulating longevity under these conditions of CSCAA supplementation. CSCAAs comprise neutral (glutamate and aspartic acid) and basic (lysine and arginine) amino acids, thus herein we study the role Avt3 and Avt4 transporters, because Avt3 exports neutral amino acids, and Avt4 exports neutral and basic amino acids.[129] Deletion of *AVT3* or *AVT4* genes abrogates the longevity extension promoted by arginine or amino acids MIX supplementation. This means that AVT transporters probably also play an important role in CLS modeling under these CSCAAs supplementation conditions. Furthermore, we also disclose that Sch9 coordinates the regulation of these transporters.

Regarding lysine supplementation, we showed that premature aging promoted by this amino acid supplementation is particularly revoked under abrogation of *SCH9* and *AVT4* genes, which suggests that in fact, besides Gcn2, Sch9 also as a role in the response to lysine supplementation.

Based on the data obtained with this project, we can propose a pathway that mediates the effects of CSCAAs supplementation on CLS. Arginine and amino acids MIX supplementation lead to phosphorylation of Sch9 that controls the activity of Gcn2, Avt3 and Avt4 promoting amino acid homeostasis (Fig. 16). Herein, we still need to disclose the crosstalk between Gcn2 and the AVT transporters. Concerning lysine supplementation, this amino acid can induce the activation of Gcn2 that consequently reduces cells longevity. Data also indicates that Sch9 and Avt4 also participates in this mechanism. Nevertheless, the link between Gcn2 and Sch9 is still undisclosed (Fig.17).

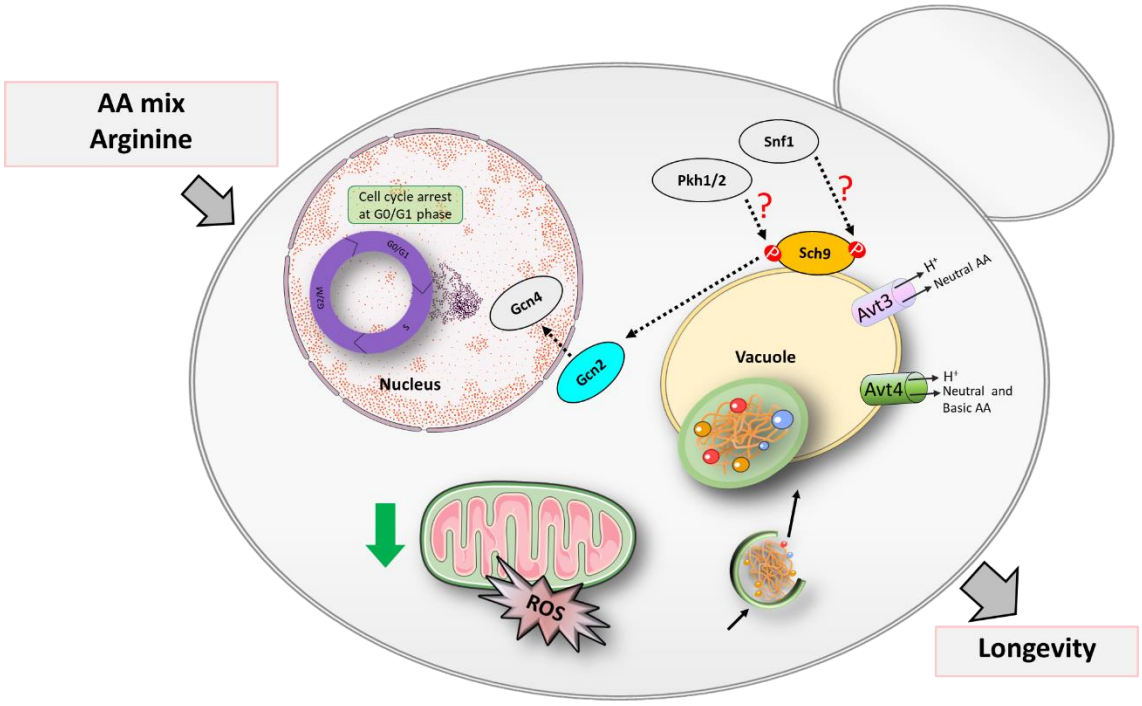

Fig.16: General effect of arginine and MIX supplementation: Arginine and MIX supplementation decrease ROS accumulation and promote G0/G1 cell cycle arrest. Supplementation with arginine and MIX also leads to Sch9 phosphorylation, which in turn modulate the activity of AVT3 and AVT4, promoting amino acids homeostasis. Sch9 also regulate de activity of GCN2.

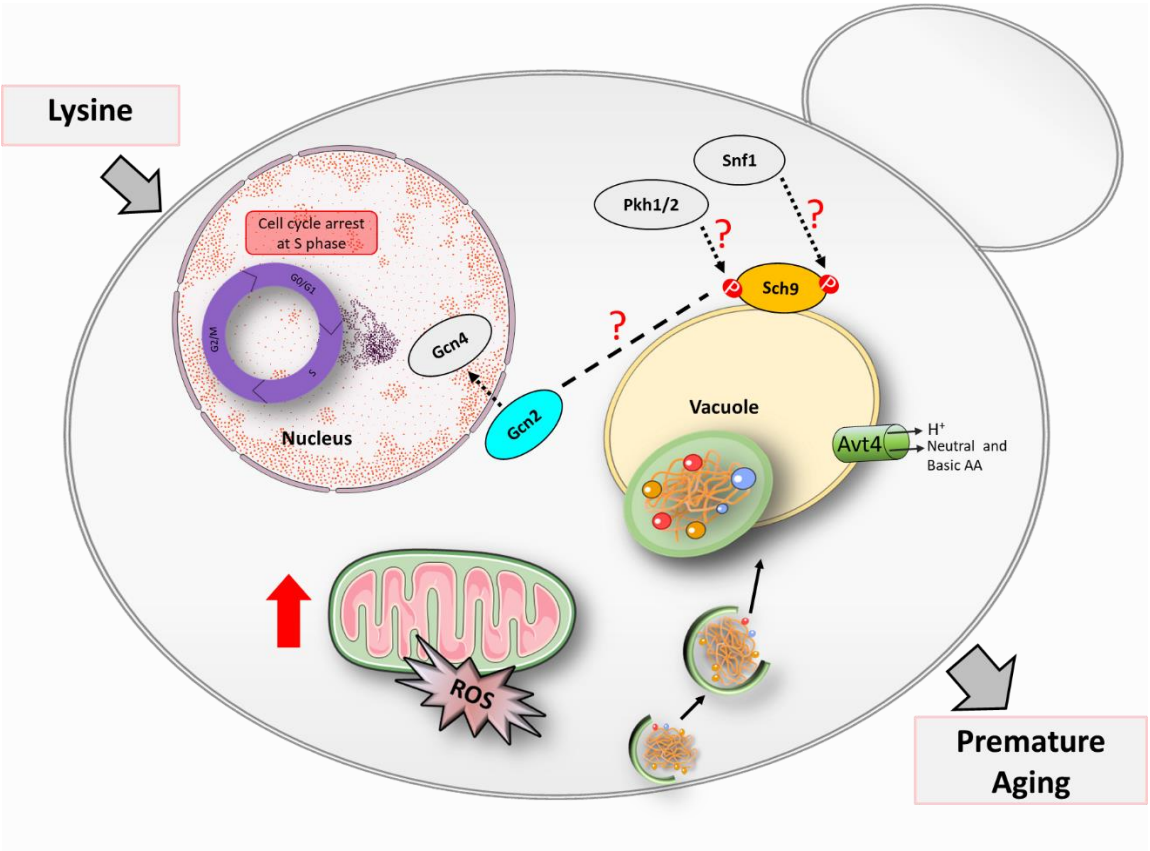

Fig.17: Lysin supplementation leads to premature aging: supplementation with lysin increase ROS accumulation and promote S-phase cell cycle arrest. Lysin supplementation also induce the activation of Gcn2 and reduce cells longevity. Sch9 and Avt4 also participates in this mechanis.

CHAPTER 6

REFERENCES

1. Vijg, J. & Kennedy, B. K. The Essence of Aging. *Gerontology* **62**, 381–385 (2016).
2. Campisi, J. *et al.* From discoveries in ageing research to therapeutics for healthy ageing. *Nature* **571**, 183–192 (2019).
3. João Pinto da Costa, Rui Vitorinob, Gustavo M. Silva, Christine Vogel, Armando C. Duarte, and T. R.-S. A synopsis on aging—Theories, mechanisms and future prospects. *Ageing Res. Rev.* **29**, 90–112 (2016).
4. Taormina, G. *et al.* Longevity: Lesson from model organisms. *Genes (Basel).* **10**, (2019).
5. Ljubuncic, P. & Reznick, A. Z. The evolutionary theories of aging revisited - A mini-review. *Gerontology* **55**, 205–216 (2009).
6. Gavrilov, L. A. & Gavrilova, N. S. Evolutionary theories of aging and longevity. *Sci. World J.* **2**, 339–356 (2002).
7. Harman, D. The aging process. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 7124–7128 (1981).
8. Lee, J. S. Cellular senescence, aging, and age-related disease: Special issue of bmb reports in 2019. *BMB Rep.* **52**, 1–2 (2019).
9. López-otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The Hallmarks of Aging. *Cell* **153**, 1194–1217 (2013).
10. Longo, V. D., Shadel, G. S., Kaeberlein, M. & Kennedy, B. Replicative and chronological aging in *Saccharomyces cerevisiae. Cell Metab.* **16**, 18–31 (2012).
11. Jessica M. Hoffman, Yang Lyu, Scott D. Pletcher, D. E. L. P. Proteomics and metabolomics in ageing research: from biomarkers to systems biology. *Essays Biochem.* **61**, 379–388 (2017).
12. Finch, C. E. & Ruvkun, G. The Genetics Of Aging. *Annu. Rev. Genomics Hum. Genet.* **2**, 435–62 (2001).
13. Jung, P. P. *et al.* Natural variation of chronological aging in the *Saccharomyces cerevisiae* species reveals diet-dependent mechanisms of life span control. *Nature* **4**, 1–11 (2018).
14. Ferraz, R. C. *et al.* IMPACT is a GCN2 inhibitor that limits lifespan in *Caenorhabditis elegans*. *BMC Biol.* **14**, 1–15 (2016).
15. Brooks-Wilson, A. R. Genetics of healthy aging and longevity. *Hum. Genet.* **132**, 1323–1338 (2013).
16. Falcón, P., Escandón, M., Brito, Á. & Matus, S. Nutrient sensing and redox balance: GCN2

as a new integrator in aging. *Oxid. Med. Cell. Longev.* **2019**, 8–10 (2019).

17. Guimarães, G. R. *et al.* Hallmarks of Aging in Macrophages: Consequences to Skin Inflammaging. *Cells* **10**, 1323 (2021).
18. Rebelo-Marques, A. *et al.* Aging hallmarks: The benefits of physical exercise. *Front. Endocrinol. (Lausanne).* **9**, 1–15 (2018).
19. McHugh, D. & Gil, J. Senescence and aging: Causes, consequences, and therapeutic avenues. *J. Cell Biol.* **217**, 65–77 (2018).
20. van der Rijt, S., Molenaars, M., McIntyre, R. L., Janssens, G. E. & Houtkooper, R. H. Integrating the Hallmarks of Aging Throughout the Tree of Life: A Focus on Mitochondrial Dysfunction. *Front. Cell Dev. Biol.* **8**, 1–12 (2020).
21. Aunan, J. R., Watson, M. M., Hagland, H. R. & Søreide, K. Molecular and biological hallmarks of ageing. *Br. J. Surg.* **103**, 29–46 (2016).
22. Vermeij, W. P., Hoeijmakers, J. H. J. & Pothof, J. Aging : not all DNA damage is equal. *Curr. Opin. Genet. Dev.* **26**, 124–130.
23. Belém Sampaio-Marques, William C. Burhans, P. L. *Yeast at the Forefront of Research on Ageing and Age-Related Diseases*. vol. 58 (Springer International Publishing, 2019).
24. Vijg, J. & Suh, Y. Genome instability and aging. *Annu. Rev. Physiol.* **75**, 645–668 (2013).
25. Kamada, Y. *et al.* Tor Directly Controls the Atg1 Kinase Complex To Regulate Autophagy. *Mol. Cell. Biol.* **30**, 1049–1058 (2010).
26. Powers, R. W., Kaeberlein, M., Caldwell, S. D., Kennedy, B. K. & Fields, S. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev.* **20**, 174–184 (2006).
27. Mesquita, A. *et al.* Growth signaling promotes chronological aging in budding yeast by inducing superoxide anions that inhibit quiescence. *Aging (Albany. NY).* **2**, 709–726 (2010).
28. Weinberger, M. *et al.* DNA replication stress is a determinant of chronological lifespan in budding yeast. *PLoS One* **2**, e748 (2007).
29. Talens, R. P. *et al.* Epigenetic variation during the adult lifespan: Cross-sectional and longitudinal data on monozygotic twin pairs. *Aging Cell* **11**, 694–703 (2012).
30. Dali-Youcef, N. *et al.* Sirtuins: The 'magnificent seven', function, metabolism and longevity. *Ann. Med.* **39**, 335–345 (2007).

31. Sampaio-Marques, B. *et al.* SNCA (α-synuclein)-induced toxicity in yeast cells is dependent on sirtuin 2 (Sir2)-mediated mitophagy. *Autophagy* **8**, 1494–1509 (2012).

32. Sampaio-Marques, B. & Ludovico, P. Sirtuins and proteolytic systems: Implications for pathogenesis of synucleinopathies. *Biomolecules* **5**, 735–757 (2015).

33. Sen, P., Shah, P. P., Nativio, R. & Berger, S. L. Epigenetic mechanisms regulating longev. **166**, 822–839 (2018).

34. Tyedmers, J., Mogk, A. & Bukau, B. Cellular strategies for controlling protein aggregation. *Nat. Rev. Mol. Cell Biol.* **11**, 777–788 (2010).

35. Hartl, F. U., Bracher, A. & Hayer-Hartl, M. Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324–332 (2011).

36. Morimoto, J. L. and R. I. The Biology of Proteostasis in Aging and Disease. *Annu Rev Biochem* 435–464 (2015)

37. Chiti, F. & Dobson, C. M. Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* **75**, 333–366 (2006).

38. Kaushik, S. & Cuervo, A. M. Proteostasis and aging. *Nat. Med.* **21**, 1406–1415 (2015).

39. Sampaio-Marques, B. & Ludovico, P. Linking cellular proteostasis to yeast longevity. *FEMS Yeast Res.* **18**, foy043 (2018).

40. Hughes, A. L., Gottschling, D. E. & Hutchinson, F. An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. *Nature* **492**, 261–265 (2013).

41. Laun, P. *et al.* Aged mother cells of *Saccharomyces cerevisiae* show markers of oxidative stress and apoptosis. *Mol. Microbiol.* **39**, 1166–1173 (2001).

42. Cuervo, A. M. & Dice, J. F. Age-related decline in chaperone-mediated autophagy. *J. Biol. Chem.* **275**, 31505–31513 (2000).

43. Tonoki, A. *et al.* Genetic Evidence Linking Age-Dependent Attenuation of the 26S Proteasome with the Aging Process. *Mol. Cell. Biol.* **29**, 1095–1106 (2009).

44. Mark S Hipp, Sae-Hun Park, F. U. H. Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends Cell Biol* **24**, 506–514 (2014).

45. Lilienbaum, A. Relationship between the proteasomal system and autophagy. *Int. J. Biochem. Mol. Biol.* **4**, 1–26 (2013).

46. Selimovic, D. *et al.* Bortezomib/proteasome inhibitor triggers both apoptosis and autophagy-dependent pathways in melanoma cells. *Cell. Signal.* **25**, 308–318 (2013).

47. Kocaturk, N. M. & Gozuacik, D. Crosstalk between mammalian autophagy and the ubiquitin-proteasome system. *Front. Cell Dev. Biol.* **6**, 128 (2018).

48. Sampaio-Marques, B., Pereira, H., Santos, A. R., Teixeira, A. & Ludovico, P. Caloric restriction rescues yeast cells from alpha-synuclein toxicity through autophagic control of proteostasis. *Aging (Albany. NY).* **10**, 3821–3833 (2018).

49. Galluzzi, L. *et al.* Molecular definitions of autophagy and related processes. *EMBO J.* **36**, 1811–1836 (2017).

50. Horst, M., Knecht, E. C. & Schu, P. V. Import into and degradation of cytosolic proteins by isolated yeast vacuoles. *Mol. Biol. Cell* **10**, 2879–2889 (1999).

51. Marisa Ponpuak, Michael Mandell, Tomonori Kimura, Santosh Chauhan, Cédric Cleyrat, and V. D. Secretory autophagy. *Curr Opin Cell Biol* **35**, 106–116 (2015).

52. Leidal, A. M., Levine, B. & Debnath, J. Autophagy and the cell biology of age-related disease. *Nat. Cell Biol.* **20**, 1338–1348 (2018).

53. An, Z. *et al.* Autophagy is required for G1/G0quiescence in response to nitrogen starvation in *Saccharomyces cerevisiae*. *Autophagy* **10**, 1702–1711 (2014).

54. Shi Q. Wong, Anita V. Kumar, Joslyn Mills, L. R. L. Autophagy in aging and longevity. *Physiol. Behav.* **136**, 277–290 (2020).

55. Ciechanover, A. & Kwon, Y. T. a. Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. *Exp. Mol. Med.* **47**, e147 (2015).

56. Pyo, J. O. *et al.* Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat. Commun.* **4**, 1–9 (2013).

57. Koga, H., Kaushik, S. & Cuervo, A. M. Protein homeostasis and aging: The importance of exquisite quality control. *Ageing Res. Rev.* **10**, 205–215 (2011).

58. Fontana, L., Partridge, L. & Longo, V. D. Dietary Restriction, Growth Factors and Aging: from yeast to humans. *Science (80-.).* **328**, 321–6 (2010).

59. Kudryavtseva, A. V. *et al.* Mitochondrial dysfunction and oxidative stress in aging and cancer. *Oncotarget* **7**, 44879–44905 (2016).

60. Fisher, S. J. A. and P. R. Mitochondria in health and disease. *Cells* **8**, 680 (2019).

61. Webb, M. & Sideris, D. P. Intimate Relations — Mitochondria and Ageing. *Int. J. Mol. Sci.* **21**, 7580 (2020).

62. van der Giezen, M. & Tovar, J. Degenerate mitochondria. *EMBO Rep.* **6**, 525–530 (2005).

63. Hoogenraad, N. Mitochondria: In sickness and in health. *Today's Life Sci.* **2**, 37–39 (1990).

64. Nicholas D. Bonawitz, Marc Chatenay-Lapointe, Yong Pan, and G. S. S. Reduced TOR Signaling Extends Chronological Life Span via Increased Respiration and Upregulation of Mitochondrial Gene Expression. *Cell Metab* **5**, 265–277 (2007).

65. Kwon, Y. Y., Lee, S. K. & Lee, C. K. Caloric restriction-induced extension of chronological lifespan requires intact respiration in budding yeast. *Mol. Cells* **40**, 307–313 (2017).

66. Poljsak, B. Strategies for reducing or preventing the generation of oxidative stress. *Oxid. Med. Cell. Longev.* **2011**, 15 (2011).

67. Paul D. Ray, Bo-Wen Huang, and Y. T. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal.* **24**, 281–990 (2012).

68. Dodig, S., Čepelak, I. & Pavić, I. Hallmarks of senescence and aging. *Biochem. Medica* **29**, 1–15 (2019).

69. Beckhauser, T. F., Francis-Oliveira, J. & De Pasquale, R. Reactive oxygen species: Physiological and physiopathological effects on synaptic plasticity. *J. Exp. Neurosci.* **2016**, 23–48 (2016).

70. Sohal, R. S. & Weindruch, R. Oxidative stress, caloric restriction, and aging. *Science.* **273**, 59–63 (1996).

71. Schriner, S. E. *et al.* Medecine: Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science (80).* **308**, 1909–1911 (2005).

72. Kwilasz, A. J. Mitochondria in the signaling pathways that control longevity and health span. *Physiol. Behav.* **176**, 139–148 (2016).

73. Ristow, M. & Zarse, K. How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). *Exp. Gerontol.* **45**, 410–418 (2010).

74. Archer, C. R., Sakaluk, S. K., Selman, C., Royle, N. J. & Hunt, J. Oxidative stress and the evolution of sex differences in life span and ageing in the decorated cricket, Gryllodes sigillatus. *Evolution (N. Y).* **67**, 620–634 (2013).

75. Milisav, I., Ribarič, S. & Poljsak, B. Antioxidant vitamins and ageing. *Subcell. Biochem.* **90**, 1–23 (2018).

76. Harman, D. Free radical theory of aging: An update - Increasing the functional life span. *Ann. N. Y. Acad. Sci.* **1067**, 10–21 (2006).

77. Wickens, A. P. Ageing and the free radical theory. *Respir. Physiol.* **128**, 379–391 (2001).

78. Ludovico, P. & Burhans, W. C. Reactive oxygen species, ageing and the hormesis police. *FEMS Yeast Res.* **14**, 33–39 (2014).

79. Mesquita, A. *et al.* Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15123–15128 (2010).

80. Bettedi, L. & Foukas, L. C. Growth factor , energy and nutrient sensing signalling pathways in metabolic ageing. *Biogerontology* **18**, 913–929 (2017).

81. Ungvari, Z. *et al.* Mechanisms of Vascular Aging. *Circ. Res.* **123**, 849–867 (2018).

82. Lin, S. J., Defossez, P. A. & Guarente, L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in saccharomyces cerevisiae. *Science (80-.).* **289**, 2126–2128 (2000).

83. Speakman, J. R. & Mitchell, S. E. Caloric restriction. *Mol. Aspects Med.* **32**, 159–221 (2011).

84. Micó, V., Berninches, L., Tapia, J. & Daimiel, L. Nutrimiraging: Micromanaging nutrient sensing pathways through nutrition to promote healthy aging. *Int. J. Mol. Sci.* **18**, 915 (2017).

85. Su-Ju Lin, Matt Kaeberlein, Alex A. Andalis, Lori A. Sturtz, Pierre-Antoine Defossez, Valeria C. Culotta§, Gerald R. Fink, L. G. Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* **418**, 334–348 (2002).

86. Michiko Kato., S.-J. L. Regulation of NAD+ metabolism, signaling and compartmentalization in the yeast *Saccharomyces cerevisiae*. *DNA Repair (Amst).* **23**, 49–58 (2014).

87. Guedes, A., Ludovico, P. & Sampaio-Marques, B. Caloric restriction alleviates alpha-synuclein toxicity in aged yeast cells by controlling the opposite roles of Tor1 and Sir2 on autophagy. *Mech. Ageing Dev.* **161**, 270–276 (2017).

88. Medvedik, O., Lamming, D. W., Kim, K. D. & Sinclair, D. A. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in *Saccharomyces cerevisiae. PLoS Biol.* **5**, 2330–2341 (2007).

89. Wierman, M. B. & Smith, J. S. Yeast sirtuins and the regulation of aging. *FEMS Yeast Res.* **14**, 73–88 (2014).

90. He, C., Zhou, C. & Kennedy, B. K. The yeast replicative aging model. *Biochim. Biophys.*

Acta - Mol. Basis Dis. **1864**, 2690–2696 (2018).

91. Dilova, I., Easlon, E. & Lin, S. J. Calorie restriction and the nutrient sensing signaling pathways. *Cell. Mol. Life Sci.* **64**, 752–767 (2007).
92. Kaeberlein, M., Burtner, C. R. & Kennedy, B. K. Recent developments in yeast aging. *PLoS Genet.* **3**, 655–660 (2007).
93. Zimmermann, A. *et al.* Yeast as a tool to identify anti-aging compounds. *FEMS Yeast Res.* **18**, 1–16 (2018).
94. Eleutherio, E. *et al.* Oxidative stress and aging: Learning from yeast lessons. *Fungal Biol.* **122**, 514–525 (2018).
95. Polymenis, M. & and Brian K. Kennedy. Cell Cycle News & Views Chronological and replicative lifespan in yeast : Do they meet in the middle ? *Cell cycle* **11**, 3531–3535 (2012).
96. Maruyama, Y., Ito, T., Kodama, H. & Matsuura, A. Availability of amino acids extends chronological lifespan by suppressing hyper-acidification of the environment in *Saccharomyces cerevisiae*. *PLoS One* **11**, 1–19 (2016).
97. Molon, M. & Zadrag-Tecza, R. Effect of temperature on replicative aging of the budding yeast *Saccharomyces cerevisiae. Biogerontology* **17**, 347–357 (2016).
98. Yuan, W. *et al.* General control nonderepressible 2 (GCN2) kinase inhibits target of rapamycin complex 1 in response to amino acid starvation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **292**, 2660–2669 (2017).
99. Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124**, 471–484 (2006).
100. Bjornsti, M. A. & Houghton, P. J. The TOR pathway: A target for cancer therapy. *Nat. Rev. Cancer* **4**, 335–348 (2004).
101. Abraham, R. T. Identification of TOR signaling complexes: More TORC for the cell growth engine. *Cell* **111**, 9–12 (2002).
102. Valter D. Longo, Gerald S. Shadel, Matt Kaeberlein, and B. K. Replicative and Chronological Aging in *Saccharomyces cerevisiae*. *Cell Metab* **16**, 18–31 (2012).
103. Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M. & Longo, V. D. Regulation of longevity and stress resistance by Sch9 in yeast. *Science (80-.).* **292**, 288–290 (2001).
104. Breitenbach, M., Laun, P. & Gimona, M. The actin cytoskeleton, RAS-cAMP signaling and

mitochondrial ROS in yeast apoptosis. *Trends Cell Biol.* **15**, 637–639 (2005).

105. Xie, Y. & Varshavsky, A. RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: A negative feedback circuit. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3056–3061 (2001).
106. Kaeberlein, M., Kirkland, K. T., Fields, S. & Kennedy, B. K. Sir2-independent life span extension by calorie restriction in yeast. *PLoS Biol.* **2**, 1381–1387 (2004).
107. Fabrizio, P. *et al.* Sir2 blocks extreme life-span extension. *Cell* **123**, 655–667 (2005).
108. Deprez, M. A., Eskes, E., Winderickx, J. & Wilms, T. The TORC1-Sch9 pathway as a crucial mediator of chronological lifespan in the yeast *Saccharomyces cerevisiae. FEMS Yeast Res.* **18**, 48 (2018).
109. Zhang, Z., Cottignie, I., van Zeebroeck, G. & Thevelein, J. M. Nutrient transceptors physically interact with the yeast S6/protein kinase B homolog, Sch9, a TOR kinase target. *Biochem. J.* **478**, 357–375 (2021).
110. Jorgensen, P. *et al.* A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes Dev.* **18**, 2491–2505 (2004).
111. Wilms, T. *et al.* The yeast protein kinase Sch9 adjusts V-ATPase assembly/disassembly to control pH homeostasis and longevity in response to glucose availability. *PLoS Genet.* **13**, 1–30 (2017).
112. Drozdova, P., Lipaeva, P., Rogoza, T., Zhouravleva, G. & Bondarev, S. Overproduction of Sch9 leads to its aggregation and cell elongation in *Saccharomyces cerevisiae*. *PLoS One* **13**, 1–16 (2018).
113. Bitterman, K. J., Medvedik, O. & Sinclair, D. A. Longevity Regulation in *Saccharomyces cerevisiae:* Linking Metabolism, Genome Stability, and Heterochromatin. *Microbiology* **67**, 376–399 (2003).
114. Swinnen, E., Ghillebert, R., Wilms, T. & Winderickx, J. Molecular mechanisms linking the evolutionary conserved TORC1-Sch9 nutrient signalling branch to lifespan regulation in *Saccharomyces cerevisiae. FEMS Yeast Res.* **14**, 17–32 (2014).
115. Liu, K., Zhang, X., Lester, R. L. & Dickson, R. C. The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in *Saccharomyces cerevisiae* including Ypk1, Ypk2, and Sch9. *J. Biol. Chem.* **280**, 22679–22687 (2005).
116. Yukiko Kabeya, Y. K. & Misuzu Baba, Hirosato Takikawa,§ Mitsuru Sasaki, and Y. O. Atg17

Functions in Cooperation with Atg1 and Atg13 in Yeast Autophagy. *Mol Biol Cell* **16**, 2544–2553 (2004).

117. Yoshiaki Kamada, Tomoko Funakoshi, Takahiro Shintani, Kazuya Nagano, Mariko Ohsumi, and Y. O. Tor-mediated Induction of Autophagy Via an Apg1 Protein Kinase Complex. *Cell Biol* **150**, 1507–1513 (29616BC).
118. Vlanti, A., Rousakis, A. & Syntichaki, P. Gcn2 and TOR converge on aging. *Aging (Albany. NY).* **5**, 584–585 (2013).
119. Van Dam, T. J. P., Zwartkruis, F. J. T., Bos, J. L. & Snel, B. Evolution of the TOR pathway. *J. Mol. Evol.* **73**, 209–220 (2011).
120. Rdøland, G. E., Tvegaŕd, T., Boye, E. & Grallert, B. Crosstalk between the Tor and Gcn2 pathways in response to different stresses. *Cell Cycle* **13**, 453–461 (2014).
121. Bröer, S. & Bröer, A. Amino acid homeostasis and signalling in mammalian cells and organisms. *Biochem. J.* **474**, 1935–1963 (2017).
122. Wu, G. Functional amino acids in nutrition and health. *Amino Acids* **45**, 407–411 (2013).
123. Bianchi, F., van't Klooster, J. S., Ruiz, S. J. & Poolman, B. Regulation of Amino Acid Transport in *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev.* **83**, (2019).
124. Rabanal-Ruiz, Y., Otten, E. G. & Korolchuk, V. I. mTORC1 as the main gateway to autophagy. *Essays Biochem.* **61**, 565–584 (2017).
125. Canfield, C. A. & Bradshaw, P. C. Amino acids in the regulation of aging and aging-related diseases. *Transl. Med. Aging* **3**, 70–89 (2019).
126. Houghton, F. D. Media composition: Amino acids and cellular homeostasis. *Methods Mol. Biol.* **912**, 97–106 (2012).
127. Wu, G. Amino acids: Metabolism, functions, and nutrition. *Amino Acids* **37**, 1–17 (2009).
128. Laura K. Fishwick, Michelle L. Marraffini, Arnold Y. Seo, Christiaan Leeuwenburgh, W. A. D. J. Amino Acid Homeostasis and Chronological Longevity in *Saccharomyces cerevisiae* John. *Subcell Biochem* **57**, 161–186 (2012).
129. Kawano-kawada, M., Kakinuma, Y. & Sekito, T. Transport of Amino Acids across the Vacuolar Membrane of Yeast: Its Mechanism and Physiological Role. *Biol. Pharm. Bull.* **41**, 1496–1501 (2018).
130. Stracka, D., Jozefczuk, S., Rudroff, F., Sauer, U. & Hall, M. N. Nitrogen source activates TOR (Target of Rapamycin) complex 1 via glutamine and independently of Gtr/Rag proteins.

J. Biol. Chem. **289**, 25010–25020 (2014).

131. Zhang, T., Péli-Gulli, M. P., Yang, H., De Virgilio, C. & Ding, J. Ego3 functions as a homodimer to mediate the interaction between Gtr1-Gtr2 and Ego1 in the EGO complex to activate TORC1. *Structure* **20**, 2151–2160 (2012).
132. Sancak, Y. *et al.* The rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science (80-.).* **320**, 1496–1501 (2008).
133. Binda, M. *et al.* The Vam6 GEF Controls TORC1 by Activating the EGO Complex. *Mol. Cell* **35**, 563–573 (2009).
134. Varlakhanova, N. V., Mihalevic, M. J., Bernstein, K. A. & Ford, M. G. J. Pib2 and the EGO complex are both required for activation of TORC1. *J. Cell Sci.* **130**, 3878–3890 (2017).
135. Bonfils, G. *et al.* Leucyl-tRNA Synthetase Controls TORC1 via the EGO Complex. *Mol. Cell* **46**, 105–110 (2012).
136. Benjamin M. Sutter1, Xi Wu, Sunil Laxman1, and B. P. T. Methionine Inhibits Autophagy and Promotes Growth by Inducing the SAM-Responsive Methylation of PP2A. *Cell* **154**, 403–415 (2013).
137. Laxman, S., Sutter, B. M. & Tu, B. P. Methionine is a signal of amino acid sufficiency that inhibits autophagy through the methylation of PP2A. *Autophagy* **10**, 386–387 (2014).
138. Tanigawa, M. & Maeda, T. An In Vitro TORC1 Kinase Assay That Recapitulates the Gtr-Independent Glutamine-Responsive TORC1 Activation Mechanism on Yeast Vacuoles. *Mol Cell Biol* **37**, (2017).
139. Masson, G. R. Towards a model of GCN2 activation. *Biochem. Soc. Trans.* **47**, 1481–1488 (2019).
140. Anda, S., Zach, R. & Grallert, B. Activation of Gcn2 in response to different stresses. *PLoS One* **12**, 1–13 (2017).
141. Inglis, A. J. *et al.* Activation of GCN2 by the ribosomal P-stalk. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 4946–4954 (2019).
142. Kim, K. *et al.* Tissue-specific roles of GCN2 in aging and autosomal dominant retinitis pigmentosa. *Biochem. Biophys. Res. Commun.* **533**, 1054–1060 (2020).
143. Murguía, J. R. & Serrano, R. New functions of protein kinase Gcn2 in yeast and mammals. *IUBMB Life* **64**, 971–974 (2012).
144. Nakamura, A. & Kimura, H. A new role of GCN2 in the nucleolus. *Biochem. Biophys. Res.*

Commun. **485**, 484–491 (2017).

145. Sood, R., Porter, A. C., Olsen, D. A., Cavener, D. R. & Wek, R. C. A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2α. *Genetics* **154**, 787–801 (2000).
146. Grallert, B. & Boye, E. GCN2, an old dog with new tricks. *Biochem. Soc. Trans.* **41**, 1687–1691 (2013).
147. Hu, Z. *et al.* Ssd1 and Gcn2 suppress global translation efficiency in replicatively aged yeast while their activation extends lifespan. *Elife* **7**, 1–24 (2018).
148. Battu, S., Minhas, G., Mishra, A. & Khan, N. Amino Acid Sensing via General Control Nonderepressible-2 Kinase and immunological Programming. *Front. Immunol.* **8**, 1–11 (2017).
149. Caballero-Molada, M. *et al.* The Gcn2–eIF2α pathway connects iron and amino acid homeostasis in *Saccharomyces cerevisiae. Biochem. J.* **475**, 1523–1534 (2018).
150. González, A. & Hall, M. N. Nutrient sensing and TOR signaling in yeast and mammals . *EMBO J.* **36**, 397–408 (2017).
151. Demaurex, N. pH homeostasis of cellular organelles. *News Physiol. Sci.* **17**, 1–5 (2002).
152. Bignell, E. *The Molecular Basis of pH Sensing, Signaling, and Homeostasis in Fungi*. *Advances in Applied Microbiology* vol. 79 (Elsevier Inc., 2012).
153. Deprez, M. A., Eskes, E., Wilms, T., Ludovico, P. & Winderickx, J. ph homeostasis links the nutrient sensing PKA/TORC1/ScH9 ménage-à-trois to stress tolerance and longevity. *Microb. Cell* **5**, 119–136 (2018).
154. Ariño, J., Ramos, J. & Sychrová, H. Alkali Metal Cation Transport and Homeostasis in Yeasts. *Microbiol. Mol. Biol. Rev.* **74**, 95–120 (2010).
155. Sato, T., Ohsumi, Y. & Anraku, Y. Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of *Saccharomyces cerevisiae*. Evidence of seven independent proton/amino acid antiport systems. *J. Biol. Chem.* **259**, 11505–11508 (1984).
156. Shimazu, M., Sekito, T., Akiyama, K., Ohsumi, Y. & Kakinuma, Y. A family of basic amino acid transporters of the vacuolar membrane from *Saccharomyces cerevisiae. J. Biol. Chem.* **280**, 4851–4857 (2005).
157. Russnak, R., Konczal, D. & McIntire, S. L. A Family of Yeast Proteins Mediating Bidirectional

Vacuolar Amino Acid Transport. *J. Biol. Chem.* **276**, 23849–23857 (2001).

158. Ishimoto, M., Sugimoto, N., Sekito, T., Kawano-Kawada, M. & Kakinuma, Y. Atp-dependent export of neutral amino acids by vacuolar membrane vesicles of saccharomyces cerevisiae. *Biosci. Biotechnol. Biochem.* **76**, 1802–1804 (2012).
159. Sekito, T., Fujiki, Y., Ohsumi, Y. & Kakinuma, Y. Novel families of vacuolar amino acid transporters. *IUBMB Life* **60**, 519–525 (2008).
160. Cools, M. *et al. Nitrogen coordinated import and export of arginine across the yeast vacuolar membrane*. *PLoS Genetics* vol. 16 (2020).
161. Kawano-Kawada, M. *et al.* Ygr125w/Vsb1-dependent accumulation of basic amino acids into vacuoles of Saccharomyces cerevisiae. *Biosci. Biotechnol. Biochem.* **85**, 1157–1164 (2021).
162. Tone, J. *et al.* Characterization of Avt1p as a vacuolar proton/amino acid antiporter in Saccharomyces cerevisiae. *Biosci. Biotechnol. Biochem.* **79**, 782–789 (2015).
163. Sekito, T. *et al.* Vacuolar transporter Avt4 is involved in excretion of basic amino acids from the vacuoles of *Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem.* **78**, 969–975 (2014).
164. Urban, J. *et al.* Sch9 Is a Major Target of TORC1 in *Saccharomyces cerevisiae*. *Mol. Cell* **26**, 663–674 (2007).
165. Hatakeyama, R. & De Virgilio, C. Unsolved mysteries of Rag GTPase signaling in yeast. *Small GTPases* **7**, 239–246 (2016).
166. Choi, K. M. *et al.* Caloric Restriction and Rapamycin Differentially Alter Energy Metabolism in Yeast. *J. Gerontol. A. Biol. Sci. Med. Sci.* **73**, 29–38 (2017).
167. Large, P. J. Degradation of organic nitrogen compounds by yeasts. *Yeast* **2**, 1–34 (1986).
168. Ashley L. Alvers, Laura K. Fishwick, Michael S. Wood, Doreen Hu, Hye Sun Chung, William A. Dunn Jr., and J. P. A. Autophagy and amino acid homeostasis are required for chronological longevity in *Saccharomyces cerevisiae. Aging Cell* **8**, 353–369 (2009).
169. Solon-Biet, S. M. *et al.* Branched-chain amino acids impact health and lifespan indirectly via amino acid balance and appetite control. *Nat. Metab.* **1**, 532–545 (2019).
170. D'Antona, G. *et al.* Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metab.* **12**, 362–372 (2010).

171. Fuchs, S. *et al.* A metabolic signature of long life in *Caenorhabditis elegans*. *BMC Biol.* **8**, 1–12 (2010).

172. Juricic, P., Grönke, S. & Partridge, L. Branched-chain amino acids have equivalent effects to other essential amino acids on lifespan and aging-related traits in drosophila. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **75**, 24–31 (2020).

173. Le Couteur, D. G. *et al.* Branched chain amino acids, aging and age-related health. *Ageing Res. Rev.* **64**, 1–46 (2020).

174. Suzuki, S. W., Onodera, J. & Ohsumi, Y. Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction. *PLoS One* **6**, 0–7 (2011).

175. Mohammad, K. & Titorenko, V. I. Yeast chronological aging is linked to cell cycle regulation. *Cell Cycle* **17**, 1035–1036 (2018).

176. Weinberger, M., Sampaio-Marques, B., Ludovico, P. & Burhans, W. C. DNA replication stress-induced loss of reproductive capacity in *S. cerevisiae* and its inhibition by caloric restriction. *Biochem. Soc. Trans.* **39**, 1466–1470 (2011).

177. Delaney, J. R. *et al.* End-of-life cell cycle arrest contributes to stochasticity of yeast replicative aging. *FEMS Yeast Res.* **13**, 267–276 (2013).

178. Hansen, M., Rubinsztein, D. C. & Walker, D. W. Autophagy as a promoter of longevity: insights from model organisms. *Nat. Rev. Mol. Cell Biol.* **19**, 579–593 (2018).

179. Torggler, R., Papinski, D. & Kraft, C. Assays to Monitor Autophagy in *Saccharomyces cerevisiae*. *Cells* **6**, 23 (2017).

180. Barros, C. D. S. *et al.* L-arginine reduces nitro-oxidative stress in cultured cells with mitochondrial deficiency. *Nutrients* **13**, 534 (2021).

181. Mudholkar, K., Fitzke, E., Prinz, C., Mayer, M. P. & Rospert, S. The Hsp70 homolog Ssb affects ribosome biogenesis via the TORC1-Sch9 signaling pathway. *Nat. Commun.* **8**, 937 (2017).

182. Swinnen, E. *et al.* The protein kinase Sch9 is a key regulator of sphingolipid metabolism in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **25**, 196–211 (2014).

183. Piper, P. W. Maximising the yeast chronological lifespan. *Subcell. Biochem.* **57**, 145–159 (2015).

184. Kaeberlein, M. & Kennedy, B. K. A new chronological survival assay in mammalian cell culture. *Cell Cycle* **11**, 201–202 (2012).

185. Weisman, L. S. Yeast Vacuole Inheritance and Dynamics. *Annu. Rev. Genet.* **37**, 435–460 (2003).

186. Mcguire, C., Stransky, L., Cotter, K., Forgac, M. & Ave, H. Regulation of V-ATPase activity. *Front. Biosci.* **22**, 609–622 (2017).

187. Rallis, C. *et al.* Amino acids whose intracellular levels change most during aging alter chronological life span of fission yeast. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **76**, 205–210 (2021).

188. Sampaio-Marques, B., Burhans, W. C. & Ludovico, P. Longevity pathways and maintenance of the proteome: The role of autophagy and mitophagy during yeast ageing. *Microb. Cell* **1**, 118–127 (2014).