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Ammonium toxicity in aging yeast cells reduces chronological life span: cell death mechanisms and nutrient sensing pathways

Toxicidade do amónio e seu impacto na morte celular e longevidade cronológica em leveduras: mecanismos e vias de sinalização

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ABSTRACT

Yeast has emerged as one of the most important model organisms to study the environmental and genetic factors affecting longevity and its exploitation has made huge contributions to the progress in understanding aging. Major advances in this research field came from dietary regimes that have been shown to increase longevity in organisms ranging from yeast to mammals. The understanding on how nutrient signaling pathways collaborate to the beneficial effects of dietary restriction can help expose new targets for therapy in the prevention of aged-related diseases.

When studying the impact of nutrient-signaling pathways in aging of yeast, by culturing *Saccharomyces cerevisiae*, the composition of culture media has proven to be an extrinsic factor affecting the chronological life span (CLS). Reducing glucose concentration in the culture medium, is an environmental modulation that was shown to be sufficient to increase CLS. Other components of the culture media and factors such as the products of fermentation have also been implicated in the regulation of CLS. Particularly, the CLS of *S. cerevisiae* is strongly affected by the concentration of the auxotrophy-complementing amino acid in the medium.

In this context, in the present work, we aimed to identify new nutrient signaling capable of regulating *S. cerevisiae* CLS and uncover the signaling pathways involved. The results obtained show that manipulation of the ammonium (NH_4^+) concentration in the culture medium also affects CLS. NH_4^+ reduced CLS of cells cultured to stationary phase under both standard amino acid supplementation and amino acid restriction conditions, in a concentration-dependent manner, a significant increase in cell survival being observed when the starting NH_4^+ concentration in the medium was decreased. In cells cultured to stationary phase with amino acid restriction or starved for auxotrophy-complementing amino acids and subsequently transferred to water, the CLS was also significantly shortened by the addition of NH_4^+, indicating that ammonium alone could induce loss of cell viability as observed in culture media. Cells starved for auxotrophic-complementing amino acids were particularly sensitive to ammonium-induced cell death and starvation for leucine in particular, largely contributed to this phenotype. Death induced by ammonium in cells starved for auxotrophic-complementing amino acids (aa-starved cells) was mediated through the regulation of the evolutionary conserved pathways PKA, TOR and SCH9 and accompanied by an initial apoptotic cell death followed by a fast secondary necrosis. Autophagy, which has been described as essential for

cell survival during nitrogen starvation and regulating amino acid homeostasis did not seem to have a role in ammonium-induced cell death. The results with aa-starved cells of *tor1*Δ, *tpk1*Δ and *sch9*Δ strains showed that NH_4^+ toxicity is mediated through the over-activation of PKA and TOR and inhibition of Sch9p, suggesting that the role of Sch9p in the process is essentially independent of the TOR-PKA pathway. Furthermore, it was shown that NH_4^+ signalling to PKA is mediated via Tor1p and Sch9p but does not depend on Mep2p. This activation of PKA by NH_4^+ signalling is not dependent on its metabolization as testing for the activity of enzymes involved in the metabolism of NH_4^+ demonstrated no correlation with NH_4^+ toxicity. In agreement, the use of the NH_4^+ non-metabolizable analog methylamine produced the same outcome as NH_4^+. As a final result, it was shown that NH_4^+ toxicity is a generalized effect in aging yeasts, not only dependent on amino acid restrictions, but also present in prototrophic strains.

In conclusion, our results point out, for the first time, a role for ammonium as an extrinsic factor affecting CLS regulation in the culture medium joining other known extrinsic factors such as glucose, acetic acid and ethanol. Also, the effects of ammonium toxicity were characterized in yeast for the first time, showing that this process shares common features with NH_4^+ toxicity in mammalian cells. The model presented in this work may be a powerful system for elucidation of conserved mechanisms and pathways of ammonium toxicity, with important implications in diverse fields extending from diseases associated with hyperammonemia and clarification of longevity regulation in multicellular organisms, to new insights for wine fermentations involving nitrogen supplementation.

RESUMO

A levedura *Sacharomyces cerevisiae*, tem vindo a destacar-se como um dos mais significativos organismos modelo no estudo de factores ambientais e genéticos que afectam a longevidade. A sua utilização na investigação dos processos de envelhecimentos tem gerado importantes contributos neste campo. Muitos dos avanços nesta área de investigação advêm do estudo da influência da dieta na longevidade, com resultados positivos no aumento da longevidade em diversos organismos, desde a levedura até aos mamíferos. O estudo da contribuição das vias de sinalização de nutrientes para os efeitos da dieta na longevidade, pode ajudar na identificação de novos alvos terapêuticos para a prevenção de doenças associadas ao envelhecimento. No estudo do impacto das vias de sinalização de nutrientes no envelhecimento de leveduras, a composição do meio de cultura figura como um dos factores extrínsecos que afecta a longevidade cronológica. Neste âmbito, encontra-se bem documentado na literatura que a redução da concentração de glucose no meio de cultura resulta no aumento da longevidade cronológica da levedura. De forma análoga, outros componentes do meio de cultura, como por exemplo alguns produtos da fermentação alcoólica, estão também envolvidos na regulação da longevidade cronológica da levedura. Adicionalmente, em estirpes auxotróficas de *S. cerevisiae*, a concentração de aminoácidos essenciais (correspondentes às marcas auxotróficas) no meio de cultura afecta particularmente a longevidade cronológica da levedura.

Neste contexto, o presente trabalho teve como objectivo a identificação de novos sinais nutricionais envolvidos na regulação da longevidade cronológica da levedura *S. cerevisiae*, bem como das vias de sinalização potencialmente envolvidas. Os resultados obtidos mostraram que a manipulação da concentração de amónio no meio de cultura afecta a longevidade cronológica. O amónio reduz a longevidade cronológica de células cultivadas até à fase estacionária, tanto em condições de suplementação *standard* de aminoácidos essenciais, como em condições de restrição, sendo a redução proporcional à concentração de amónio no meio extracelular. Em consonância, observou-se um aumento significativo na sobrevivência das células quando se reduziu a concentração inicial de amónio no meio de cultura. Por outro lado, em células cultivadas até fase estacionária em condições de restrição de aminoácidos essenciais ou esfomeadas para os mesmos aminoácidos e posteriormente transferidas para água, observou-se um decréscimo significativo na longevidade cronológica da levedura após a adição de amónio, sugerindo que o amónio, por si só, é

responsável pela perda de viabilidade celular, tal como observado para células mantidas no meio de cultura. As células esfomeadas para aminoácidos essenciais mostraram ser especialmente sensíveis à morte induzida pelo amónio, tendo o esfomeamento em leucina contribuído particularmente para este fenótipo. A morte induzida pelo amónio em células esfomeadas para aminoácidos essenciais, foi mediada pela vias de regulação PKA, TOR e SCH9, sendo esta morte inicialmente apoptótica seguida por uma extensa necrose secundária. A autofagia, um processo muitas vezes descrito como essencial à sobrevivência durante o esfomeamento em azoto e responsável pela regulação da homeostase de aminoácidos, não parece ter um papel na morte celular induzida pelo amónio. Os resultados com células das estirpes *tor1*Δ, *tpk1*Δ e *sch9*Δ esfomeadas para aminoácidos, mostraram que a toxicidade do amónio é mediada através da sobre-activação das cinases PKA e TOR, e pela inibição da Sch9p, sugerindo que esta última tem uma acção independente da via TOR-PKA. Verificou-se ainda que a activação da cinase PKA pelo amónio é mediada via Tor1p e Sch9p, porém independente da Mep2p. Não se observou relação significativa entre a toxicidade do amónio e a actividade das enzimas envolvidas no seu metabolismo, indicando que a activação da proteína PKA, através da sinalização pelo amónio, não depende da metabolização deste. Em concordância, verificou-se também que a metilamina, um análogo não metabolizável do amónio, induziu morte celular. Os efeitos tóxicos do amónio foram ainda observados em estirpes prototróficas de *S. cerevisiae*, indicando que a toxicidade do amónio é um efeito generalizado em células de levedura envelhecidas, não estando dependente apenas de condições de restrição em aminoácidos.

Em conclusão, os estudos desenvolvidos no presente trabalho de tese, permitiram identificar, pela primeira vez, o amónio como um factor extrínseco envolvido na regulação da longevidade cronológica de *S. cerevisiae*, juntando-se, assim, a outros factores já conhecidos como a glucose, o ácido acético e o etanol. Os efeitos da toxicidade do amónio foram ainda caracterizados, também pela primeira vez, em leveduras, demonstrando que este processo possui semelhanças com o descrito para a toxicidade do amónio em células de mamíferos. O modelo de *S. cerevisiae* apresentado neste trabalho, poderá assim vir a constituir uma importante ferramenta na elucidação de mecanismos conservados e vias envolvidos na toxicidade do amónio, podendo ser útil em diversas áreas de investigação tais como no estudo de doenças associadas à hiperamonémia e na clarificação da regulação da longevidade em organismos multicelulares. Sob o ponto de vista biotecnológico, os resultados obtidos poderão também ser relevantes na definição de estratégias de suplementação de azoto em fermentações alcoólicas, muito em particular no sector vínico.

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LIST OF ABBREVIATIONS

AAC	ADP/ATP carrier
AC	Adenylate cyclase
ADP	Adenosine Diphosphate
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
ANT	Adenine nucleotide transporter
ATG	Autophagy-related genes
ATO	Ammonium transporter outward
ATP	Adenosine triphosphate
BCAA	Branched side chain amino acids
cAMP	Adenosine 3',5'-cyclic monophosphate
CatD	Cathepsin D
CFU	Colony Forming Units
CLS	Chronological life span
CNC	Central nervous system
CNTF	Ciliary neurotrophic factor
COX	Cytochrome oxidase activity
CR	Calorie restriction
Cvt	Cytoplasm to vacuole targeting pathway
Cyt C	Cytochrome C
DAPI	4,6-diamido-2-phenylindole
DHE	Dihydroethidium
DHR	Dihydrorhodamine
DR	Dietary restriction
EDTA	Ethylenediaminetetraacetic acid
endo G	Endonuclease G
ER	Endoplasmatic reticulum
ERCs	Extrachromosomal ribosomal DNA circles
FMG	Fermentable-growth-medium induced pathway
GAAC	General amino acid control pathway
GDH	Glutamate dhydrogenase
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GS	Glutamine synthetase
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid
HMGB1	Mammalian high mobility group box-1
IAP	Inhibitor apoptosis protein
IGF-1	Insulin-like growth factor
LMP	Lysosomal membrane permeabilization

MAC	Mitochondrial apoptosis-inducing channel
MAPK	Mitogen-activated protein kinase
MEP	Ammonium permease
MOMP	Mitochondrial outer membrane permeabilization
MPTP	Mitochondrial permeability transition pore
MSX	Methionine sulfoximine
NCR	Nitrogen catabolite repression
NDP	Nitrogen discrimination pathway
NH_4^+	Ammonium
$(NH_4)_2SO_4$	Ammonium sulphate
O_2^-	Superoxide anion
PAS	Phagophore assembly site
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDS	post-diauxic shift
PE	phosphatidylethanolamine
PI	Propidium iodide
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PTP	Permeability transition pore
RIP	Receptor interacting protein
RLS	Replicative life span
ROS	Reactive oxygen species
RP	Ribosomal protein
RTG	Retrograde response pathway
S6K	Ribosomal S6 protein kinase
SC	Synthetic complete
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrilamide gel electophoresis
SODs	Superoxide dismutases enzymes
STRE	Stress-responsive elements
TCA	Tricarboxylic acid cycle
TCA	Trichloroacetic acid
TOR	Target of rapamycin
TORC1	TOR-1 complex
TORC2	TOR-2-complex
UCD	Urea cycle disorders
VDAC	Voltage dependent anion channel
VPS	Vacuolar protein sorting-associated
YPD	Yeast extract, peptone, dextrose
Z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

CHAPTER 1

General Introduction

This chapter comprises parts from the following publication:

- Santos, J., Leão, C., Sousa, M.J. *Growth Culture Conditions and Nutrient Signaling Modulating Yeast Chronological Longevity*. Oxidative Medicine and Cellular Longevity, vol. 2012, Article ID 680304, 10 pages, 2012.

1. General Introduction

1.1. Cellular pathways governing aging

Aging is common to most living organisms ranging from bacteria, a unicellular prokaryotic organism, to multicellular eukaryotic organisms like humans. It is a complex biological process that involves accumulation of damage at diverse components of the organism leading ultimately to the loss of function and demise [1-3]. In the aging process, cellular activities compromised are modulated by a network of nutrient and energy sensing signaling pathways that are highly conserved among organisms. These pathways include the insulin/insulin-like growth factor 1 (Ins/IGF-1), the protein kinase/target of rapamycin (TOR) and adenylate cyclase /protein kinase A (AC/PKA) pathways [4]. Pioneering studies using mutations on key genes of these pathways have shown an increase by threefold or more in the life span of model organisms like yeast [5, 6], fruit flies [7], worms [8, 9] and mice [10, 11]. Many of these mutations which extend life span decrease the activity of the nutrient signaling pathways mimicking a starvation state during which oxidative stress responses are induced, reducing the levels of reactive oxygen species (ROS) and oxidative damage to macromolecules [12]. Accordingly, it has been shown in different aging models (yeast, flies, worms, fish, rodents, and rhesus monkeys) that reducing growth factors/nutrients intake has profound positive effects in extension of life span and also improves overall health by delaying or reducing aged-related diseases in mammals including diabetes, cancer and cardiovascular diseases (reviewed in [4]). One of the first models to implicate growth/nutrient-sensing signaling with longevity was the nematode worm *Caenorhabditis elegans*. In this model, it was shown, that the recessive mutation in *age-1*, coding for phosphatidylinositol 3-Kinase (PI3K) extend lifespan significantly [8]. Also in *C. elegans*, the insulin/IGF-1 pathway was linked to longevity by the discovery that mutating the gene coding for an insulin/IGF-1 receptor ortholog, *DAF-2*, doubled its life span [9]. This life span extension was dependent on the reduction of activity of Daf-2 and consequently of its downstream effector PI3K (encoded by *age-1*), and the subsequent activation of Daf-16, a Forkhead FoxO family transcription factor (FOXO), which regulates several genes involved in stress response, antimicrobial activity, and detoxification of xenobiotics and free radicals [4, 9, 13]. Another pathway involved in longevity regulation in *C. elegans* is the conserved TOR-S6K (Ribosomal S6 protein Kinase) pathway. This pathway interacts with the insulin/IGF-1 pathway converging on the worm

ortholog of regulatory associated protein of mTOR, Daf-15, to regulate larval development, metabolism and longevity [14] and so down-regulation of its activity results in extended life span [4].

The fruit fly *Drosophila melanogaster* is a more complex model, allowing studies based on sex differences. As in *C. elegans*, reducing the activity of the insulin/IGF-1 pathway mediates cellular protection mechanisms and the extension of life span in this organism. Mutations in the insulin-like receptor favour the extension of life span yielding dwarf sterile flies with females showing up to 85% extension of adult longevity [15]. Down regulation of the TOR pathway in flies, similarly to *C. elegans*, was shown to increase life span when inactivated pharmacologically with rapamycin or with overexpression of dominant-negative forms of S6K or TSC1 or TSC2, which encode negative regulators of TOR [16, 17].

Identical outcomes for genetic or pharmacologic manipulation of insulin/IGF-1 and TOR pathways and for dietary restriction regimes were observed in *D. melanogaster* and *C. elegans*, as well as in yeasts, establishing the evolutionary conserved roles of these pathways in determining life span and implicating them as mediators of the protective effects of dietary restriction in different species [4, 18].

In mammals, hormones of the endocrine system, the growth hormone, insulin-like growth factor-1 (IGF-1) and insulin pathways are key players in the hormonal control of aging in association with an increase of antioxidant defenses and increased stress resistance (reviewed in [19]). Deficiency in levels of circulating growth hormone has been shown to enhance antioxidant defenses and stress resistance, reduce tumor burden and to increase insulin sensitivity (reviewed in [20]). Enhanced insulin sensitivity is a common feature of long-living mutant mice and in humans, studies of centenarian populations strongly correlates this increase in insulin sensitivity with longevity [21, 22]. IGF-1 and insulin also modulate TOR activity through Akt kinase which is a downstream effector of the insulin/IGF-1 pathway [23] and inhibition of mTOR pathway by rapamycin [24] or deletion of its downstream effector S6K, increases mice life span [25]. The life span extension due to the deletion of S6K was accompanied by slower progression of age-related pathologies and in particular slower loss of insulin sensitivity [25].

Another pathway involved in longevity regulation is the AC/PKA pathway that is conserved from yeast to mammals. Down regulation of the Ras/AC/PKA pathway was first shown in yeast to

have a major effect on life span extension [4, 6, 26]. Only recently, studies correlating AC/PKA pathway with aging and age-related diseases started to emerge in mammals. Deletion of the mouse AC type 5, which mediates PKA activity by modulating cAMP levels, was reported to significantly increase life span, as it does in yeast [27], and improve cardiac stress resistance [28]. Likewise as described for yeast [26], deletions of PKA subunits in mice have recently been shown to increase life span while protecting against age-related deleterious changes such as weight gain, hypertrophic liver and cardiac dysfunction [29]. Although the subunits deleted are the regulatory subunits (RIIβ) and not the catalytic subunits like in yeast (TPK), loss of RIIβ in mice causes a concomitant and compensatory decrease in catalytic subunits showing a mechanistic association between loss of these subunits and life span extension [29]. This converging result in such divergent models suggests a highly conserved role for PKA in longevity and opens the possibility for new therapeutic targets for aging and obesity.

In mammals the Ras proteins do not directly signal to PKA through AC [30] as it occurs in yeast [31] however, a recent study reported that homozygous deletion of Ras-GRF1 promotes both median and maximum longevity in mice [32]. Ras-GRF1 is a guanine nucleotide exchange factor (GEF) responsible for activating Ras by favouring its GTP-bound state [33] suggesting that the cause of longevity extension of the Ras-GRF1 deletion could be the reduction of Ras activity [34]. Therefore, the Ras pathway appears as a conserved pathway in the aging process from yeast to mammals [34].

1.2. The particular case of yeast as a cell aging model

Yeast has emerged as a highly exploited model to study the environmental and genetic factors affecting longevity. In particular, the genetic tools now available make yeast one of the best established experimental model organisms for screening genes involved in the regulation of fundamental cellular process including the pathways controlling life span. Two yeast life span models have been characterized: replicative life span (RLS) and chronological life span (CLS) (reviewed in [35]). RLS is defined as the total number of times a single mother cell can undergo a mitotic event and originate daughter cells before senescence [36]. RLS is accurately measured by moving and counting small daughters away from the mothers via microscopic manipulation and

simulates aging of mitotically active mammalian cells [35, 37, 38]. On the other hand, CLS defines the length of time non-dividing yeast cells remain viable [39] thus simulating aging of the pos-mitotic mammalian cells [40, 41]. This viability is assessed by cells re-entering the cell cycle after transfer from the depleted medium or water to nutrient complete-medium [35]. In CLS, two types of metabolic yeast cells can be studied: post-diauxic or stationary phase cells. Both metabolic state cells are grown in synthetic complete (SC) medium but while post-diauxic cells are kept in the culture medium, the stationary phase ones are transferred to water (extreme calorie restriction) after 3 days of growth. Some protocols for post-diauxic cells could also use cells grown in YPD (yeast extract, peptone, dextrose) medium [35, 42] instead of SC medium.

The two paradigms of aging in yeast, CLS and RLS, have become useful tools to compare the aging process in proliferating and non-proliferating cells as well as to serve as models to study the mechanics of the aging process in mitotic and post-mitotic cells of multicellular organisms [38].

The yeast *Saccharomyces. cerevisiae* divides by budding and therefore undergoes asymmetrical cell division, with the mother cell retaining more volume than the daughter cell. In this asymmetric division mother cells retain most of the age-associated damage, thus sacrificing individual replicative capacity while daughter cells retain full replicative potential [43, 44]. One of the aging factors affecting RLS is the accumulation of extrachromosomal ribosomal DNA circles (ERCs) [45]. These circular DNA molecules are self-replicating units formed in the nucleus by homologous recombination between adjacent rDNA repeats which segregate asymmetrically to the mother-cell nucleus during cell division. During each division ERCs replicate leading to an exponential accumulation in the mother cell and consequently to cell senescence [44, 45]. This finding in yeasts came in large part from the study of important age-related proteins called sirtuins. Sirtuins are NAD^+-dependent protein deacetylases involved in chromatin silencing and known to mediate longevity in yeast, nematodes, flies and mammals [40, 44, 46]. Deletion of *SIR2* decreases RLS and its overexpression increases RLS showing that Sir2p mediates RLS in yeast most probably by regulating rDNA recombination and ERCs formation [47]. ERCs appear to be an aging factor specific to yeast, although without relevance in non-dividing yeast cells (CLS) and so far without a role in aging of multicellular eukaryotes [46, 48].

Another factor known to decrease longevity both in CLS and RLS is accumulation of oxidative damage due to the production of reactive oxygen species (ROS). Deletion of the yeast antioxidant defense enzymes superoxide dismutases (SOD), reduces significantly CLS [39] and RLS [49]. However, overexpression of cytosolic (SOD1) and mitochondrial (SOD2) superoxide dismutases increased longevity of non-dividing cells [50] while it decreased RLS.

Although studies show several similarities but also major differences between CLS and RLS mechanistic regulation, these two models are interconnected as RLS decreases in chronologically aged cells [48]. In addition both aging models are regulated by nutrient-signaling kinases as screenings for long-lived mutants identified the same gene mutations in both paradigms [27, 51-53].

1.2.1. Glucose-signaling pathways involved in yeast longevity

The most common dietary regimes used to study the interaction between nutrient signaling pathways and longevity include: dietary restriction (DR) in which the intake of nutrients, but not necessarily calories, is reduced without causing malnutrition; and calorie restriction (CR), a regime in which only calories are reduced without compromising other nutrients, for instance amino acids and vitamins [54, 55].

In yeast, when studying both RLS and CLS, several results correlating environmental growth conditions and longevity emerged. Many studies, including those using calorie restriction (CR), showed that reducing the glucose or amino acids concentrations of the culture media is sufficient to increase replicative and chronological life span. The composition of culture media has proven to be an extrinsic factor affecting chronological life span but this is still giving rise to different interpretations on longevity regulation.

The manipulation of nutrient-signaling pathways for the study of aging regulating mechanisms, as previously mentioned, can be accomplished by genetic manipulations of key components of these pathways or by DR and calorie restriction CR. In yeast, the vast majority of protocols for CR are based on the decrease of the glucose concentration in the medium from the standard 2% to 0.5 or 0.05%. The latter (0.05% glucose) is considered extreme calorie restriction as

well as the one achieved by transferring cells grown in 2% glucose to water [35, 56]. The first studies to report glucose as an agent affecting life span were conducted by Granot and co-worker who showed that addition of glucose to stationary phase cells previously transferred to water leads to a reduction of CLS [57]. The authors further demonstrated that glucose, in the absence of other complementing nutrients, induces apoptotic cell death accompanied by an increase in ROS production [58]. Further studies in yeast have revealed that the major nutrient-signaling pathways TOR, SCH9 and Ras/AC/PKA are all involved in longevity regulation by glucose [6, 46, 59]. These pathways promote cell division and growth in response to nutrients while inhibiting the general stress response and autophagy. *SCH9* was one of the first genes to be implicated in CLS [27]. Sch9p is the yeast closest homolog of the mammalian AKT/PKB and S6K, and its deletion leads to an increase in both CLS and RLS [27, 53]. Sch9p is a kinase that mediates PKA activation in the fermentable-growth-medium-induced (FGM) pathway and also mediates regulation of many of the TORC1 controlled processes [60-62]. Sch9p was first described as having a partially redundant role with PKA pathway, since deletion of *SCH9* could be compensated by increased activity of PKA and vice versa [63], and later as a direct target of TOR complex 1 (TORC1) regulation [61]. More recently, TORC1 was also identified as a target for regulating longevity in both CLS and RLS [51, 52]. The TOR pathway responds to nitrogen and carbon sources, mainly to control cell growth, through the regulation of processes such as translation initiation, ribosome biogenesis, mRNA and amino acid permeases stability, transcription of nutrient-regulated genes and stress response genes, actin cytoskeleton organization and autophagy [64, 65]. Reduction of TORC1-Sch9p signaling was shown to promote longevity by increasing the expression of stress-response genes in a Rim15p-dependent manner [59] as *RIM15* deletion reduced the life span extension of the long-lived *sch9*Δ cells [27]. Alternatively, a recent study proposes a Rim15p-independet mechanism for life span extension in reduced TORC1-Sch9p signaling [66]. This study shows that in *tor1*Δ cells, CLS is reduced if mitochondrial respiration is uncoupled. The authors suggest that during growth, mitochondrial ROS signaling down regulates both the mitochondrial membrane potential and ROS accumulation of stationary phase cells to promote their longevity [66]. This is in agreement with previous data showing that pre-adaptation to respiratory growth can also promote extension of CLS [67]. CR also promoted CLS extension by doubling the life span of the long-lived *sch9*Δ and *tor1*Δ cells by a Rim15p partially dependent mechanism. In fact, cells with a triple mutation in Rim15p downstream transcription factors (*msn2*Δ *msn4*Δ *gis1*Δ) do not display a reduction of CR promoted

CLS extension when compared to the long-lived *sch9*Δ and *tor1*Δ cells suggesting the involvement of additional Rim15p independent transcriptional factors [59].

Another pathway involved in aging is the other major nutrient-signaling pathway Ras/AC/PKA responsible for the link between glucose availability and the control of growth, proliferation, metabolism, stress resistance, and longevity [6, 27, 50, 68]. Deletion of *RAS2* or a reduced activity of adenylate cyclase (Cyr1p), which is activated by the Ras proteins, causes life span extension and stress resistance [50]. Mutation in the *CYR1* gene increases both RLS and CLS while deletion of *RAS2* decreases RLS [53, 69]. Rim15p also mediates *ras2*Δ life span extension by enhancing cellular protection against oxidative stress through the activation of *SOD2* [50] indicating that Rim15p is a common denominator of the pathways Ras/AC/PKA, Sch9p and TOR. In addition, deletion of *MSN2/4* in *ras2*Δ cells leads to life span reduction indicating that Msn2p/4p and Gis1p transcription factors controlled by Rim15p are also required for CLS extension. Nevertheless, the Rim15p downstream transcription factors (Msn2p, Msn4p and Gis1p) appear to have different roles in *sch9*Δ and *ras2*Δ cells given that only the abrogation of *GIS1*, and not of *MSN2/4*, was shown to almost completely abolish the life span expansion of *sch9*Δ cells [27, 59]. Therefore, Sch9p and Ras2p seem to differentially modulate the common downstream effectors, which is also corroborated by the higher stress resistance and increased CLS exhibited by *ras2*Δ *sch9*Δ double knockout cells in comparison to the single deletion mutants [59, 70].

More recently, the correlation between glucose signaling, oxidative stress and aging was further addressed in a study showing that increasing glucose from the standard 2% to 10%, promotes a shortening of CLS accompanied by increased levels of intracellular superoxide anion ($O_2^{\cdot-}$), decreased levels of hydrogen peroxide (H_2O_2), reduced efficiency of stationary phase G0/G1 arrest and activation of DNA damage [12]. On the other hand, CR by reducing glucose or by deletion of *SCH9* or *TOR1* extends CLS and diminishes superoxide anion levels promoting at the same time a more efficient G0/G1 arrest [12]. These and other results point to superoxide levels as one of the key factors regulating aging [71] which is in agreement with the aforementioned results showing that reduction of signaling pathways leads to the activation of oxidative stress responses mediated by Rim15p [59]. Nevertheless, an alternative activation of oxidative stress responses independent of Rim15p [59], and mediated by H_2O_2 has been also reported [12]. Furthermore, high levels of H_2O_2,

which respond to glucose in an inversely dose-dependent manner, promotes activation of SODs, leading to a reduction in superoxide anion levels and therefore to CLS extension [12, 72].

1.2.2. Amino acid metabolism in the regulation of the yeast chronological life span

In nature, yeast cells enter a resting or quiescent state in the absence of favorable nutritional conditions. When inadequate carbon, nitrogen, sulfur or phosphorus levels are sensed by yeast cells, growth ceases and cell cycle is arrested as a survival strategy. In natural environments, yeast are prototrophs capable of synthetizing most of their metabolites from simple carbon and nitrogen sources, whereas laboratory strains commonly have auxotrophic markers that confer a nutrient-limiting growth phenotype useful for genetic manipulation. These markers are usually genes involved in the biosynthesis of specific amino acids or nucleotides. Amino acids are important nutrients that can also be recycled by autophagy. This recycling process maintains amino acid homeostasis and is crucial for cell survival under nitrogen starvation leading to rapid loss of viability in autophagy-defective mutants [73] and therefore has been implicated in CLS regulation. Curiously, it was demonstrated that prototrophic and auxotrophic strains display different responses to nutrient starvation [74]. Starvation of "natural" nutrients such as phosphate and sulphate leads to an arrest in G0/G1 cell cycle phase of prototrophics cells, while auxotrophic cells failed to arrest the cell cycle upon starvation of "supplemental" nutrients (auxotrophic nutrients) [74]. It was also observed that auxotrophic cells limited for leucine or uracil consume glucose at a much faster rate, exhausting it from the medium, than prototrophic cells limited for phosphate, sulfate or ammonium that spare glucose [75]. These findings clearly reveal a failure of auxotrotophic cells in regulating nutrient sensing in response to starvation of "supplemental" nutrients [76]. Furthermore, limiting levels of auxotrophy-complementing amino acids, in the growth medium, induce an early arrest in G2/M phase, negatively affecting chronological longevity and leading to a premature aging phenotype [77]. In accordance, reduction of total amino acid levels, including essential ones (auxotrophic amino acids), in the medium also decreases CLS [78]. Starvation for leucine in non-dividing leucine auxotrophic cells induces a rapid loss of viability [76]. Nevertheless, this phenotype is partially dependent on the carbon source present in the starvation medium but not in that used in the growth medium. For example the presence of ethanol/glycerol or galactose in the starvation medium

increases CLS in contrast to starvation in glucose [76]. However not all essential (auxotrophic) amino acids have the same effect on CLS. In fact, methionine starvation of methionine auxotrophic cells has no effect on viability [79]. Another study also reported that from extra supplementation of the auxotrophic-complementing amino acids lysine, histidine and leucine, the latter has a more pronounced positive effect in CLS in both autophagy-competent and autophagy-deficient strains [80]. Authors pointed out that the enhanced sensitivity of yeast cells to leucine starvation is correlated to the high levels of leucine codon, the most frequent amino acid codon [80]. CLS is extended by the presence of non-essential amino acids, particularly isoleucine and its precursors threonine and valine, via the general amino acid control (GAAC) pathway. The authors proposed a mechanism for CLS regulation by the branched side chain amino acids (BCAA) leucine, isoleucine and valine, in which low levels of these amino acids induce the GAAC pathway therefore shortening CLS and vice versa [80].

Starvation for non-essential amino acids was reported to extend RLS [81] and starvation for preferred amino acids such as asparagine or glutamate induced CLS extension in direct proportion to the nature of the amino acid removed [52].

1.2.3. Impact of products of fermentation in the yeast chronological life span

Ethanol is the main product resulting from alcoholic fermentation and it is used as a carbon source during the diauxic shift and post-diauxic phase. Nevertheless, ethanol is known to negatively affect the metabolic activity of the yeast cells by inhibiting cell growth and fermentation [82]. It is also known to cause among others the damage of cell membranes by increasing membrane fluidity [83, 84] and the inhibition of transport systems across the plasma membrane. The severity of the effects is dependent on the alcohol concentration and at high ethanol levels it results in cell death [82, 85, 86]. Recently, ethanol was described as an apoptotic inducer [87] and has also been implicated as an extrinsic factor in aging, significantly decreasing CLS of severely calorie restricted strains (CR in water), known for their life span extension in this condition [88]. In contrast to wild type cells, long-lived *sch9*Δ cells consume all the ethanol from the medium during chronological aging, further supporting ethanol as a modulator of aging [88].

A recent study on the genetic expression profile of long-lived *tor1*Δ*, sch9*Δ and *ras2*Δ cells revealed an up-regulation of genes involved in the metabolism of glycerol. In contrast to wild type cells that accumulate ethanol and rapidly deplete glycerol, those long-lived mutant cells accumulate glycerol whereas ethanol was early depleted. These observations suggest that inhibition of Tor1p/Sch9p mediates a metabolic switch from biosynthesis and release of ethanol to activation of glycerol biosynthesis and its consequent release [70]. Glycerol, unlike glucose and ethanol [56, 59, 88], does not promote aging or cell death and so this metabolic change extends CLS [70, 89]. In calorie restricted cells, ethanol is completely consumed before the beginning of viability decline. Conversely, non-calorie restricted cells were unable to completely consume ethanol before viability decline. The authors suggested a correlation between ethanol accumulation and loss of peroxisome function in non-calorie restricted cells since ethanol suppresses the synthesis of certain proteins localized to peroxisomes [90].

Acetic acid is a byproduct of fermentative metabolism in yeast accumulating in the medium during fermentation of glucose to ethanol and is also one factor described to affect CLS [91, 92]. After sugar is depleted in 2% glucose standard conditions, a shift in metabolism occurs from fermentation to respiration and the metabolization of ethanol also leads to the production and accumulation of acetic acid. Acetic acid is a well-known inducer of apoptotic cell death leading to ROS production [93, 94]. In a recent study, Burtner and co-workers identified acetic acid as an important extracellular factor affecting CLS in SC medium [91]. The authors showed that cells grown for 48 hours under extreme calorie restriction conditions (0.05% glucose concentration), known to extend CLS, rapidly loss viability if transferred to cell-free supernatants of 2% standard glucose-depleted medium, indicating that cell-extrinsic aging factors were present in the SC depleted medium [91]. Although several other organic acids also accumulate in the culture medium during chronological aging, only acetic acid was identified as being sufficient to cause chronological aging [91]. In the same study it was also shown that buffering of aging cultures to pH 6 is sufficient to increase CLS, neutralizing the toxic effect of acetic acid. Actually, the acetate anion is not readily taken up from the environment by glucose grown yeast cells, but the protonated acetic acid can cross the plasma membrane resulting in intracellular acidification [95]. This negative effect of acetic acid in CLS was diminished by mutational inactivation of conserved signaling pathways namely deletion of *SCH9* and *RAS2* conferring resistance via unknown mechanisms [91]. *SCH9* and *RAS2* mutant cells are known to have a more frequent growth arrest in G1 phase when compared to the

wild-type, promoted by the reduction in growth signaling in these mutants [96]. In accordance, nutrient-depleted stationary phase cells are continuously subjected to acetic acid-induced growth signals, even in the absence of glucose, that promote cell cycle progression and consequently replication stress due to the lack of favorable conditions [97]. These and other results show that acetic acid, as glucose, activates Sch9p and RAS pathways and seems to mediate cell death by promoting the accumulation of superoxide anion ($O_{2}^{\cdot-}$) in consequence of down regulation of SODs and other oxidative stress defenses by the activated pathways [12]. The long-lived *ade4*Δ cells (Ade4p is involved in the purine *de novo* biosynthetic pathway) do not accumulate acetic acid in the culture medium when compared to the wild type cells, while the short-lived *atg16*Δ cells (Atg16p is involved in the autophagic process) accumulate acetic acid at higher concentrations than the wild type cells, inversely correlating the amount of acid release from cells and the extension of CLS [98]. Buffering the growth media to pH 6.0 of the short-lived *atg16*Δ cells and the wild type strain, also dramatically increase CLS to the same levels obtained for the CR growth condition and for the long-lived *ade4*Δ cells, indicating that pH neutralized the toxic effects of acetic acid. Overall the results demonstrate that acetic acid can have an important impact on CLS through a cell extrinsic mechanism that is dependent on media pH [98].

1.2.4. Nutrient and stress signaling pathways in the regulation of survival: TOR, PKA and SCH9 pathways

Regulation of metabolic survival is mediated by environmental conditions in all living organisms. Flexibility in adaptation to environmental conditions is conserved from yeasts to mammals and is mediated by complex nutrient signaling pathways. In yeast these pathways, through complex signaling cascades, control the necessary metabolic changes that take place when a shift in environmental conditions occurs, thus allowing cells to stimulate proliferation in optimal conditions and also to induce cell cycle arrest and enter into a quiescent state in nutrient exhaustion conditions. Mainly, three major nutrient signaling pathways control and regulate cell metabolism: TOR (Target of rapamycin), PKA (protein kinase A) and Sch9p [99].

TOR pathway

In *S. cerevisiae*, TOR signaling responds to nitrogen and possibly to carbon sources. This pathway controls cell growth by activating anabolic processes and inhibiting catabolic processes and mRNA degradation [64, 65]. The yeast Tor proteins (Tor1p and Tor2p) are highly conserved serine/threonine kinases that were first described as the target of antifungal and immunosuppressive agent rapamycin [100]. The Tor proteins form two distinct multiprotein complexes entitled TOR1 and TOR2 complex (TORC1 and TORC2, respectively), with distinctive subunit composition (TORC1 consisting of Tor1/2p, Lst8p, Kog1p, and Tco89p, and TORC2 consisting of Tor2p, Lst8p, Avo1p, Avo2p, Avo3p, Bit61p and Bit2p). This difference in composition is responsible for making only TORC1 rapamycin sensitive, presumably due to Avo1p masking the FRB domain of Tor2p in TORC2, which is the FKBP-rapamycin binding site [101]. TORC1 can be assembled by integrating Tor1p or Tor2p, while TORC2 only contains Tor2p. Although Tor2p can substitute Tor1p in the TORC1 complex due to its redundant role, the contrary is not true, since Tor2p possesses additional functions that cannot be fulfilled by tor1p and hence the lethality in deleting *TOR2* [64, 65, 101, 102].

TORC1 signaling - Phosphatase effector branch

TORC1 signaling is nowadays quite well described due to the rapamycin specific inhibitory effect on this complex. A big part of TORC1 signaling is mediated by several type 2A or type 2A-related protein phosphatases, namely PP2As (Pph21p and Pph22p), Sit4p (Suppressor of Initiation of Transcription-4), Tap42p (Type 2A-Phosphatase Associated protein of 42kDa) and Tip41p (TAP42-interacting protein of 41kDa) [64, 103]. Combined together as complexes (Tap42-phophatase complexes) these proteins regulate the activity of several transcription factors whose target genes are controlled by TORC1 (Figure 1). Tap42p is directly phosphorylated by TORC1, which enables the interaction of this essential protein with the catalytic subunits of type 2A (and 2A-like) protein phosphatases, and consequently formation of the complexes, in favourable nutrient conditions [64, 103, 104]. Tip41p is also part of the complex and has recently been proposed to have a similar role to Tap42p in transducing the signal within the complex [104, 105].

Nitrogen metabolism regulation

One set of genes regulated by TORC1 in a Tap42-phosphatase-dependent manner belongs to one of the nitrogen metabolism pathways. TORC1 regulates the transcription of genes of the nitrogen metabolism by controlling several of its transcription factors in response to nutrients. Under rich nitrogen sources conditions, Tap42p-Sit4p phosphatase complex is attached to TORC1 [106] and the transcription factor Gln3p, whose activity regulates Nitrogen catabolite repression (NCR)-sensitive genes, is sequestered in the cytoplasm via Ure2p TORC1- dependent phosphorylation [107, 108]. Treatment with rapamycin or nitrogen limiting conditions which inhibit TORC1, result in the dissociation of the Tap42p-Sit4p phosphatase complex from TORC1, subsequent release of the complex in the cytoplasm followed by its disassembly, leading to Gln3p dephosphorylation via Sit4p. Gln3p then dissociates from Ure2p and successfully enters in the nucleus, activating NCR genes. [104, 106, 107].

Another nitrogen pathway regulated by TORC1 via Tap42-phophatase complex is the general amino acid control pathway (GAAC). The GAAC is a stress response pathway that, in reaction to amino acid starvation, activates the protein kinase Gcn2p to phosphorylate the eukaryotic initiation factor-2 (eIF2p) reducing its activity and thus lowering global translation while at the same time, preferentially stimulating translation of *GCN4* mRNA. Gcn4p is a transcription factor that activates transcription of genes under the control of GAAC, many of which involved in amino acid biosynthesis. TORC1 negatively regulates GAAC by indirectly phosphorylating, via Tap42p-Sit4p phosphatase, the Gcn2p and thus inactivating this kinase [104, 109]. A recent study has revealed that Gcn4p not only activates transcription upon amino acid depletion but also in response to rapamycin treatment, activating several genes in conjunction with Gln3p that are required for secondary nitrogen sources adaptation and thus having a major role as an effector of the TOR pathway [109].

Also targets of TORC1 regulation are the transcription factors RTG1/RTG3 involved in the Retrograde Response Pathway (RTG). This pathway controls target genes that code for intermediates of the tricarboxylic acid cycle (TCA) that assure high levels of α-ketoglutarate, a precursor involved in the biosynthesis of glutamate [64, 110]. In good nitrogen growth conditions the RTG pathway is inactive and Rtg3p and Rtg1p are sequestered in the cytoplasm due to hyperphosphorylation by

Mks1p, which is a phosphoprotein controlled by TOR. Activation of RTG pathway by rapamycin treatment or limiting nitrogen conditions triggers Rtg3p partial dephosphorilation followed by Rtg1p/Rtg3p translocation into the nucleus. This translocation is dependent on the association of dephosphorylated Mks1p to Rtg2p and thus inactivation of the former protein [104, 111].

Amino acids uptake by permeases is also subjected to TORC1 regulation and is dependent on the nitrogen composition of the medium. Rich nitrogen sources favor the delivery of constitutive high-specificity permeases to the plasma membrane such as Tat2p (tryptophan permease) and Hip1p (histidine permease), while targeting nitrogen-responsive broad-specificity permeases like Gap1p (general amino acid permease) to degradation in the vacuole. Poor nitrogen sources reverse this fluidity. TORC1 regulation of the stability of these permeases is mediated by protein kinase Npr1 (nitrogen permease reactivation kinase). TORC1 promotes phosphorylation and inactivation Npr1p in a Tap42p-Sit4p dependent manner, preventing Npr1 to protect Gap1p from ubiquitination and favouring Tat2p stabilization [64, 104].

Regulation by other nutrients

TORC1 also mediates signal cascades via Tap42-phophatase complex, in response to nutrients other than nitrogen and so regulating autophagy, stress response genes and ribosomal protein mRNA [64]. In stressful conditions deriving from oxidative stress, nutrient depletion and also heat and osmotic shock, the redundant transcription factors Msn2p and Msn4p activate the expression of more than 150 genes involved in the stress response pathway [112]. These transcription factors are subjected to control of TORC1 in a Tap42p-PP2Ap dependent manner, being phosphorylated and retained in the cytoplasm. Following TORC1 inactivation, the transcription factors accumulate in the nucleus and proceed to activate the stress response genes [105]. TORC1 regulation upon stress response transcription factors can also be mediated following another route independent of Tap42-phophatase complex, which will be addressed in the next section (Sch9 effector branch). Recent studies have shown that the catabolic process of autophagy is also negatively regulated by TORC1 through Tap42p-PP2Ap complex [113]. TORC1 exhibits its negative effects on autophagy by phosphorylating Tap42p which subsequently activates the PP2A catalytic

subunit. However, the downstream effector of PP2Ap that exerts the negative regulation is not yet known [113, 114].

Sch9p effector branch

The other known direct downstream effector of TORC1 responsible for the mediation of TORC1 signaling, is a serine/threonine kinase named Sch9p (Figure 1). Sch9p is a substrate for TORC1 suffering direct phosphorylation on several residues in the C-terminus and essential for TORC1 regulation of entry into G0 phase, ribosome biogenesis, translation initiation and stress response [61, 104]. The three nuclear RNA polymerases (Pol I, Pol II and Pol III) responsible for Ribosome biogenesis are tightly regulated by TORC1 and nuclear localization of TORC1 with binding to the 35S and 5S rDNA chromatin has been described as necessary for their transcription by RNA polymerase I and RNA polymerase III, respectively, under favorable conditions [115]. RNA polymerase II is responsible for the transcription of ribosomal protein (RP) genes and genes coding for proteins involved in the assembly of ribosomal subunits [116]. The transcription of these genes (RP genes) is regulated by the zinc finger-containing transcription factor Sfp1p which is a direct target of TORC1. Sfp1 activates RP gene expression in favourable conditions and down-regulates expression, by translocating to the cytoplasm, in response to cell stress and nutrient limitation [117]. RNA polymerase I, II and III regulation by TORC1 involves key factors such as the Rrn3p essential initiation factor for RNA polymerase I, the RNA polymerase II transcription repressors Stb3p and Dot6p/Tod6p, and the negative regulator of RNA polymerase III transcription, Maf1p [115, 116]. Besides directly regulating ribosome biogenesis, TORC1 also regulates this process via Sch9p. Sch9p is involved in the regulation of Maf1p capacity to bind and inhibit RNA polymerase III by direct phosphorylation of this repressor [116]. Similarly it was demonstrated that Sch9p phosphorylates and inhibits the activity of the repressors (Stb3p and Dot6p/Tod6p) of RNA polymerase II. As for Sch9p effects on RNA polymerase I it was observed to promote rRNA species processing from the 35S transcript into the 25S, 18S and 5.8S rRNA and to recruit the polymerase to the rDNA locus [61, 104, 116]. Previous studies also demonstrated that Sch9p controls expression of translation initiation and elongation factors [60] and recent studies have demonstrated that eIF2α phosphorylation is dependent on Sch9p [61]. TORC1 control on stress response is not only

mediated by the phosphatase complex as mentioned above but also by the Sch9p effector [118]. Sch9p directly phosphorylates the kinase Rim15p in a TORC1 dependent manner [119], thus preventing its nuclear translocation and the consequent activation of transcription factors Gis1p and Msn2p and Msn4p that are involved in the post-diauxic shift (PDS) element-driven gene expression and stress-responsive elements (STRE) gene expression, respectively [60, 104, 119].

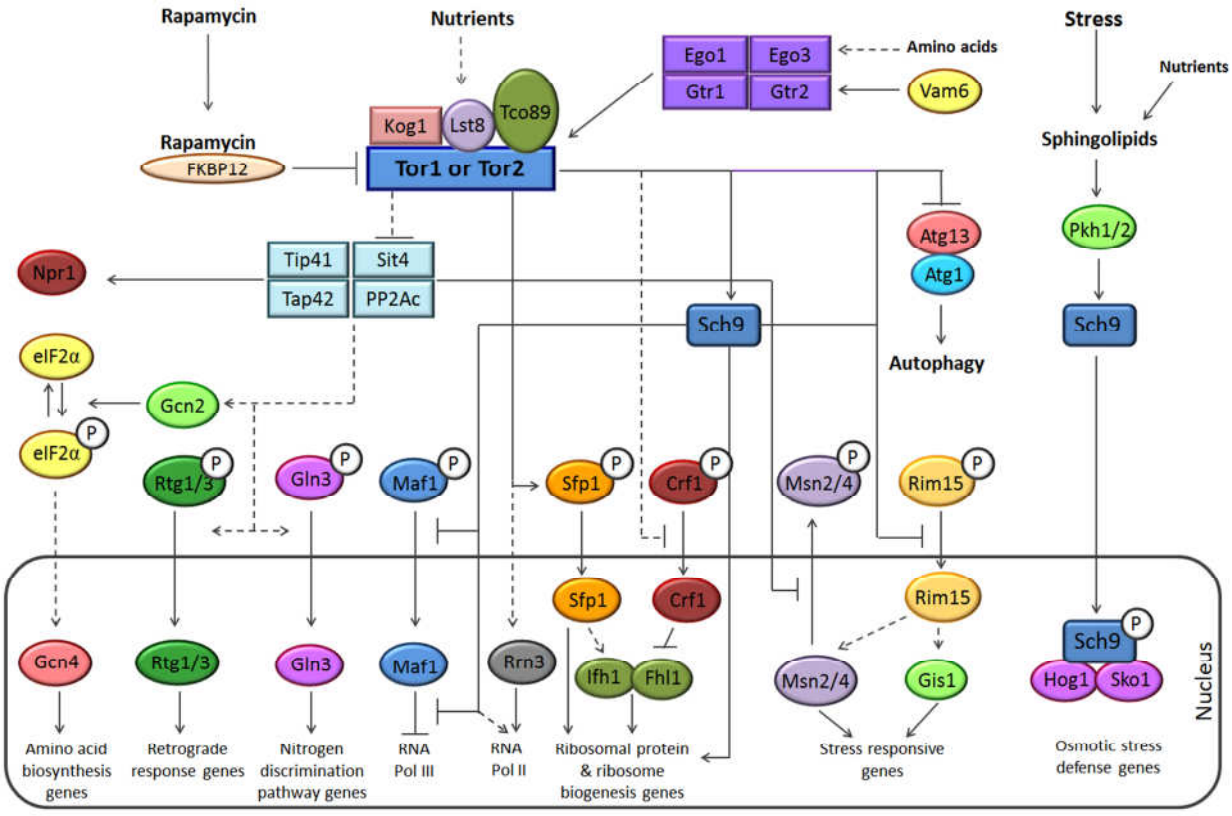

Figure 1.TORC1 signaling pathway in *S. cerevisiae*. The vast majority of the processes regulated by the rapamycin-sensitive TORC1 complex are mediated via the Tap42-Sit4/PPA2c or the Sch9 branches. This pathway responds to nutrients resulting in the stimulation of protein synthesis and the inhibition of stress response genes, autophagy and several pathways that allow growth on poor nitrogen sources. Adapted from [104].

PKA pathway

The protein kinase A (PKA) pathway is involved in the regulation of metabolism, stress response and proliferation, and this regulation is dependent on a fermentative growth [104, 120]. Activation of PKA pathway requires a rapid fermentable sugar but also a complete medium with all the essential nutrients present in order to trigger its downstream effectors. Because of these requirements for a sustainable activation of the PKA pathway, and in addition to the glucose activated cAMP-PKA pathway, Thevelein and co-workers named the activation of PKA pathway by other nutrients, the 'fermentable-growth-medium induced pathway' or FGM pathway [120-122]. In favorable conditions of fermentative growth, PKA upregulates a variety of processes such as glycolysis, cell growth and cell cycle progression, while at the same time downregulating stress resistance and gluconeogenesis and mobilizing carbohydrate glycogen and stress protectant trehalose reserves, in a process known as high PKA phenotype. In a low PKA phenotype, like under respirative growth or in stationary phase, PKA downregulation causes a variety of characteristics such as accumulation of the carbohydrates trehalose and glycogen, induction of stress-responsive element- and postdiauxic shift-controlled genes, induction of autophagy and increased stress resistance, and in the case of nutrient starvation growth arrest and subsequent entrance into G0 [120, 121, 123]. PKA is a conserved serine/theonine kinase formed by two catalytic subunits, encoded by the partially redundant genes *TPK1, TPK2* and *TPK3,* and by two regulatory subunits, encoded by *BCY1* [124, 125]. In its inactive form, PKA forms a hetero-tetramer between the catalytic and the regulatory subunits, and activation occurs by the binding of cyclic AMP (cAMP), a secondary messenger, to the regulatory subunit Bcy1p, causing its dissociation and leaving the catalytic subunits free to interact and regulate their downstream targets [60, 104, 124, 125].

cAMP-dependent regulation of PKA

In order to respond to glucose, PKA is regulated by upstream intracellular glucose sensing system and extracellular glucose detection system (Figure 2) [104]. For glucose intracellular signaling to occur, glucose is required to be transported into the cell and consequently phosphorylated by hexokinases (Hxk1p, Hxk2p and Glk1p). Glucose phosphorylation will then induce cAMP-PKA activation through the Ras proteins (Ras1p and Ras2p) which are small GTP-binding

proteins that when active in the GTP-bound state will stimulate the activity of the adenylate cyclase Cyr1p to produce cAMP [31, 104, 121, 123]. The Ras-GTP/Ras-GDP ratio is controlled through the GTPase-activating proteins Ira1 and Ira2, and the guanine nucleotide exchange factors (GEFs) Cdc25p/Sdc25p. Ira proteins stimulate GTP hydrolysis and keep Rasp in the GDP-bound inactive state while GEFs promotes GTP loading on Rasp and thus favouring the GTP-bound active state [31, 123, 126].

The extracellular glucose detection system consists of a G protein-coupled receptor (GPCR) system, composed of Gpr1 and Gpa2 [104, 123, 127]. *GPR1* codes for a seven-transmembrane G protein–coupled receptor that belongs to the GPCR superfamily and interacts with GTP-binding protein Gpa2, which belongs to the heterotrimeric G protein α subunit (Gα) protein family [104, 123, 127]. Extracellular glucose activates Gpr1p stimulating the formation of the GTP-bound active form of Gpa2p which will in turn increase cAMP production levels through adenylate cyclase (Cyr1p) stimulus [104, 123, 126]. The Gpr1p-Gpa2p module displays a low affinity for glucose and sucrose but interestingly does not respond to any other sugar. The switch from respiration to fermentative growth only occurs in high glucose concentrations with cAMP production levels having a major role in this switch, which relates to the low affinity of the Gpr1p-Gpa2p module for glucose [104, 128]. The extracellular detection system and the intracellular sensing system are described as being two distinct processes but they are also interdependent with glucose phosphorylation being required for the GPCR system to further continue to activate adenylate cyclase to produce cAMP [128]. The levels of cAMP are also subjected to negative control via PKA itself. Two phosphodiesterases, encoded by *PDE1* and *PDE2*, are responsible for the hydrolysis of cAMP to AMP [104].

cAMP-activated PKA targets

PKA regulation is responsible for 90% of transcriptional changes that occur upon addition of glucose to glucose starved cells, in which multistress response is one of the downregulated processes [104, 123, 126]. This process is mediated by PKA through two transcription factors, Msn2p and Msn4p, which in unfavourable conditions of PKA activity bind to stress responsive elements (STRE) in the promoter of their targets genes and thus promoting the general stress-response (Figure 2) [112]. In this group of STRE controlled genes, are genes involved in the

protection against stress such as heat, oxidative and osmotic stress, carbohydrate metabolism and growth regulation [104, 123, 126]. PKA regulation possibly occurs from direct phosphorylation of the two transcription factors impeding their nuclear localization and thus promoting their sequestration in the cytoplasm. Besides direct phosphorylation and inhibition of the transcription factors, PKA also regulates Msn2p/Msn4p through other routes by negatively controlling other protein kinases such as Yak1 and Rim15 [104, 112, 129]. Yak1p is a PKA antagonist responsible for inhibiting growth and is regulated by the Msn2p/Msn4p transcription factors in a positive feedback loop. PKA inhibits Yak1p possibly by direct phosphorylation via a yet unknown mechanism thus maintaining a cytoplasmic localization of Yak1p. PKA inhibition favors nuclear translocation of Yak1p, where it will activate Msn2p and thus favor transcription of the STRE response genes [130]. Rim15p is also negatively regulated by PKA being phosphorylated and inactivated in the cytoplasm. Rim15p is a kinase responsible for the accumulation of glycogen and trehalose, proper G1-cell cycle arrest upon nutrient starvation, the induction of several stress response genes and activation of Gis1p-dependent PDS-element driven transcription [131, 132]. The majority of the Rim15p controlled gene expression is mostly mediated by the three transcription factors Gis1p, Msn2p and Msn4p [60, 104, 131]. Upon PKA inactivation both kinases Yak1p and Rim15p, enter the nucleus and activate their targets, which in turn have also been liberated from the direct negative control of PKA.

PKA has also been described to mediate the transcription of Ribosomal protein (RP) genes in a positive manner. The transcription factor Sfp1p, which has been described above as a TORC1 target for regulation of RP genes expression, is also a target of PKA. PKA contributes to the maintenance of Sfp1p in the nucleus acting in a redundant way with TORC1 [104, 117]. Other direct targets of PKA mediation are the metabolic enzymes fructose-1,6-bisphosphatase (Fbp1p) involved in gluconeogenesis, which is negatively regulated, and the 6-phosphofructo-2-kinase (Pfk2p) and pyruvate kinase (Pyk1p and Pyk2p) involved in glycolysis, which are positively regulated by PKA in favorable conditions of glucose availability [104, 133, 134]. Another important effector of PKA signaling is the neutral trehalase (Nth1p) responsible for the breakdown of the stress protectant trehalose. Trehalase activity is mediated by direct phosphorylation of PKA and so after PKA activation by glucose addition to glucose starved cells, trehalase activity increases just a few minutes after, thus being extensively used to monitor nutrient-induced activation of the PKA pathway [133, 135, 136]. Autophagy is another process negatively regulated by PKA in its active state. PKA directly

phosphorylates Atg1p and Atg13p proteins necessary for the autophagy process to take place, demonstrating to be an autophagy regulator [137]. The authors also state that this regulation occurs independently of TORC1 regulation of autophagy indicating that both pathways independently control autophagy [137].

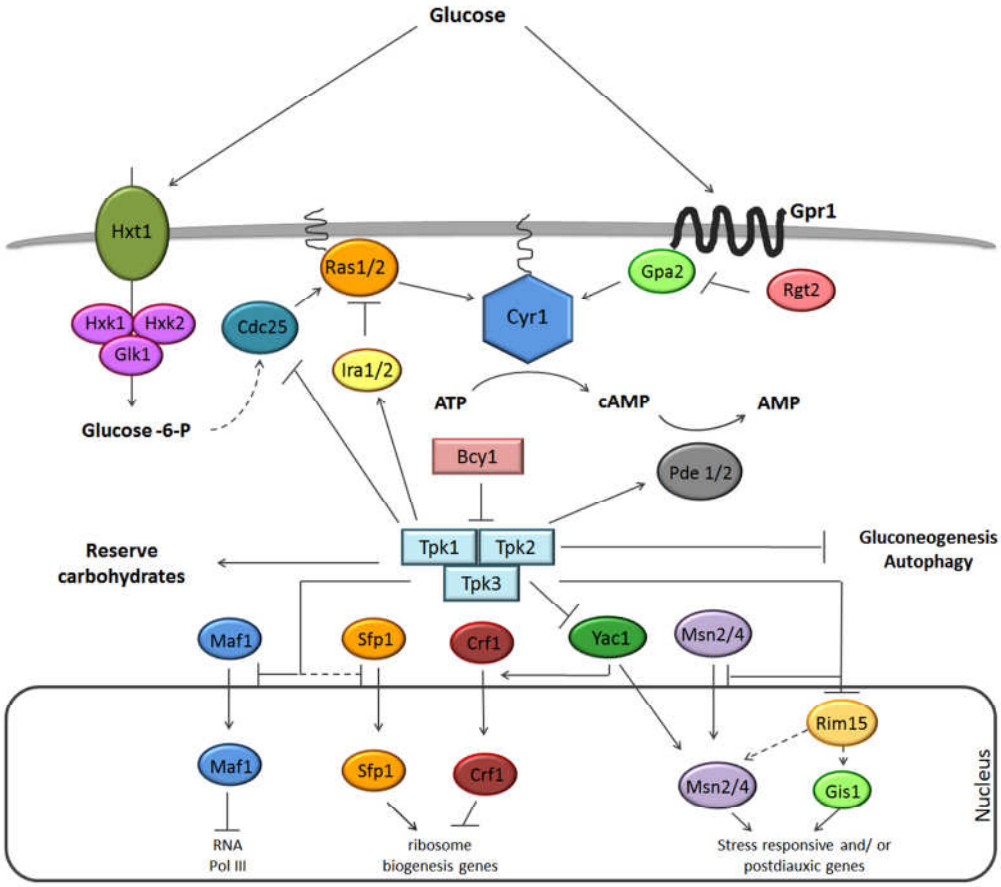

Figure 2. cAMP-PKA signaling pathway in *S. cerevisiae*. cAMP synthesis by adenylate cyclase for PKA activation requires two sensing systems: (i) extracellular detection of glucose via the Gpr1–Gpa2 system and (ii) intracellular detection of glucose, which requires uptake and phosphorylation of the sugar. PKA upregulates a variety of processes such as glycolysis, cell growth and cell cycle progression, while at the same time downregulating stress resistance and gluconeogenesis .Adapted from [104].

cAMP-independent regulation of PKA

As mentioned previously, in order for PKA to become active it is necessary the availability of a rapid fermentable sugar but also a complete medium, indicating that PKA also responds to other essential nutrients for its activation [62, 120]. The lack of any other essential nutrient in the presence of glucose causes cell cycle arrest with characteristics of a "low PKA phenotype" whereas the readdition of the missing nutrient rapidly activates PKA. Nitrogen and phosphate induced activation of PKA seems to follow the above mentioned 'fermentable-growth-medium induced pathway' or FGM pathway in which cAMP increase is not a mediator in the signaling cascade [62, 120, 121, 123] but the free catalytic subunits of PKA are still necessary for mediation in this pathway [138]. Another kinase, Sch9p, has also been described to mediate response in amino acid and ammonium induced activation of the FMG pathway but not in phosphate induced activation [62]. Due to the homology between the catalytic domains of Sch9p and the catalytic subunits of PKA [63], and to the common targets shared by the two kinases, the Sch9p kinase has been proposed to act in parallel with PKA [60].

In the cAMP-independent regulation of PKA, the activation of the FMG pathway occurs through plasmas membrane sensors known as transceptors because of their double functions as active transporters and receptor sensors. There are three described transceptors that activate this pathway: the amino acid transceptor, Gap1p; the ammonium transceptors, Mep1p and Mep2p; and the phosphate transceptor, Pho84 [121, 123]. Synthesis and activity of the Gap1p and the Meps transceptors are subjected to tight control by the nitrogen catabolite repression (NCR) pathway. The regulation of the transceptors by this pathway is addressed in section (1.5). Rapid PKA activation of nitrogen-starved cells by amino acid addition requires Gap1p as an amino acid sensor but not further metabolization of the amino acids. In *gap1*Δ strains, addition of the amino acid L-citrulline, fails to active trehalase when transported by other amino acid carriers, showing that amino acid-induced activation of PKA is mostly dependent on Gap1p [139]. Recent work has also shown that transport is not necessary for Gap1p to activate signaling since amino acid analogs that are not transported are still able to trigger signaling [140]. Mep1p but predominantly Mep2p can also activate PKA in response to the addition of ammonia to nitrogen-starved cells and as for Gap1p this activation in not dependent on a cAMP increase [135]. The non-metabolizable ammonium analog

methylamine, was able to activate trehalase in a Mep2p expressing strain while being unable to do the same in a triple Mep deletion strain although it can diffuse through the plasma membrane, showing that trehalase activation is in fact Mep2p-dependent [123, 135]. As referred above the Gap1p and Mep2p induced PKA activation involves the kinase Sch9p, however the underlying mechanism behind the signaling is not yet known. A recent study has identified different putative interactors for Gap1p and Mep2p that appear to be good candidates to unravel the signaling cascade between the transceptors and PKA. The study also demonstrated that the signaling and transporting functions can be separated and can act independently of each other conforming that signaling does not require transport. Using deleted strains in these putative interactors the authors verified that variations (increase or decrease) in transport are not necessarily correlated to an increase or decrease in signaling [141].

SCH9 pathway

As introduced above, Sch9p is a kinase that mediates PKA activation in the FMG pathway and also mediates many of the TORC1 controlled processes [60-62]. Sch9p was first described has having a partially redundant role with PKA pathway, since deletion of Sch9p could be compensated by increased activity of PKA and vice versa [63], and later as a direct target of TORC1 regulation, perceiving Sch9p as a downstream effector of TORC1 and an integrating part of the PKA signaling cascade. Nevertheless, over the past few years a new, more relevant role has emerged for Sch9p as recent studies propose an antagonistic and independent effect of Sch9p in the regulation of some of TORC1 and PKA mediated processes [60, 118, 142]. Roosen and co-workers verified that PKA and Sch9p act through separate but partially overlapping signaling cascades in a parallel manner, having both synergistic and opposite effects in the expression of common target genes [60]. Sch9p has been implicated in the shift in signaling that occurs upon the alteration of the carbon source as it became clear that in strains lacking Sch9p, cells cannot perceive the signal of starvation of the fermentable carbon source, displaying phenotypic characteristics associated with higher PKA activity in these conditions and reverse phenotypic characteristics in fermentable carbon sources [60, 62]. Sch9p displays a similar function to the PKA pathway in fermentative growth but activates stress response genes during diauxic shift and respiratory growth [60, 118], negatively regulating nuclear

accumulation of the Rim15p kinase during the former condition and positively regulating Gis1-dependent PDS gene expression, independently of Rim15p itself, in the latter conditions [60, 118]. Rim15p is the converging signaling effector of PKA, TORC1 and Sch9p and a regulator of the entrance into G0 [143] In other stressful conditions like in osmotic stress, Sch9p also has an independent role in the activation of osmostress-responsive genes, acting as a chromatin-associated transcriptional activator, being recruited to the promoter region of these genes in a Hog1p dependent manner. Hog1p is a High-Osmolarity Glycerol MAP kinase involved in the osmoregulatory signaling cascade [118, 142]. A recent study also emphasized the independent role of Sch9p by showing that it acts independently of TORC1 to regulate genes encoding for proteins involved in respiration, such as TCA cycle proteins, involved in fatty acid metabolism and mitochondrial ribosomal proteins, and the authors propose a mechanism in which during favorable conditions, the TORC1-dependent Sch9p effector branch is predominant, leading to translation while repressing the stress response genes, and alternatively in unfavorable conditions, when TORC1 is inactive, Sch9p is necessary to activate the expression of the stress response genes [118]. Also in the same study Sch9p emerged as a regulator of the nitrogen metabolism, being able to act independently of TORC1 to reduce the basal expression of genes belonging to the nitrogen discrimination pathway (NDP) like, Gap1p and Mep2p and to the GAAC pathway like Gcn4p [118]. Recently, Sch9p has also been described as being involved in the regulation of autophagy when Yorimitsu and co-workers found that Sch9p and PKA cooperate in this regulation, demonstrating that simultaneous inactivation of both kinases is sufficient to trigger autophagy induction, while TORC1 is activated. This study proposes that the three pathways act in parallel to regulate autophagy since inactivation of all three kinases revealed an additive stimulation of autophagy [144].

1.3. Programmed cell death

Programmed cell death (PCD) is an active form of cell death, genetically regulated, opposite to accidental cell death or classical necrosis that occurs during cell injury. It is often referred as a suicide process that cells undergo during development and homeostasis of adult tissues, embryogenesis and disease control in multicellular organisms. PCD includes, among others,

programmed necrosis, autophagic cell death and apoptosis, the latter being the most common form of PCD [145]. Cells undergoing apoptosis are characterized by severe morphological changes such as the reduction of cellular volume, chromatin condensation and nuclear fragmentation and engulfment by resident phagocytes.

1.3.1. General aspects in multicellular organisms

Due to recent advances in the biochemical and genetic characterization of cell death, a new terminology has been proposed for PCD, that replaces morphological criteria for molecular criteria, and so delineates PCD in the following categories: extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophe [146].

Extrinsic apoptosis, based on molecular criteria, is defined as a caspase-dependent subroutine that is activated by extracellular stress signals and that is possible to suppress by caspases inhibitors such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) and viral inhibitors of caspases like cytokine response modifier A (CrmA) [146, 147]. Caspases are cysteine proteases that cleave their substrates following an aspartic acid residue and have key mediating functions in this PCD process. These proteases can be divided into initiator caspases (pro-caspases 2, 8, 9 and 10) and effector caspases (pro-caspases 3, 6 and 7). Initiator caspases activate effector caspases by cleaving inactive pro-forms of effector caspases, which are then responsible for triggering the apoptotic process [148, 149]. Extracellular signals are sensed and subsequent propagated by specific transmembrane receptors, namely death receptors (e.g., TNFR1, Fas/CD95 and TRAIL-R) and dependence receptors The signal is produced upon the interaction of specific ligands, called death activators, with the transmembrane receptors that in turn recruit proteins that form a multiprotein complex that will then activate the caspase cascade [146].

On the other hand, intrinsic apoptosis is activated by intracellular stressful signals (DNA damage, oxidative stress, overload of cytosolic Ca^{2+}, accumulation of unfolded proteins in the endoplasmatic reticulum (ER), etc.) which generate pro-survival and pro-death signals that culminate in a mitochondria-controlled mechanism [150]. In a scenario where cells cannot cope with stress,

pro-death signals prevail and will generate the mitochondrial outer membrane permeabilization (MOMP) that can be triggered by members of the BCL-2 protein family such as BAK and BAX, at the outer mitochondrial membrane, or it can be triggered at the inner mitochondrial membrane resulting from a multiprotein complex formation known as mitochondrial permeability transition pore (MPTP) [150, 151]. Upon MOMP, a series of events are triggered that lead to mitochondrial membrane potential ($\Delta\Psi m$) dissipation, arrest of ATP synthesis and $\Delta\Psi m$-dependent transport activities, release of proteins from the mitochondrial intermembrane space into the cytosol like cytochrome *c* (cyt *c*), apoptosis inducing factor (AIF), endonuclease G (Endo G), HtrA2/OMI and Smac/DIABLO, and inhibition of the respiratory chain leading to overproduction of reactive oxygen species (ROS) [152, 153]. Once released into the cytosol, cyt *c* promotes the formation of the apoptosome, by assembling with the adaptor protein Apaf1 and the pro-caspase 9, and upon formation the apoptosome will trigger the caspase 9-caspase 3 proteolytic cascade. This caspase cascade activation is further facilitated by the inhibition of antiapoptotic proteins members of the Inhibitor apoptosis protein (IAP) family, which is executed by the mitochondrial released Smac/DIABLO and HtrA2/OMI. In a caspase-independent manner, on the other hand, apoptosis-inducing factor (AIF) and endonuclease G (endoG) after mitochondrial release relocate to the nucleus and mediate large-scale DNA fragmentation [154].

Autophagic cell death has been described in cases where cell death occurs with massive cytoplasmic vacuolization, in the absence of chromatin condensation. Initially, this expression merely meant that autophagy was present during cell death but was not the causing agent of death [145, 146]. Nevertheless, involvement of autophagy in cell demise was discovered in the developmental program of *Drosophila melanogaster* and in some cancer cells, especially in cells lacking essential apoptotic modulators like BAX and BAK or caspases [155-157]. All the same, in most cases autophagy is considered a protective response to cope with stress, where dying cells activate autophagy in an attempt to re-establish homeostasis and survival [158]. The new molecular criteria for the definition of autophagic cell death postulate that this type of death should be suppressed by chemical inhibitors of the autophagic pathway and by genetic manipulation of the pathway which leads to inhibition of the autophagic process, in order to access the actual occurrence of an autophagic death [146].

Necrosis, in the classical view, is an accidental, uncontrolled death that occurs upon major cellular injury. The main morphological characteristics of necrosis include increase in cell volume and swelling of organelles with subsequent loss of plasma membrane integrity and leakage of cell content, and also at the molecular level, DNA is randomly degraded, ATP-levels are depleted and overproduction of ROS occurs [159-161]. However, in the past decade evidences have emerged indicating that necrosis can be regulated process controlled by a set of signal transduction pathways and catabolic mechanisms [159, 160]. Although the complete mechanism in yet unknown, some key players in the regulation of necrosis have been identified such as receptor interacting protein (RIP) kinases (Rip1p and Rip3p), calpains, cathepsins and cyclophilin D. Activation of death receptors is known to stimulate necrosis, when caspases are inhibited, by interacting and activating Rip1p that in turn phosphorylates and activates Rip3p [145, 160]. This cascade will then affect mitochondria and production of ROS initiating a number of events including mitochondrial alterations, such as nitroxidative stress by nitric oxide and mitochondrial membrane permeabilization, lysosomal and nuclear changes, lipid degradation and increases in the cytosolic concentration of calcium, which culminates in activation of non-caspase proteases like calpains and cathepsins [145, 160, 162]. Recently, it was discovered that Rip3p can regulate necrosis in a Rip1p independent manner and so, due to the lack of a biochemical denominator, identifying a necrotic process still relies on negative criteria like absence of apoptotic or autophagic markers [145, 146].

1.3.2. Apoptosis and necrosis in yeast: pathways and key molecular components

PCD processes, involving tightly regulated mechanisms, were thought to be exclusive of multicellular organisms for many years until apoptosis-like, autophagic and necrotic cell death pathways were detected in yeast cells. It was shown that yeast also possesses a coordinated suicide program that can help the community to deal with external stressful conditions and ensure a rapid adaptability. Actually, in yeast colonies, single cells undergo an altruistic death promoting the long-term survival of the whole colony [163]. However this process can also be imposed, triggered by competing yeast strains or higher eukaryotes [164]. The “group selection theory” postulates that individual cells sacrifice themselves in benefit of the whole population and so programmed cell death seems to clean the population over time, suggesting that aging in yeast and possibly in higher

organisms may be programmed [42, 196]. This death in chronological aging cultures provides the population with nutrients and the DNA damage and mutation frequency that derives from superoxide eases the appearance of adaptive regrowth mutants that assure the survival of the group. These mutants turn up when almost all the population is dead (90-99%) and grow under conditions that are normally not permissive for growth [71].

Apoptosis in unicellular *S. cerevisiae* yeast cells was first described by Frank Madeo and co-workers in 1997, in *CDC48* defective cells (*cdc48*^{S565G}) that showed many of the markers associated with apoptosis, like DNA fragmentation, phosphatidylserine externalization and chromatin condensation [165]. Soon after, the same authors described that accumulation of reactive oxygen species (ROS) by depletion of glutathione or exposure to low external doses of H_2O_2 led to apoptosis in wild-type cells, while depletion of ROS or hypoxia prevented cell death. Therefore a key role for ROS in the regulation of yeast apoptosis emerged [166]. In the early and mid-2000s a vast number of other stimuli that could induce apoptosis were discovered, including acetic acid [94], sugar [58], osmotic stress [167], UV radiation [168], amino acid starvation in auxotrophic cells [169], DNA damage [170] and chronological aging [171], among others. The most well studied external inducers of apoptosis in yeast are the acetic acid and H_2O_2 compounds. Studies by Ludovico and co-workers discovered that acetic acid induced apoptosis through a mitochondria-mediated apoptotic pathway similar to the mammalian intrinsic apoptotic pathway, resulting in cytochrome *c* release and ROS production [93]. Another important breakthrough in yeast apoptosis studies was the identification of metacaspase Yca1p, the only yeast ortholog of mammalian caspases identified so far, and its implication in H_2O_2 induced apoptosis [172]. The discovery that yeast possesses some orthologs of mammalian apoptosis regulators such as cytochrome *c*, AIF, HtrA/OMI and endonuclease G supports the existence of conserved primordial apoptotic machinery in yeast similar to that found in higher eukaryotic organisms [173, 174], that has proven to be useful in unraveling unknown features of mammalian proteins of the intrinsic apoptotic pathway [175].

Necrosis in yeast is up till now an extremely recent field not yet generally well-known. The fate of an apoptotic yeast cell in culture is in fact a necrotic end with loss of plasma membrane integrity since it will not be phagocytosed by neighboring yeast cells. This phenomenon is known as secondary necrosis. In chronological aged yeast cells, death exhibits biochemical and morphological markers of both apoptosis and necrosis [171], nevertheless several studies have shown the

appearance of necrotic markers without apoptotic characteristics, suggesting an additional type of necrotic cell death, distinguishable from secondary necrosis and that should be classified as primary necrosis [176-178]. Although further studies are necessary for establishment of necrotic machinery in yeast, it is already known that mitochondria plays an important role in this process and some of the biochemical changes that occur during necrosis include, mitochondrial outer membrane permeabilization (MOMP), dissipation of mitochondrial potential, ATP depletion, overproduction of reactive oxygen species (ROS) and nuclear release of Nhp6Ap, yeast homolog of the mammalian high mobility group box-1 (HMGB1) protein [176]. A recent study showed the involvement of pro-apoptotic mitochondrial Nuc1p, yeast homolog of mammalian endonuclease G (EndoG), displaying vital functions as an anti-necrotic protein. Deletion of *NUC*1, led to increasing rates of necrosis upon aging or peroxide treatment [179]. Another finding that further postulates that necrosis is also a well-controlled molecular process in yeast was the discovery of anti-necrotic characteristics of yeast ortholog of mammalian cathepsin D, Pep4p. The propeptide of this vacuolar protease promoted survival of chronological aged cells by specifically reducing necrosis [180].

The involvement of cellular organelles

Mitochondria involvement in apoptosis

As aforementioned mitochondria has an established role in apoptosis. One of the most studied apoptotic inducers is acetic acid and its effects in the apoptotic cascade have been extensively characterized. Mitochondria is also an active executer in acetic acid induced apoptosis, leading to the release of cyt *c* into the cytosol, production of ROS, reduction in oxygen consumption and in mitochondrial membrane potential and decrease in cytochrome oxidase activity (COX) linked to a specific decrease in the amount of COX II subunit [93, 181]. However the events that follow cyt *c* release are so far unknown since downstream components have not yet been identified in yeast. Although a relationship between cyt *c* release and caspase activation has been established for hyperosmotic shock induced apoptosis [167], this is not the case for most apoptotic scenarios in yeast and so the precise role of cyt *c* release, as it has been clearly demonstrated for the mammalian caspase activation, still requires further investigation. Another important step in

mammalian mitochondria-dependent apoptosis is the formation of the above mentioned MOMP. Though the molecular composition of the pore is not completely defined, it has been proposed that its major components/regulators are the adenine nucleotide transporter (ANT), the voltage dependent anion channel (VDAC) and cyclophilin D [151]. In yeast, Pereira and co-workers demonstrated that the absence of ADP/ATP carrier (AAC) proteins (yeast orthologs of ANT) protects cells exposed to acetic acid, by inhibiting cyt *c* release [182] and Pavlov and coworkers demonstrated that Bax expressed in yeast cells induced the formation of mitochondrial apoptosis-induced channel (MAC) [183]. The absence of AAC proteins and cyt *c* release just partially prevented acetic acid-induced cell death, suggesting that alternative cyt *c*-independent pathways are involved [182]. The translocation from the mitochondria to the nucleus of intermembrane space mitochondrial proteins released in response to acetic acid, such as Aif1p, could possibly mediate this alternative pathways [184]. In fact, other stimuli, besides acetic acid, can stimulate yeast Aif1p to translocate from mitochondria to the nucleus, such as aging and H_2O_2 [173]. As in mammalian cells, yeast Aif1p effects are dependent on yeast cyclophilin-A and Aif1p possesses the ability to degrade DNA. However, unlike mammalian cells in which this mechanism is caspase-independent, in yeast Aif1p pro-apoptotic activity is partially metacaspase-dependent since deletion of *YCA1* gene partially suppressed cell death in cells overexpressing Aif1p during H_2O_2 exposure [184]. Besides its death function, Aif1p also has vital functions within the mitochondrial intermembrane space with its redox function being necessary for proper oxidative phosphorylation [185]. Another mitochondrial protein that translocates to the nucleus upon apoptotic stimuli is the yeast Nuc1p, ortholog of the mammalian pro-apoptotic endonuclease-G (EndoG). This protein was found to trigger apoptosis independently of the Aif1p and metacaspase, while depending on proteins involved in the permeability transition pore formation (yeast orthologs of ANT and VDAC), nuclear import (karyopherin Kap123p) and in phosphorylation of histone H2B. These results showed a EndoG-cell death route from mitochondria release to nuclear import with consequent DNA fragmentation [179]. Resembling Aif1p, Nuc1p also has dual functions, demonstrated by the deletion of this gene, which inhibited apoptotic death during mitochondrial respiration and enhanced necrotic death during fermentation [173, 179].

During mammalian apoptosis, mitochondrial fragmentation (fission) is commonly observed at early stages and is mediated by fission machinery regulated by the dynamin related protein-1

(Drp1p). This machinery has also been identified in yeast as a complex of three proteins, Dnm1p, Mdv1p/Net2p, and Fis1p.

Like for mammals, mitochondria have emerged as a central organelle in yeast PCD. The finding that proteins within this organelle regulate apoptosis in a similar way in both unicellular and multicellular organisms is indicative of an evolutionary conserved mechanism and so yeast has proven to be a valuable help to unravel the mechanisms of severe human diseases associated with mitochondria-dependent apoptosis [174].

Vacuole involvement in apoptosis

Other organelles besides mitochondria have also been described to be involved in PCD regulation. In mammalians, lysosomal membrane permeabilization (LMP) causes the release of hydrolases from the lysosome lumen into the cytosol [186]. Once in the cytosol these hydrolases, namely CatD, initiate a mitochondrial apoptotic cascade [187]. Recent studies have demonstrated that yeast vacuoles, the yeast equivalent to mammalian lysosomes, also play a role in cell death. As in mammalian cells, the yeast ortholog Pep4p is released into the cytosol without rupture of the vacuolar membrane, during H_2O_2 [188] and acetic acid exposure [189] leading to degration of nucleoporins and mitochondria. However, anti-apoptotic and anti-necrotic functions have been identified for Pep4p in yeast. Deletion of this vacuolar protease, causes increased cell death during acetic acid exposure [189] and results in combined apoptotic and necrotic cell death during chronological aging [180]. Also, a pro-survival role of CatD during apoptosis has also been observed in mammalian cells [190, 191]. The involvement of Pep4p in the degradation of mitochondria and the importance of this degradation to the cell death cascade reveals a complex regulation between mitochondria and the vacuole.

Peroxisome involvement in apoptosis

Peroxisomes also seem to have a role in PCD, since it was discovered that deletion of *PEX6*, which codes for a peroxisomal membrane protein involved in protein import to the peroxisome, induced a necrotic cell death process accompanied by an increased accumulation of ROS in yeast

cells exposed to acetic acid or entering early stationary phase. The fact that peroxisomes harbour antioxidant enzymes, like catalase and glutathione peroxidase to protect the cell against oxidative damage could be one explanation for the decrease in viability observed in *pex6Δ* mutant cells [178].

Signaling pathways

The signaling pathways regulating cell death in yeast are still a matter deprived of extensive study. Indeed, most studies have focused on the identification of different apoptotic triggers and the components/regulators of apoptotic death. The involvement of the major nutrient pathway Ras/AC/PKA in acetic acid induced apoptosis was first described in *Candida albicans* as it was observed that apoptotic induced cell death, during acetic acid treatment, was largely delayed or even supressed in strains deleted in several key components (*ras1*Δ, *cdc35*Δ, *tpk1*Δ, and *tpk*2Δ) of this signaling pathway whereas mutations in *RAS1* or *PDE2* that increased signaling, enhanced apoptosis [192]. In *S. cerevisiae* this major nutrient signaling pathway was also proven to be involved in acetic acid induced apoptosis since deletion of the *RAS2* gene decreased cell death during the exposure to this acid [91, 192]. Gourlay and co-workers have also shown the involvement of Ras/AC/PKA pathway in apoptotic cell death induced by accumulation of stable actin aggregates that lead to hyper activation of this pathway and consequently to the production of ROS in mitochondria. Moreover, these authors found that deletion of *TPK3*, which codes for one of PKA catalytic subunits, prevented ROS production in actin aggregating strains and that Tpk3p seems to regulate mitochondria function by activating several downstream targets [193, 194].

As TOR pathway is another major nutrient signaling pathway, its involvement in PCD seems inevitable. Indeed, TOR is involved in apoptotic cell death induced by acetic acid exposure, that causes severe intracellular amino acid starvation, regulating death by the two downstream mediator phosphatases Pph21p and Pph22p but not Sit4p [195]. The association of the TOR pathway with PCD in yeast came from CLS studies where the outcome of its inactivation was the extension of life span [52]. During aging in yeast, apoptotic cell death occurs [171] suggesting that cell death has evolved in microorganisms as a survival strategy beneficial to the group [71]. The nutrient signaling pathways Sch9, TOR and Ras/AC/PKA are key elements in this program and its activation reduces

cell protection as it raises the levels of superoxide production leading to cell damage and death [12, 42, 196]. Deletion of key genes in these pathways such as *SCH9*, *RAS2* or *TOR1* increase longevity up to 3-fold and point to an "aging program" that blocks cell protection and accelerates cell death, naturally connecting aging and apoptosis [27, 50, 52, 71].

1.4. Autophagy and its role in cell survival

In order to maintain viability during starvation periods, yeasts undergo a degradative process of their own cellular components by a self-eating process via the vacuole, named autophagy [197, 198]. Autophagy is a highly conserved porcess in eukaryotic cells and consists of the formation of a double-membrane vesicle in the cytosol, the autophagosome [199], which enfolds cellular components that are delivered into the vacuole upon fusion of the autophagosome with its membrane. Once inside the vacuole the cellular components engulfed are exposed to a variety of hydrolytic enzymes that degrade their macromolecules, generating an internal pool of molecules ready to be recycled [197, 198]. Although under starvation conditions mainly nitrogen starvation but also carbon source starvation or auxotrophic amino acid starvation, autophagy is drastically induced, it also occurs under normal growth conditions degrading damaged or aged organelles [200]. Selective autophagy of organelles is essential for cellular homeostasis and includes mitochondria (mitophagy), peroxisomes (pexophagy) and ribosomes (ribophagy) [198, 201, 202]. Another autophagy-related pathway of selective autophagy, active under normal growth conditions, involves the transport of the precursors of the hydrolases aminopeptidase I (prApe1) and α-mannosidase (prAms1) synthesized in the cytosol into the vacuole by a pathway so-called cytoplasm to vacuole targeting (Cvt) which consists of the only known biosynthetic function of autophagy [114]. The involvement of autophagy in several conditions ranging from cell stress and starvation to growth reveals the importance of autophagy regulation in to the cell's response to specific metabolic requirements.

1.4.1. Basic mechanisms and molecular components

Autophagy in yeast starts in a define location with the biogenesis of membranes called the phagophore assembly site (PAS). This PAS is not yet fully known and it is still not clear if it represents the site of autophagosome formation or the phagophore itself that will then mature into an autophagosome [203]. At the PAS, several specific autophagy proteins are recruited to initiate the formation of the autophagosome. The autophagy-related genes (*ATG*) where almost all screened in yeast and so far 33 genes have been identified that code for proteins having functions at several steps in the non-selective and selective autophagy-related pathways [204]. Of these Atg proteins, seventeen are core components, making part of all autophagy-related pathways and the other sixteen proteins have more specific roles. In spite of being isolated in yeast orthologues of almost all Atg proteins have now been identified in multicellular organisms, revealing that both the molecular machinery and the mechanism of autophagy are highly conserved [198, 205]. The assembly of the PNAS, called nucleation, starts with the recruitment of Atg17p that in turn recruits Atg13p and Atg9p. Together they activate class III phosphoinositide 3-kinase (PI3K) complex formed by the two vacuolar protein sorting-associated (VPS) 34 and 15 and the two Atg proteins 14 and 6 [200]. The next step is the formation of a complex between Atg13p and Atg1p however this step is highly regulated by the nutrient signaling pathways [137, 144]. Under rich nutrient conditions, the TOR kinase hyperphosphorylates Atg13p reducing its affinity for Atg1p. On the other hand under starvation conditions TOR kinase is inactive and Atg13p becomes hypophosphorylated and is now capable of forming a complex with Atg1p [206]. More recently, the involvement of the PKA pathway in the regulation of autophagy by interaction with the Atg13p/Atg1p complex was discovered. The PKA kinase directly phosphorylates Atg13p in a TOR-independent manner, and it is by itself responsible for inhibiting autophagy during TOR inactivation and vice versa, which means that these two pathways independently regulate autophagy [137]. After the formation of the Atg13p/Atg1p complex, Atg18p and Atg2p are recruited to join the PI3K complex and to mediate the retrograde transport of Atg9p to periphery sites where it is thought to recruit membranes to the PAS for the biogenesis of the autophagosome. For vesicle expansion, it is necessary the conjugation between Atg5p, Atg12p and Atg16p and also Atg8p with phosphatidylethanolamine (PE), which is dependent on an ubiquitin-like conjugation systems involving Atg3p, Atg4p, Atg7p, and/or Atg10p [114, 200]. The Atg8p-PE then associates with the expanding double membrane autophagosome in both

surfaces while the Atg5p/Atg12p/Atg16p complex only associates with the outer membrane. After fusion of both ends of the expanding double membrane, Atg4p is activated to cleave Atg8p-PE and Atg5p/Atg12p/Atg16p complex releasing these complexes from the surface of the outer membrane but leaving Atg8p-PE intact in the inner membrane. After removal of the complexes from the autophagosome surface, it is now ready to dock and fuse with the vacuolar membrane. Once inside the vacuole, the autophagic body is degraded by lipase Atg15p and by vacuolar hydrolases such as Pep4p and finally the products from autophagy degradation are then released back into the cytosol through permeases such as Atg22 [114, 200].

1.4.2 Autophagy in cell survival and aging

Due to its role in cellular homeostasis, autophagy is also an important process in aging and in cell death and is regulated by the same major nutrient signaling pathways as aging. During aging, damaged proteins and organelles accumulate and an effective “clean up” process like autophagy is necessary for cell survival. Although the contribution of autophagy to CLS is presumed since autophagy is induced in long-lived deletion strains of the TOR/SCH9 and PKA pathways, only recently a number of studies confirmed this contribution. Deletion of genes involved in the autophagic pathway shortened the CLS, demonstrating that autophagy is required for extension of CLS [80, 89, 98]. Also a relation between the amino acid pool and autophagy is critical for CLS, as amino acids extend CLS in autophagy-deficient as well as autophagy-competent yeast cells [80]. This result is in agreement with previous results for nitrogen starvation, where it was demonstrated that autophagy-defective mutants could not maintain the amino acid pool needed for protein synthesis [207]. The same authors in a recent work also showed that autophagy is essential for maintaining mitochondrial functions during nitrogen starvation and that mitochondria dysfunction is the major cause of starvation-induced cell death in autophagy-defective mutants [208]. Future studies in yeast will further clarify the involvement of autophagy in CLS and the role of mitochondria as several short-lived mutants are in mitochondria related genes [89, 98].

Even though most studies point to a positive role for autophagy in survival during aging, some studies point to the involvement and contribution of autophagy in cell death [209, 210]. A

study using proteotoxic stressed cells evaluated the role of autophagy in CLS and demonstrated that excessive autophagy can lead to cell death and shortening of CLS. These results evidence the balance of autophagy levels as a key point in survival [210].

1.5. Ammonium utilization and its toxicity

Ammonium toxicity has been well described in animals and plant systems [211], however little is known about a possible toxic role of ammonium in yeast. Production of ammonia in yeast colonies has even been described as a mechanism of protection from cell death during colony development [163]. To date, only one report of ammonium toxicity in yeast is known and refers to steady-state chemostat cultures limited for potassium [212]. In this study, excess ammonium was found to be toxic for *S. cerevisiae*, under potassium limitation, resulting in amino acid excretion similar to the detoxifying mechanism found in mammals. The authors described that ammonium toxicity in yeast is related to a "leak current" of ammonium ions that enter the cell through potassium channels, in limiting potassium conditions, and this influx causes an excess of internal ammonium that becomes toxic for the cell. To cope with this ammonium excess, cells excrete amino acids possibly through the Ssy1p-Ptr3p-Ssy5p (SPS)-system of amino acid transporters, which were found to be strongly up-regulated in this condition, or by directly excreting ammonium via the Ato (Ammonium Transporter Outward) transporters [212].

1.5.1. A brief introduction to ammonium toxicity in mammals

In mammals ammonium toxicity is associated to Urea Cycle Disorders (UCD) in which the inability to detoxify ammonium through the urea cycle in liver cells, leads to high levels of ammonium in the blood, a condition known as hyperammonemia [213]. This condition of excess ammonium in the blood can ultimately reach the central nervous system (CNS), where ammonium toxicity will cause the most damages. Hyperammonemia can result in neurological disorders such as hepatic encephalopathy and other metabolic encephalopathies, which display high mortality rates [129]. The main alteration caused by hyperammonemia in the CNS is astrocyte swelling that can

lead to brain edema with increased intracranial pressure and brain herniation [213, 214]. Astrocytes are the first line of defence of the brain against ammonium neurotoxicity by metabolizing ammonium as it reaches the brain. Possibly as the only brain cells to express glutamine synthetase (GS), astrocytes detoxify ammonium by synthesizing glutamine leading to an increase in the intracellular levels of glutamine [213, 214]. Excess of glutamine can enter into mitochondria and be degraded back to ammonium resulting in the generation of reactive oxygen-nitrogen species, mitochondrial permeability transition (mPT) and also activation of mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappaB (NF-κB) [215]. Ammonium toxicity in the brain also alters neurotransmitters system by promoting glutamate extracellular release in a pH and Ca^{2+} dependent manner by astrocytes, by inhibiting glutamate uptake in astrocytes due to down regulation of protein levels of GLT-1 and GAST transporters and by an excess in depolarization of glutamatergic neurons [213, 215].

Pedriatic patients are more susceptible to ammonium toxicity than adults and prolonged exposure to high levels of ammonium can permanently damage the CNS causing cortical atrophy, ventricular enlargement, demyelination and other alterations in neurons and oligodendrocytes, while no major structural damage to neurons is observed in adult patients [216]. Ammonium triggers apoptosis in neurons of new-borns or infants mediated by activation of caspases and calpain, during which calpain cleaves the cyclin-dependent kinase 5 activator p35 to p25, forming the complex cdk5/p25 which has been described to cause neuronal death [213]. During ammonium exposure the brain also activates defensive mechanisms to protect itself from damage. Among these endogenous defensive mechanisms is the activation of ciliary neurotrophic factor (CNTF), which is a cytokine-like protein expressed only in astrocytes, involved in injury survival in the brain. This neurotropic factor is up-regulated in response to ammonium exposure by p38 MAPK activation but its expression is also controlled by intracellular signaling occurring in both oligodendrocytes and neurons via SAPK/JNK and Erk1/2 MAPK activation. The protective effect of CNTF was only observed in oligodendrocytes in the presence of ammonium, through the involvement of JAK/STAT, SAPK/JNK and c-Jun pathways [216].

1.5.2. Ammonium as a nitrogen source

Ammonium has a central role in nitrogen metabolism in *S. cerevisiae* being involved in both catabolic and biosynthetic pathways [217]. In yeast, nitrogen sources need to be converted into glutamate and glutamine prior to their use. However not all nitrogen sources are equally preferred and yeast can select the nitrogen sources through nitrogen catabolite repression (NCR) mechanism also known as nitrogen discrimination pathway (NDP). As mentioned above, this pathway enables yeast to repress genes that code for proteins required for the use of poor nitrogen sources, when in the presence of sufficient quantities of rich nitrogen sources like glutamine [104]. This transcriptional regulation is performed by four GATA family transcription factors, in partnership with the TOR pathway, of which two are activators, Gln3p and Gat1p and two are repressors, Dal80p and Gzf3p [104, 107, 218]. As previously described in section 1.2.4., under favorable conditions, the TORC1 and Tap42p-Sit4p phosphatase form a complex that prevents the transcription of genes of NCR by sequestrating Gln3p in the cytoplasm and promoting its binding to the cytoplasmic protein Ure2. When nitrogen becomes limiting, the Tap42p-Sit4p complex detaches from TORC1, becoming active, and dephosphorylates Gln3p which can now translocate to the nucleus where it will activate the transcription of NCR genes [104, 107]. These NCR genes include genes coding for Gln1 (Glutamine synthetase), Glt1 (Glutamate synthase), Gdh1 and Gdh2 (Glutamate Dehydrogenase), Gap1 (General amino acid permease), Mep2 (Ammonium permease), enzymes involved in nitrogen source metabolism (Dal3p and Put1p), and the transcription factors Dal80p and Dal82p [108]. As for the second activator, Gat1p, it responds to nitrogen availability in a similar way to Gln3p, although it does not seem to be as regulated as Gln3p. Ure2p restriction of Gat1p to the cytoplasm is much weaker than the one imposed on Gln3p and also Sit4p is not required for nuclear localization of Gat1p. The expression and regulation of the repressor transcription factors is achieved by overlapped and auto regulation between the activators and the repressors. The activator Gln3p strongly regulates Dal80p and Gat1p whereas Gzf3p is weakly regulated. On the other hand, expression of Gzf3p is NCR sensitive and regulated by Dal80p in the presence of one of the two activators [107].

The genetic background of strains also plays a major role in the perception of quality of the nitrogen source in question and even ammonium can have a dubious quality. Early studies with strains of the ∑1278b genetic background demonstrated ammonium as a preferred nitrogen source

whereas more recent studies using S288C genetic background strains, demonstrated that ammonium was not a preferred source of the latter strain, being unable to block the Gap1p transport [218]. A recent study, using strains from the Sigma and the TB background, demonstrated that regulation of the NCR differed at the level of the GATA activators Gln3p and Gat1p and the presence or absence of the repressor Gzf3p in the strain background [107]. For strains with the S288C genetic background like BY strains, ammonium does not act as a repressor of the NCR pathway and thus allows the transcription of genes regulated by this pathway such as *GAP1* [218].

Ammonium membrane transporters system in yeast belongs to the highly conserved Mep/Amt/Rh superfamily found in all forms of life [211] and comprises three permeases (Mep1p, Mep2p and Mep 3p) with different kinetics properties [219]. From the three, Mep2 protein displays the highest affinity for ammonia (K_m 1–2 μM), followed by Mep1p (K_m 5–10 μM) and Mep3p (K_m 1.4–2.1mM). When ammonium is available in concentrations below 5 mM as the only nitrogen source, the Mep proteins are indispensible for growth in these conditions [219]. Mep proteins, more specifically Mep2p, have also been described to be involved in the retrieval of ammonium after catabolic ammonium leakage [220]. As mentioned above, Mep proteins are subjected to NCR control and so, in the presence of good nitrogen sources all three genes are repressed, on the other hand, in the presence of limiting ammonium concentrations or poor nitrogen sources, *MEP2* expression is much higher than *MEP1* or *MEP3* expression. This difference in expression is due to different transcription controls exerted by the two general nitrogen regulatory factors, Gln3p and Gat1p. *MEP2* expression is controlled by both Gln3p and Gat1p, and the contribution of each individual regulatory factor is dependent on the nitrogen source available. In the presence of poor nitrogen sources, both regulatory factors activate transcription and one can even compensate for the absence of the other. Also, both factors are needed for basal *MEP2* expression in high ammonium concentration grown cells. By the contrary, when good nitrogen sources are available, only Gln3p is required for *MEP2* expression. As for *MEP1* and *MEP3*, Gln3p is the essential regulatory factor for these genes expression in all sources of nitrogen, however Gat1p still down-regulates its expression in poor nitrogen sources [123, 219]. Recent studies have attributed yet another role for Mep1 and Mep2 proteins, demonstrating that they can also function as sensors to activate major nutrient-signaling pathways. In nitrogen starvation conditions, in the presence of a fermentable sugar, Mep2p acts as an ammonium sensor enabling pseudohyphal growth in diploid yeast cells [123, 220]. Also, Mep2p and Mep1p to some extent act as nutrient transceptors for ammonium-induced activation of

the PKA pathway. Ammonium re-supplementation to nitrogen starved cells induced PKA activation, measured by trehalase activity, in a Mep1p,2p-dependent manner [135].

Another set of ammonium transporters belonging to the YaaH family have been reported to be outward transporters, involved in ammonia production in *S. cerevisiae* [221].These membrane transporters designated as Ato1p, Ato2p and Ato3p seem to be ammonium/ H^+ antiporters, excreting ammonium and importing protons [211, 221].

1.6. General objectives and work plan of the thesis

The elucidation of the mechanisms responsible for aging and the regulators involved in these processes are a major area of current research. The budding yeast *S. cerevisiae* has contributed vastly as a model system to the understanding of the mechanisms involved in aging. Understanding and discovering new modulators of yeast longevity contributes to unravel pathways and regulators that proved to be conserved among eukaryotic organisms. In this context, we aimed to identify new nutrient signaling capable of regulating chronological life span (CLS) of *S. cerevisiae.*

The work developed in the scope of this thesis is organized in four chapters:

In chapter 1, an introduction to the theme is made regarding the main contribution of the aging models to the understanding of the cellular pathways involved in aging and focusing in the particular case of yeast as a model. For this organism growth culture conditions and nutrient signaling modulating chronological longevity are discussed and mechanisms of cell death during are described. A brief introduction to ammonium toxicity in mammals is given as well.

In chapter 2, the materials and methods used in this work are described.

In chapter 3, results are presented in five sections. The results presented in the section 3.1 show ammonium as a new modulator regulating CLS in *S. cerevisiae* contributing to CLS decrease in amino acid restriction conditions. In section 3.2, results show the characterization of the death process induced by ammonium. In section 3.3, results show that ammonium toxicity is not dependent on its metabolization. In section 3.4, the results point out the involvement of the major

nutrient-signaling pathways (TOR, PKA and SCH9) in ammonium induced-cell death. Section 3.5 presents the results showing the contribution of auxotrophy-complementing amino acids in the regulation of CLS by ammonium and the results related with the strain background involvement in ammonium toxicity.

In chapter 4, a more thoughtful discussion is made of the results and futures perspectives of this work are highlighted.

CHAPTER 2

Materials and methods

2. Materials and methods

2.1. Strains

Several *Saccharomyces cerevisiae* strains were used throughout this study, including one prototrophic (CEN.PK113-7D) and four auxotrophic strains (W303-1A; CEN.PK2-1C; BY4741 and BY4742). Knockouts of BY4742 strain in: *AIF1*, *ATG8*, *CPR3, MEP1, MEP2, RAS2*, *RIM13*, *SCH9*, *TOR1, TPK1, TPK2, TPK3* and *YAC1* genes were also used. All strains used are summarized in table 1.

Table 1.*Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
CEN.PK113-7D	*MAT* α MAL2-8^c SUC2	P.Koetter
W303-1A	*MAT* a ura3-52 *trp1Δ*2 leu2-3,112 his3-11 ade2-1 can1-100	Euroscarf
CEN.PK2-1C	*MAT* a ura3-52 trp1-289 leu2-3,112 *his3Δ*1 MAL2-8^c SUC2	Euroscarf
BY4741	*MAT* a *his3Δ*1 *leu2Δ*0 *met15Δ*0 *ura3Δ* 0	Euroscarf
BY4742	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0	Euroscarf
*aif1*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YNR074c::kanMX4	This study
*atg8*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YBL078c::kanMX4	This study
*cpr3*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YML078w::kanMX4	This study
*mep1*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YGR121c::kanMX4	This study
*mep2*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YNL142w::kanMX4	This study
*ras2*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YNL098c::kanMX4	This study
*rim13*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YMR154c::kanMX4	This study
*sch9*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YHR205w::kanMX4	This study
*tor1*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YJR066w::kanMX4	This study
*tpk1*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YJL164c::kanMX4	This study
*tpk2*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YPL203w::kanMX4	This study
*tpk3*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YKL166c::kanMX4	This study
*yca1*Δ	*MAT* α *his3Δ*1 *leu2Δ*0 *lys2Δ*0 *ura3Δ*0 YOR197w::kanMX4	This study

2.2. Culture media and growth conditions

For experiments with stationary phase cells with or without restriction of auxotrophy-complementing amino acids (low and high amino acids conditions, respectively), cells were cultured at 26 °C, 150 rpm, for 72 hours, in defined minimal medium (SC medium) containing 0.17% yeast nitrogen base without amino acids and without ammonium sulphate (Difco, BD), 2% D-glucose and supplemented with 100 mg/l uracil. The concentrations of ammonium sulphate ($(NH_4)_2SO_4$) and auxotrophy-complementing amino acids in the medium were manipulated and the different conditions of supplemented nitrogen are presented in table 2.

Table 2. Nitrogen supplement to SC growth medium.

	Low amino acids				**High amino acids**	
Nitrogen						
Ammonium sulphate	0.01%	0.1%	0.5%	1%	0.5%	1%
Leucine	60 mg/l	60 mg/l	60 mg/l	60 mg/l	300 mg/l	300 mg/l
Lysin	10 mg/l	10 mg/l	10 mg/l	10 mg/l	50 mg/l	50 mg/l
Histidine	10 mg/l	10 mg/l	10 mg/l	10 mg/l	50 mg/l	50 mg/l

After 72 hours of growth, cells were collected by centrifugation and resuspended at a cell density of about 3.8 x 10^7 cells/ml, in: A) growth medium (exhausted medium) without adjusting pH - pH 2.9; B) growth medium (exhausted medium) with pH adjusted to 7.0; C) water (pH 7.0), after being washed three times; D) water with ammonium sulphate (0.5% or 1%, pH 7.0.), after being washed three times. For experiments at pH 7.0, no changes in pH were observed throughout the experiment. Viability of stationary 3 day old cultures (72 hours of growth) was considered to be 100% of survival and this was considered day 0 of the experiment. Cell viability was assessed by Colony Forming Units (CFU) at day 0 (100% viability) and in subsequent days, as indicated. Culture aliquots were diluted, spread on YPD (2% glucose, 2% agar, 1% peptone and 0.5% yeast extract) agar plates and incubated for 2 days at 30 °C before counting.

For experiments with amino acid and nitrogen-starved cells, cells were first cultured at 26 °C and 150 rpm, in the defined minimal medium described above, supplemented with 0.5% $(NH_4)_2SO_4$, appropriate amino acids and base (50 mg/l histidine, 50 mg/l lysine, 300 mg/l leucine and 100 mg/l uracil) and 2% D-glucose, to exponential phase (OD_{600} = 1.0–1.5). These cells were harvested and resuspended in nitrogen-starvation medium (N-) containing 4% glucose and 0.17% yeast nitrogen base without amino acids and $(NH_4)_2SO_4$, or in amino acid-starvation medium (aa-) containing the same components as N-starvation medium plus 0.5% $(NH_4)_2SO_4$. After 24 hours, cells were collected by centrifugation and resuspended at a cell density of about 3.8 x 10^7cells/ml in: A) starvation medium (N- or aa-), without adjusting pH (pH 2.7); B) resuspended in starvation medium (N- or aa-) with pH adjusted to 7.0; C) resuspended in water (pH 7.0), after being washed three times; D) resuspended in water with $(NH_4)_2SO_4$ (0.5%, pH 7.0.), after being washed three times, at cell density of about 3.8 x 10^7 cells/ml. Viability of 24 hours starved cultures was considered to be 100% of survival and this was considered day 0 of the experiment. pH 7.0 was maintained throughout the experiment in cultures with adjusted pH. Cell viability of culture aliquots was assessed by CFU at day 0 (100% viability) and in subsequent days, as indicated. Diluted samples were incubated for 2 days at 30 °C on YEPD agar plates.

In section 3.5, stationary phase cells were cultured at 26 °C, 150 rpm, for 72 hours, in minimal K medium [222] supplemented with the following auxotrophy-complementing amino acid concentrations: 1) high (300 mg/l leucine, 50 mg/l histidine, 50 mg/l lysine); 2) low (60 mg/l leucine, 10 mg/l histidine, 10 mg/l lysine); 3) low concentration reduced to half (30 mg/l leucine, 5 mg/l histidine, 5 mg/l lysine) and 4) low concentration reduced to one quarter (15 mg/l leucine, 2.5 mg/l histidine, 2.5 mg/l lysine) and with 100 mg/l uracil. After 72 hours of growth, cells were collected by centrifugation, washed three times, and resuspended at a cell density of about 3.8 x 10^7 cells/ml in: A) water (pH 7.0); B) water with $(NH_4)_2SO_4$ (0.5%, pH 7.0.) and C) water with $(NH_4)_2SO_4$ (1%, pH 7.0.). Viability of stationary phase 3 day old cultures was considered to be 100% of survival and this was considered day 0 of the experiment. pH 7.0 was maintained throughout the experiment in cultures with adjusted pH. Cell viability was assessed by CFU as described above. In the experiments where the influence of the strain background on ammonium toxicity was assessed, cells were grown in SC medium with high or low concentrations of auxotrophy-complementing amino acids and with or without 0.5% $(NH_4)_2SO_4$. Viability of stationary phase 3 day old cultures was

considered to be 100% of survival and this was considered day 0 of the experiment. Cell viability was assessed by CFU as described above.

2.3. Construction of mutant strains and plasmid transformation

To construct knockout strains in the following genes: *AIF1*, *ATG8*, *CPR3, MEP1, MEP2, RAS2*, *RIM13*, *SCH9*, *TOR1, TPK1, TPK2, TPK3* and *YAC1*, we used genomic DNA from the respective deleted strains from Euroscarf as template and amplified by PCR, the disruption cassette using the respective primers summarized in table 3. The resulting fragment was transformed into wild-type *S. cerevisiae* strain BY4742 by the lithium acetate method. Briefly, 5 µg DNA of the disruption cassette were mixed with 50 µg of freshly denatured salmon sperm DNA (10 mg/ml, boiled for 20 min in a water bath, then chilled in ice/water) and added to the cell pellet of exponential grown cells (2.9 x 10^9 cells/ml). After mixing with caution, 300 ml of freshly prepared sterile 40% PEG 4000 were added and carefully mixed. Cells were first incubated for 30 minutes at 30 °C with constant agitation and after for 15 min at 42 °C, then 800 ml sterile water were added, mixed and cells were collected by centrifugation. Cells were resuspended in 1 ml YPD and incubated for 2–3 h at 30 °C. Then cells were collected by centrifugation, resuspended in 200 ml YPD and plated onto YPD plus G418 plates (200 mg/ml G-418). Plates were incubated at 30 °C until colonies appeared [223]. Colonies were then screened by colony PCR using the verification primers summarized in table 3.

S. cerevisiae BY4742 strain was transformed as described above with plasmids pUG35 and pUG35- *NHP6A-EGFP* [177], kindly provided by Frank Madeo (University of Gratz, Austria), and was cultured as described above for aa-starved cells, in medium lacking uracil.

Table 3. Primers used in the construction of knockout strains.

Gene	**Sequence (5' – 3')**	
Gene disruption	**Forward**	**Reverse**
AIF1	GCTGCTGCAGACTAGAAACG	CCAGCGGCCTTCTTTGTTTC
ATG8	CCAATTGGTGATGAAGAACAATCTA	TTGAATTTCTTCCTATTTCTGATGC
CPR3	AGGACCATACCTTCTTGAAGGGTGA	ACTGAGGCAAACCAGAATAAAAGTTG
MEP1	CAGAGATTGCGATAACGATAAGATT	ATTACCGGTTGGTGTACTCAAATAA
MEP2	TCGGTCTCTTCTTACTGCTGTTACT	GAAACAGTGAAAGATAAGGCAAAAA
RAS2	CAAGCGTAACGCAATCCGGC	ATGATATTGCCCAAAGTTTCC
RIM13	ACAACATAAACTTGAGGAGAAGTGG	GGTTTCCAAGAATTCTACTTCCTTC
SCH9	TACTTATTCACATTACGGGTCCAAT	TTCGGATGATATAACCGACCTATAA
TOR1	TTGAATCCTAATTTCTTGCTCAATC	AAGGCATATATTGATGCTCAAAAAG
TPK1	CGTATCCCTTTACTTGAAAACTTGA	AGAAAATCAAAGACAGAAGCGTAGA
TPK2	TACAATTCTGGCCTTCTTACCTAAA	TAATTTTTGCACTGAGATCATGAGA
TPK3	GGTGAACCACTTTCTTTTTAGTGAA	TCTTCTTATTGTAGCAGGCTCACTT
YCA1	AATAGTGGACGAAATCCATCTTGTA	CTTAATTTTTCTTTGGTTGAGGTGA
Verification	**Forward**	**Reverse**
AIF1	GATGAGCATTGTACAGCTTA	GTTAATTCTCACCGTCCCC
ATG8	CTGGACAAGAAACCAGAACC	CAAACGAACAGGTCAGAGAG
CPR3	CTTACAAAGAGGCAAGGGTC	CATAGCCCTGAAGCTGTAC
MEP1	CTTCTACGGTTGTCCTTTGC	CGATATTTCTGCGGTTACCG
MEP2	GTAATTCATGTCGGCCATCG	CCATTTCAGTGCAGTTTCGAG
RAS2	GGAAACAAGGTTCACATCAGC	TTGTTATTCCAGGTGGAAC
RIM13	GGATAGCTTACTTGAGGCAG	GAGATGGATGGGTCAAGTTG
SCH9	GTTGCTTAAAGGGTGGATCG	CGATGGGATGACAGTTAAGC
TOR1	GAAATTGGTTGCAGAGGTGG	GTCGAAACTGAACGATCTCC
TPK1	GTGCTGCTATTCGTTCTTGC	CGACTTGTTTGGAGCCACCA
TPK2	CCGCCTCAAGATAAACCAGC	CTACAACTACGAAGCGTTGC
TPK3	TCCAGGTACGAGTGATTTAGG	GAATCTGCGGTAGTCTGGTA
YCA1	GGATCTTATTGGCCGAGTTG	GGTCACTCCAAAGAAGGATG

2.4. Ammonium and ATP Determination

Ammonium in the culture media was quantified by Dr. José Coutinho (University of Trás-os-Montes e Alto Douro, Portugal) as previously described [224]. ATP measurements were performed according to [225]. Briefly, cells were collected by centrifugation and the pellet was frozen with liquid nitrogen and stored at -80 °C. For the ATP assay, the pellet was mixed with 200 µl of 5% trichloroacetic acid (TCA) and vortexed for one minute, twice, with a one minute interval on ice. This mix was centrifuged for one minute, at 4 °C, and 10 µl of the supernatant were added to 990 µl of reaction buffer (25 mM HEPES, 2 mM EDTA, pH 7.75). 100 µl of this mixture was added to 100 µl of Enliten Luciferin/Luciferase Reagent (Promega) and luminescence was measured on a ThermoScientific Fluoroskan Ascent FL. Protein quantification was determined using the Bradford assay (Bio-Rad, Germany) according to the manufacturer's instructions.

2.5. Measurements of cell death markers

For the detection of chromatin changes, cells were stained with 4,6-diamido-2-phenyl-indole (DAPI, Sigma). Cells ($3x10^5$ cell/ml) were harvested, washed, suspended in DAPI solution (0.5 mg/ml in PBS (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.46mM KH_2PO_4, pH 7.4)) and then incubated in the dark for 10 min at room temperature. Stained cells were washed twice with PBS and visualized by epifluorescence microscopy. DNA strand breaks were assessed by TUNEL with the 'In Situ Cell Death Detection Kit, Fluorescein' (Roche Applied Science) as described previously [94]. Briefly, $1x10^7$ celsl/ml were fixed with 3.7% formaldehyde followed by digestion of the cell walls with lyticase. Cytospins were made and after the slides were rinsed with PBS, incubated in permeabilization solution (0.1%, v/v, Triton X-100 and 0.1%,w/v, sodium citrate) for 3 minutes on ice, rinsed twice with PBS, and incubated with 10 µl of TUNEL reaction mixture (terminal deoxynucleotidyl transferase and FITC-dUTP) for 60 minutes, at 37 °C. Finally, the slides were rinsed three times with PBS and a coverslip was mounted with a drop of anti-fading agent Vectashield (Molecular Probes, Eugene, OR, U.S.A.) and with 2 µl of 50 µg/ml propidium iodide (PI, Molecular Probes, Eugene, OR) solution in Tris buffer (10 mM, pH 7.0) with $MgCl_2$ (5 mM) and RNase (0.5 µg/ml). Cells were visualized by epifluorescence microscopy. For quantification of the

number of TUNEL positive cells, at least 400 cells from three independent assays were counted. For the nuclear release of the necrotic marker Nhp6Ap–EGFP, cells were also visualized by epifluorescence microscopy. For the later assays, at least 300 cells of three independent experiments were evaluated.

To measure DNA content, 10^6 cells were stained with SYBR Green I as described [226] and staining was assessed by flow cytometry. Plasma membrane integrity was assessed by incubating 10^6 cells with 5 mg ml^{-1} PI (Molecular Probes, Eugene, OR) for 10 minutes at room temperature followed by flow cytometry measurements of PI-stained cells. Intracellular reactive oxygen species were detected by dihydrorhodamine (DHR)-123 staining or dihydroethidium (DHE) (Molecular Probes). For DHR-123, cells were incubated with 15 mg/mL of dye for 90 minutes at 30 °C in the dark, washed in PBS and evaluated by flow cytometry. For DHE, cells were incubated with 5 μM and after incubation for 10 min at 30 °C cells were washed once with PBS and evaluated by flow cytometry. Phosphatidylserine exposure was detected by FITC Annexin-V (BD Pharmingen) staining as described previously [94]. The cell walls were digested with 3% (v/v) glusulase (NEE-154 Glusulase; Perkinelmer) and 7 U/ml lyticase (Sigma) for 40 minutes, at 28 °C. For intracellular calcium measurements, 10^6 cells previously washed with PBS were stained with 10 μM FLuo3 AM (Molecular Probes, Eugene, OR) for 2 hours at 30 °C in the dark, subsequently washed in PBS and assessed by flow cytometry. Positive controls for apoptosis involved treatment of cells with 160 mM acetic acid for 200 minutes, at pH 3.0 and 3 mM H_2O_2 at pH 3.0. For the necrotic marker Nhp6Ap–EGFP, no nuclear release was observed in the presence of 3 mM H_2O_2.

2.6. Epifluorescence microscopy and flow cytometry

In the experiments employing epifluorescence microscopy a Leica Microsystems DM-5000B microscope was used, with appropriate filter settings and a 100x/1.3 oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. Flow cytometry analysis in the above described experiments was performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488-nm blocking filter, a 550-nm/long-pass dichroic and a 525-nm/bandpass. Red fluorescence was collected through a

488-nm blocking filter, a 590-nm/long-pass dichroic and a 620-nm/bandpass. Thirty thousand cells per sample were analyzed.

2.7. Treatments

Methionine sulfoximine (MSX,Sigma), an irreversible inhibitor of glutamine synthetase, was dissolved in sterile water at a concentration of 100 mM and stored at 4 °C. MSX was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 1 mM. Wortmannin (Sigma), a PI3K inhibitor, was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 6 μM or 23 μM. Glutamate was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 5 mg/ml. Adenosine 3′,5′-cyclic monophosphate (cAMP, Sigma) was added to aa-starvation or N-starvation medium or to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 4 mM α-ketoglutaric acid potassium salt (Sigma) was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 5 mg/ml. Cyclosporin A (Sigma) was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0.), at the concentration of 120 μg/ml. For inhibition of YCA1, zVAD-fmk from a 20 mM stock in 1:1 DMSO/ethanol was added twice (at T0 and T1) to a final concentration of 40 μM, to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0). Rapamycin (Sigma) was suspended in ethanol at a stock concentration of 1 mg/mL and stored at −20°C. Rapamycin was added during the beginning of the 24 hour amino acid starvation period (aa-starved cells) or added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0) at the concentration of 0.2 μg/ml.

2.8. Western Blot analysis

Western blot analysis was performed according to [227]. Briefly, protein lysates were separated on 12.5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (hybond-P; Amersham). The membranes were blocked with 5% non-fat milk in PBS containing 0.05% Tween 20, for 1 h at room temperature. Membranes were then incubated overnight at 4 °C with primary antibodies directed against yeast Atg8p and Pgk1p, rabbit polyclonal anti-Aut7 (1:200; Santa Cruz

Biotech) and mouse monoclonal anti-PGK1 (1:5000; Molecular Probes) respectively, followed by one hour incubation at room temperature with secondary antibody Peroxidase-AffiniPure Goat Anti-Rabbit IgG (1:10000; Jackson ImmunoResearch).

2.9. Enzyme assays

Glutamine synthetase (Gs) assay was performed according to [228]. Glutamate dehydrogenase activity was determined according to [229]. Briefly, cell extracts were prepared by adding to the cell pellet a roughly equal volume of 0.5 mm diameter glass beads in the presence of 0.1 M potassium phosphate buffer (pH 6.0), followed by vigorous mixing during 1 minute intervals interspersed with periods of cooling in ice. The NADP-dependent GDH activity was determined by following the disappearance of NADPH at 340 nm. Trehalase activity was determined according to [230]. Briefly, crude enzyme extracts were obtained by ressuspending the cell pellet in ice-cold 50 mM MES/KOH buffer (pH 7.0) containing 50 μM $CaCl_2$, and adding a roughly equal volume of 0.5 mm diameter glass beads, followed by vigorous mixing during 1 minute intervals interspersed with periods of cooling in ice. The extracts were then dialyzed overnight at 4 °C in a dialysis cellulose membrane (Cellu Sep H1, Orange). The dialyzed extract was then used to assess trehalase activity by measuring the liberated glucose with glucose oxidase assay (GOD, Roche). Protein quantification was determined using the Bradford assay (Bio-Rad, Germany) according to the manufacturer's instructions.

2.10. Statistical analysis

Data are reported as mean values of at least three independent assays and presented as mean ± SD. The arithmetic means are given with SD with 95% confidence value. Statistical analyses were carried out using Two-way ANOVA. *P <0.05 was considered statistically significant.

CHAPTER 3

Results and discussion

The results presented in this chapter were partially published as follow:

Paper:

- Santos, J., Sousa, M.J. and Leao, C. *Ammonium is toxic for aging yeast cells, inducing death and shortening of the chronological lifespan.* PLoS One, 2012. 7(5): p. e37090.

National congresses:

- Santos, J.; Sousa, M. J.; Leão, C. "Extrinsic factors influencing cell viability in chronological aging yeast." XIX Jornadas de Biologia de Leveduras "Prof. Nicolau van Uden" FCT/UNL, C. Caparica,15 to 16 June 2012
- Santos, J.; Sousa, M. J.; Leão, C. "Ammonium is toxic for amino acid-starved yeast cells under extreme calorie restriction, inducing cell death through the regulation of PKA, TOR and Sch9 activities." MicroBiotec'11, Braga, Portugal, 1-3 December, 2011
- Santos, J.; Sousa, M. J.; Leão, C. "Ammonium Induces Cell Death and Inhibits Autophagy Induction in Amino Acid-Starved Cells of *Saccharomyces cerevisiae*", XVIII Jornadas de Biologia de Leveduras "Professor Nicolau van Uden" University Técnica de Lisboa, Lisbon, 18-19 June 2010

International congresses:

- Santos, J.; Leão, C; Sousa, M. J. "Ammonium as an extrinsic factor inducing loss of survival in chronologically aged yeast cells." 9th IMYA (International Meeting on Yeast Apoptosis), Rome, 17-20 September, 2012
- Santos, J.; Sousa, M. J.; Leão, C. "Ammonium blocks chronological lifespan extension of extreme calorie restriction in amino acid-starved yeast cells associated to Tpk1- and Tor1-dependent necrotic cell death induction." 8th IMYA (International Meeting on Yeast Apoptosis), Canterbury, 2-6 May, 2011
- Santos, J.; Sousa, M. J.; Leão, C. "Ammonium Induces Cell Death and Inhibits Autophagy Induction in Amino Acid-Starved Cells of Saccharomyces cerevisiae", "Yeast, an evergreen model- tribute to P. Slonimski" Sapienza University of Rome, Rome, 22-25 September, 2010
- Santos, J.; Sousa, M. J.; Leão, C. "Chronological life-span extension is blocked by ammonium in amino acid starved cells, associated to autophagy inhibition and to cell death induction", 28th SMYTE (Small meeting on yeast transport and energetic), Gurgaon India, 23rd - 27th September, 2010
- Santos, J.; Sousa, M. J.; Leão, C. "Ammonium induces cell death in amino acid-starved yeast cells", 7th IMYA (International Meeting on Yeast Apoptosis), Gratz, 9-13 September, 2009

Section 3.1

Ammonium as a yeast chronological life span modulator

Section 3.1. Ammonium as a yeast chronological life span modulator

It is well established that the composition of the culture medium can modulate the chronological life span (CLS) of yeast cells and therefore culturing cells in different media generates different outcomes in CLS [35]. In this line, cells grown in rich YPD medium are known to present an extension of CLS when compared to cells grown in synthetic complete (SC) medium [35, 90]. Also, manipulation of single components of the culture medium is known to extend CLS such as the reduction of glucose concentration (known as caloric restriction - CR) or manipulation of amino acids supply [35, 56, 77, 78, 80]. Particularly, the CLS of *Saccharomyces cerevisiae* is strongly affected by the concentration of the auxotrophy-complementing amino acids in the medium. Cells of the auxotrophic *S. cerevisiae* strain BY4742 cultured with an insufficient supply of essential amino acids display reduced lifespan compared with cells grown with increased amino acid supplementation in the medium [77]. Taking into account that manipulating glucose and auxotrophy-complementing amino acid concentration in the medium largely influences CLS, we investigated the effects of ammonium, which is a commonly used nitrogen source, on yeast CLS. In the present section, we present the results regarding the influence of different ammonium concentrations on yeast CLS, either in cells aged in the culture medium under standard or limiting auxotrophy-complementing amino acid conditions (3.1.1), or in amino acid-starved cells after transferred to water (3.1.2).

3.1.1. Ammonium induces CLS shortening of yeast cells aged in the culture medium

To determine the effect of ammonium (NH_4^+) on the survival of chronologically aged *S. cerevisiae* cells, we manipulated NH_4^+ concentration in the culture medium both under auxotrophy-complementing amino acid restriction (low amino acid concentrations), and in standard amino acid supplementation conditions (high amino acid concentrations). For that, we reduced the standard concentration of $(NH_4)_2SO_4$ in the culture medium five- or fifty-fold (from 0.5% to 0.1 and 0.01 %, respectively) in amino acid restriction conditions, and increased the starting concentration of $(NH_4)_2SO_4$ from 0.5% to 1%, either with or without amino acid restriction. The results presented in Figure 3 show that reducing the starting concentration of $(NH_4)_2SO_4$ in the culture medium improved

the survival of chronological aging cells in amino acid restriction conditions. In contrast, when the initial $(NH_4)_2SO_4$ concentration in the culture medium, either with or without restriction of amino acids, was increased to 1%, there was a decrease in cell survival, although loss of cell viability was much faster for cells grown with amino acid restriction (Figure 3).

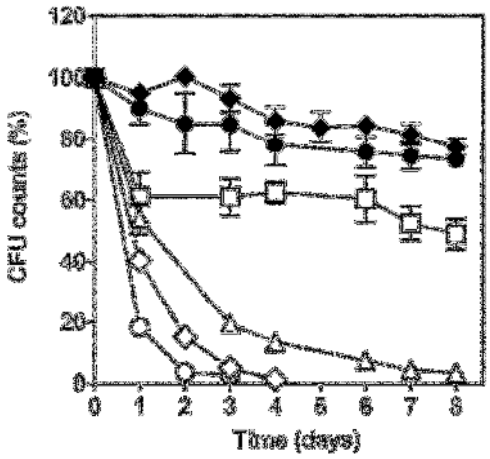

Figure 3. Survival of wild-type *S. cerevisiae* (BY4742) stationary phase cells grown in media supplemented with low (open symbols) and high (dark symbols) concentrations of auxotrophy-complementing amino acids, and with 0.01% (□); 0.1% (△); 0.5% (◇,◆) or 1% (○,●) $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml. Values are means ± SEM (n=3). $P < 0.001$ (low concentrations *vs* high concentrations of auxotrophy-complementing amino acids). Statistical analysis was performed by two-way ANOVA.

To further clarify the effect of NH_4^+ on CLS and to investigate if it was in fact responsible for the observed loss of cell viability in the culture media, we tested whether adding NH_4^+ to yeast suspensions in water induces loss of cell viability, as it had been reported for glucose [57, 58]. Cells were grown in SC medium plus 0.5% $(NH_4)_2SO_4$ with or without amino acid restriction in the medium for 72 hours and then transferred to water without NH_4^+ (pH 7.0), water with NH_4^+ (pH 7.0), or to the exhausted medium as a control. Figure 4 represents schematically the methodology used. As shown in Figure 5, cells grown with or without amino acid deprivation exhibited a longer CLS after they were transferred to water compared to cells transferred to exhausted culture medium that maintained a

pH of 2.6-2.9, although loss of cell viability again occurred much faster for cells grown with amino acid restriction.

In both cases, addition of NH_4^+ to water reduced cell survival in proportion to its concentration, mimicking its effect in the depleted media, and supporting that NH_4^+ can be responsible for triggering loss of cell viability.

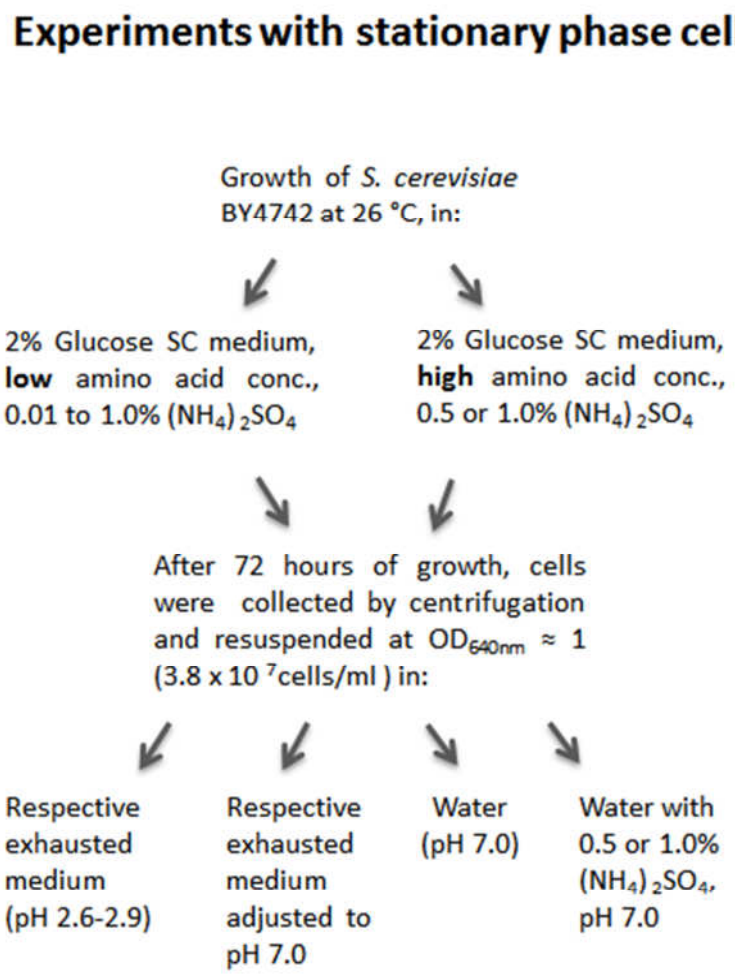

Figure 4. Scheme of the methodology used in experiments with the stationary phase cells.

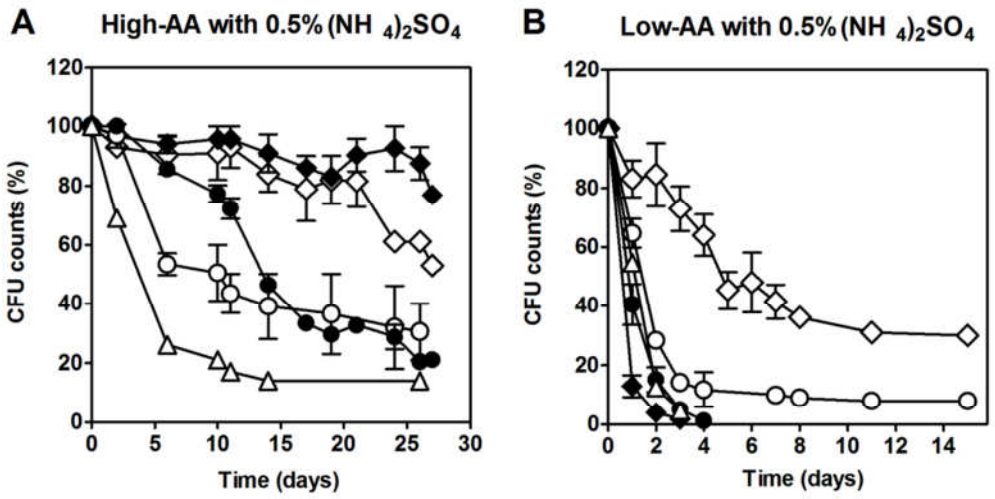

Figure 5. Survival of wild-type *S. cerevisiae* (BY4742) stationary phase cells grown in media supplemented with: (A) high (High-AA) and (B) low (Low-AA) concentrations of auxotrophy-complementing amino acids, and supplemented with 0.5% $(NH_4)_2SO_4$. After 72 hours of growth, cells were transferred to: (◇) water (pH 7.0); (○) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (△) water with 1% $(NH_4)_2SO_4$ (pH 7.0); (●) exhausted medium (pH 2.6-2.9); (◆) exhausted medium (pH 7.0). Values are means ± SEM (n=3-5). (A) and (B) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$), $P < 0.001$ (H_2O *vs* 1% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

It has been reported that medium acidification limits survival of yeast cells during chronological aging in SC medium and that the longer survival observed in water can be, at least in part, attributed to the differences in pH [91, 231, 232]. To assess whether acidification could play a role in the NH_4^+ -induced loss of cell viability, we measured cell survival in media adjusted to pH 7.0 (see schematic of methodology in Figure 4). As shown in Figure 5A, for cells grown without amino acid restriction, when the depleted medium was adjusted to pH 7.0, there was an increase in CLS compared to exhausted medium without pH adjustment, which is consistent with results previously described for similar conditions [91, 97]. However, transferring cells cultured with insufficient supply of amino acids with 0.5% $(NH_4)_2SO_4$ to the respective exhausted medium adjusted to pH 7.0 did not lead to a significant difference in CLS relative to the CLS of cells in the exhausted acidic medium, suggesting that in this condition, acidification is not the main cause of cell viability loss.

Due to the amino acid restriction, these cells do not complete glucose exhaustion [77] due to growth limitation (Figure 6A). Moreover, not only glucose, but also NH_4^+ is not completely depleted from the medium in amino acid restriction conditions (Figure 6B), thus suggesting that the presence

of unused NH_4^+ could contribute to loss of cell viability since decreasing its concentration in the medium increased CLS in amino acid restriction conditions (Figure 3).

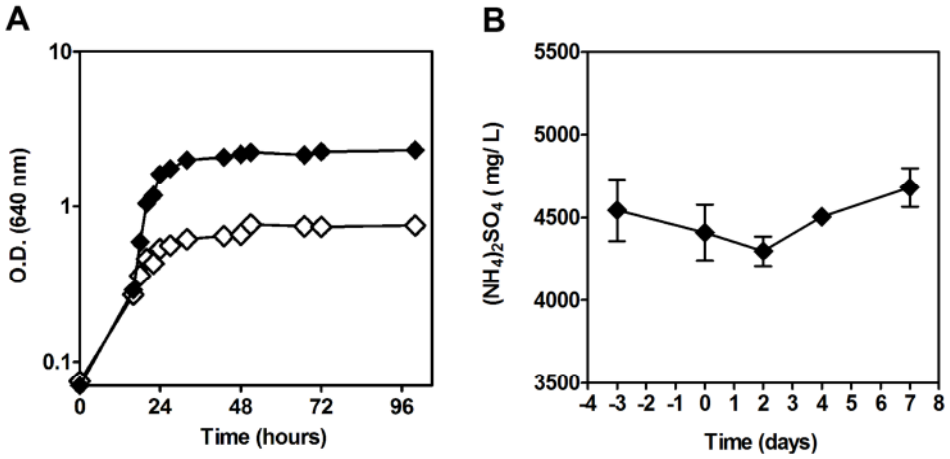

Figure 6. (A) Growth curves of wild-type *S. cerevisiae* (BY4742) cultured in SC media supplemented with: (◇) low and (◆) high concentrations of auxotrophy-complementing amino acids, and supplemented with 0.5% $(NH_4)_2SO_4$. (B) Quantification of $(NH_4)_2SO_4$ in SC medium supplemented with low concentrations of auxotrophy-complementing amino acids and 0.5% $(NH_4)_2SO_4$ during culture of wild-type *S. cerevisiae* (BY4742) cells; day -3 represents the day of culture inoculation and day zero represents the beginning of aging experiments.

Additionally, we also tested ammonium effects in cells transferred to water after being cultured in media with different NH_4^+ concentrations with or without amino acid (Figure 7). We observed that raising the initial $(NH_4)_2SO_4$ concentration in the culture medium from 0.5% (Figure 5A and 5B) to 1% (Figure 7A and 7B), either with or without amino acid restriction decreased cell survival both in water and water with $(NH_4)_2SO_4$. On the other hand, for cells cultured under amino acid restriction, reducing the initial $(NH_4)_2SO_4$ concentration from 0.5% (Figure 5B) to 0.1% (Figure 7C) or to 0.01% (Figure 7D) increased CLS in water and in water with $(NH_4)_2SO_4$. These results show that the NH_4^+-induced reduction in CLS observed in water positively correlates with the concentration of NH_4^+ in the growth medium, indicating that culture conditions pre-determined the cellular response to NH_4^+. For cells cultured with insufficient supply of amino acids and 1%, or 0.1% $(NH_4)_2SO_4$ and then transfered to the respective exhausted medium with or without pH adjustment,

pH had no significant influence on CLS (Figure 7A, 7B and 7C). In contrast, cells cultured with 1% cells $(NH_4)_2SO_4$ without amino acid restriction and cells cultured under amino acid restriction conditions with the lowest $(NH_4)_2SO_4$ concentration (0.01%), after transfer to the respective exhausted medium adjusted to pH 7.0, exhibited an increase in CLS when compared with cells transfered to exhausted medium without pH adjustment (Figure 7A and 7D). The results for 0.01% $(NH_4)_2SO_4$ (Figure 7D) seem to suggest that the ammonium concentration is low enough not to affect negatively the cell viability.

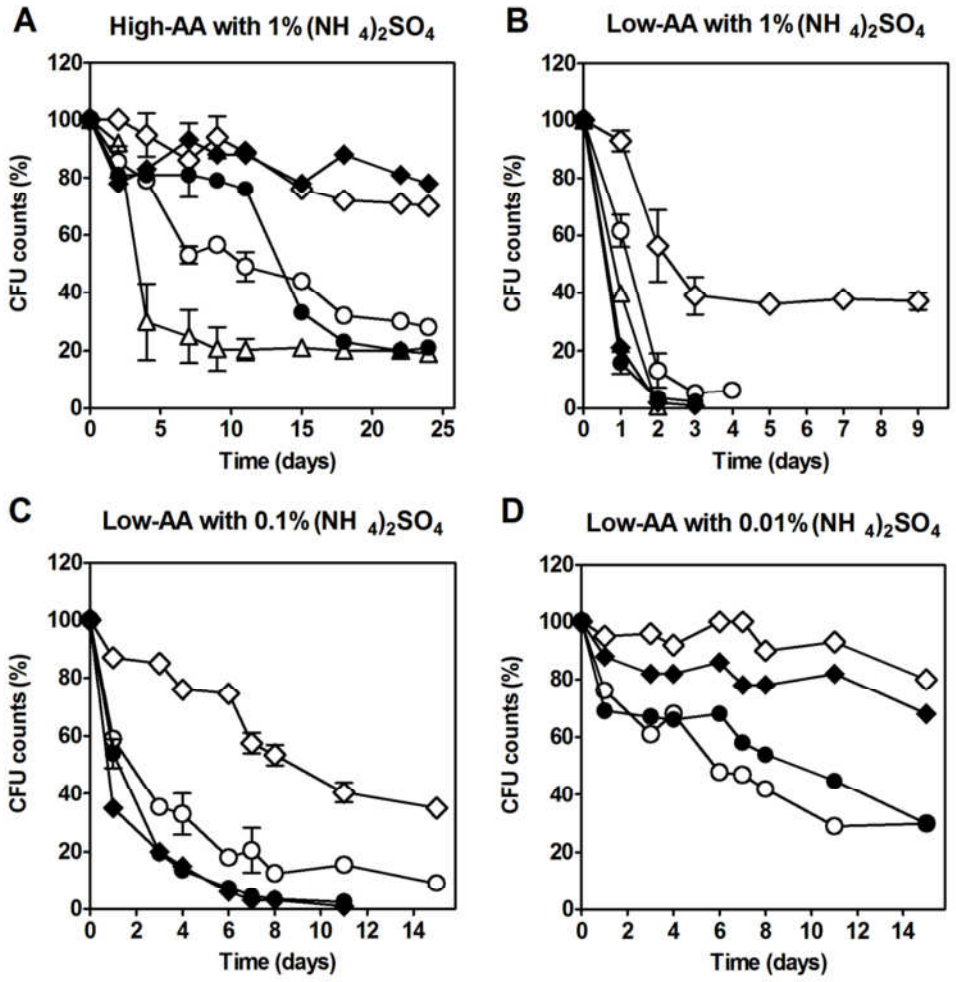

Figure 7. Survival of wild-type *S. cerevisiae* (BY4742) stationary phase cells grown in media supplemented with low (Low-AA) and high (High-AA) concentrations of auxotrophy-complementing amino acids, and with (D) 0.01%; (C) 0.1%; or (A, B) 1% $(NH_4)_2SO_4$. After 72 hours of growth, cells were transferred to: (◇) water (pH 7.0); (○) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (△) water with 1% $(NH_4)_2SO_4$ (pH 7.0); (●) exhausted medium (pH 2.6-2.9); (◆) exhausted medium (pH 7.0). Values are means ± SEM (n=3-5). (A) and (B) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$), (H_2O *vs* 1% $(NH_4)_2SO_4$); (C) and (D) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

3.1.2. Ammonium induces CLS shortening in amino acid-starved cells, after transfer to water

During the above experiments in which NH_4^+ emerged as an extrinsic factor in CLS regulation, a condition for exploring the toxic effects of ammonium also emerged from the observations that this toxic effect of NH_4^+ was more severe in amino acid restriction conditions. Following this line of thought a conventional nitrogen starvation protocol [135] was adapted to accommodate the following conditions in SC glucose starvation medium: i) lack of the auxotrophy-complementing amino acids and presence of NH_4^+ (aa-starved cells) or ii) lack of the auxotrophy-complementing amino acids and of NH_4^+ (N-starved cells). For that, cells were grown to mid exponential phase in SC medium with 2% glucose and then starved for 24 hours in both types of starvation media. Cells were subsequently transferred to water (pH 7.0), with and without NH_4^+ or to the respective 24 hour starvation medium (final pH 2.7-2.9) that was or was not adjusted to pH 7.0. The initial pH did not significantly change during the assay, except for cells transferred to starvation media at pH 7.0, which reached a final pH around 5.0. Figure 8 represents schematically the methodology used. The results presented in Figure 9 show that both aa-starved and N-starved cells survived for a longer period of time in water relative to those in starvation medium (pH 2.7-2.9). Addition of NH_4^+ to water induced a rapid loss of cell viability and shortening of CLS only for aa-starved cells (Figure 9A). Cells transferred to starvation medium that was adjusted to pH of 7.0 also exhibited a rapid decrease in cell viability, indicating that the NH_4^+ effect is not due to the acidification of the medium (Figure 9A). In contrast to aa-starved cells, N-starved cells survived for a longer period when transferred to the starvation medium adjusted to pH 7.0 (Figure 9B)

To eliminate the possibility that the reduced survival of aa-starved cells induced by the addition of $(NH_4)_2SO_4$ to water was due to sulphate and not to ammonium itself, a similar experiment with aa-starved cells was performed in water to which NH_4OH was added instead of $(NH_4)_2SO_4$. As shown in Figure 10, similar results were obtained, confirming that the cell death phenotype is due to ammonium.

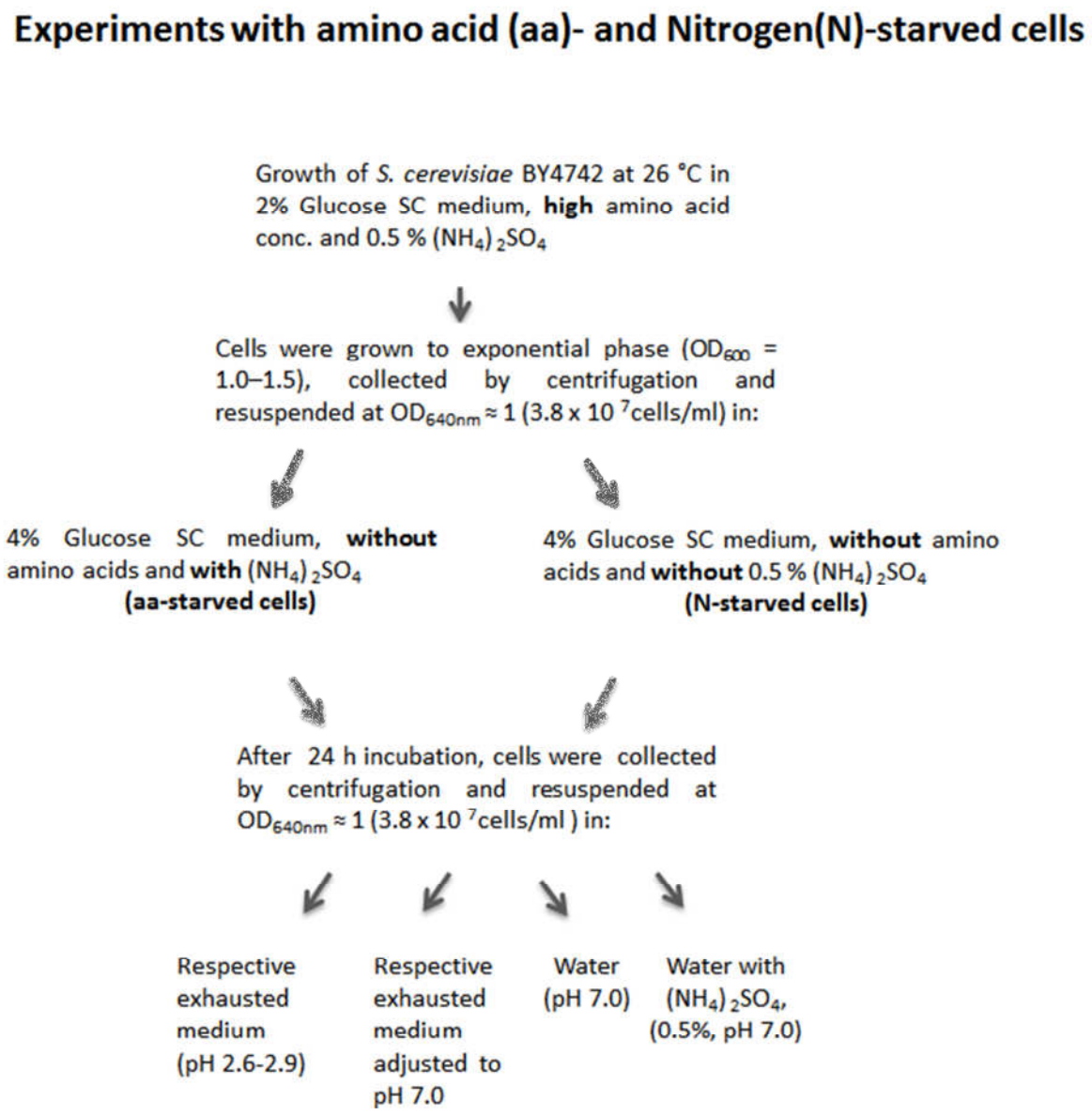

Figure 8. Scheme of the methodology used in experiments with amino acid (aa-) and nitrogen (N-) starved cells.

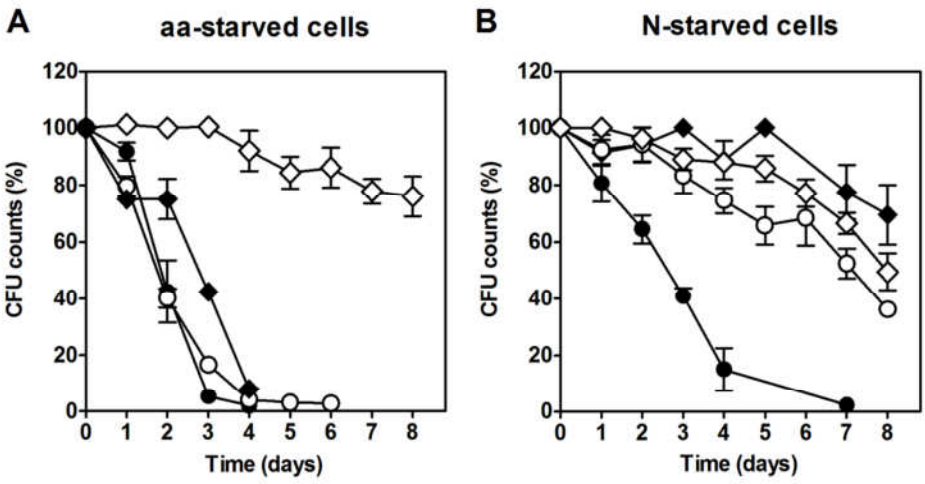

Figure 9. Survival of wild-type *S. cerevisiae* (BY4742) (A) amino acid-starved cells (aa-) or (B) nitrogen starved cells (N-), after transfer to: (◇) water (pH 7.0); (○) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (●) starvation medium (pH 2.7-2.9) and (◆) starvation medium (pH 7.0). In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml. Values are means ± SEM (n=8). $P < 0.001$ (aa-starved H_2O *vs* aa-starved 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

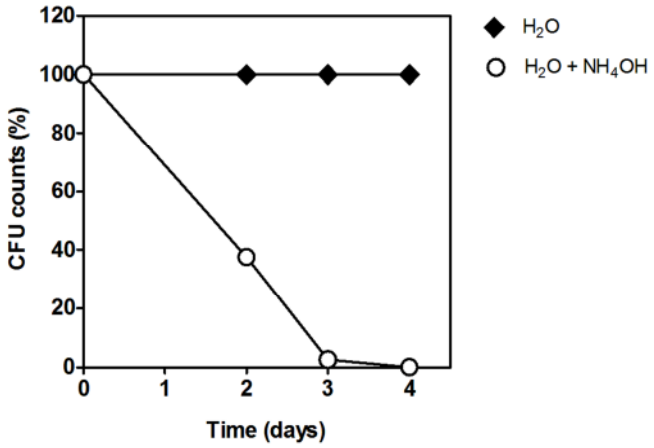

Figure 10. Survival of wild-type *S. cerevisiae* (BY4742) aa-starved cells, in water or water with 0.5% NH_4OH. Starting cell density was about 3.8 x 10^7 cells/ml. Values are means ± SEM (n=3). $P < 0.001$ (aa-starved H_2O *vs* aa-starved 0.5% NH_4OH). Statistical analysis was performed by two-way ANOVA.

3.1.3. Addition of potassium does not revert ammonium toxicity

As discussed in the Introduction, NH_4^+ toxicity was previously described in steady-state chemostat cultures of yeast under limiting potassium concentration [212]. To determine if the ammonium toxicity we observed in our experiments depends on potassium concentration, we repeated the experiments after adding potassium to water at a concentration that according to this earlier study [212] abolished NH_4^+ toxicity. The results obtained show that addition of potassium did not alter the NH_4^+-induced loss of cell viability (Figure 11), showing that potassium, at least at the concentration used, does not revert NH_4^+ toxicity in cells previously starved for amino acids.

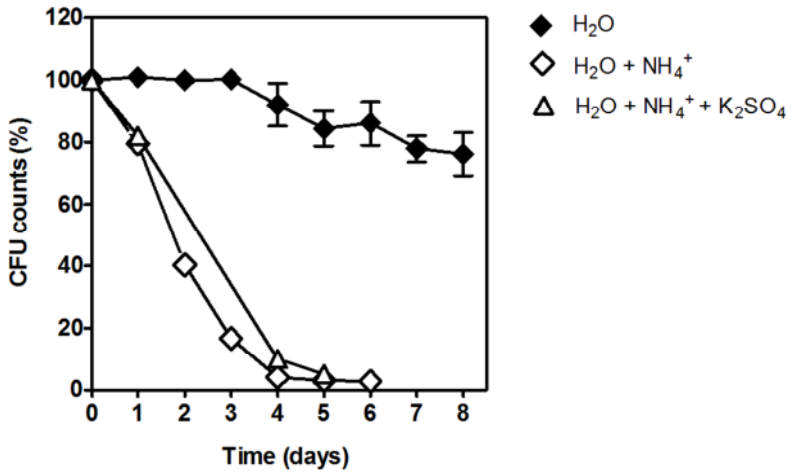

Figure 11. Survival of wild-type *S. cerevisiae* (BY4742) aa-starved cells, in water, water with 0.5% $(NH_4)_2SO_4$ and water with 0.5% $(NH_4)_2SO_4$ supplemented with 13 mM K_2SO_4. Starting cell density was about 3.8 x 10^7 cells/ml. Values are means ± SEM (n=3). $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$); (H_2O *vs* 0.5% $(NH_4)_2SO_4$ + K_2SO_4). Statistical analysis was performed by two-way ANOVA.

3.1.4. Effect of ammonium on the cell cycle of aa- and N-starved cells

To further explore the different responses from aa- and N-starved cells presented above we evaluated, by flow cytometry, the cell cycle of cells in the two conditions after 24 hours of starvation and after 5 days upon transfer to water and water with ammonium (Figure 12). Previous studies showed that cells starved for auxotrophic-complementing amino acids in otherwise complete medium, fail to properly arrest in G0 [76, 77]. In accordance, aa-starved cells in our study also do not seem properly arrested in G0, presenting a smaller percentage (71%) of cells arrested in G0/G1 in comparison to N-starved cells that show 85% of G0/G1 cells. However, this scenario of cell cycle arrest failure does not seem to account for the differences in the observed loss of cell viability, since the small differences in cell cycle phase percentages between water and water with ammonium could not probably account for the major loss of cell viability induced by ammonium in aa-starved cells (Figure 9).

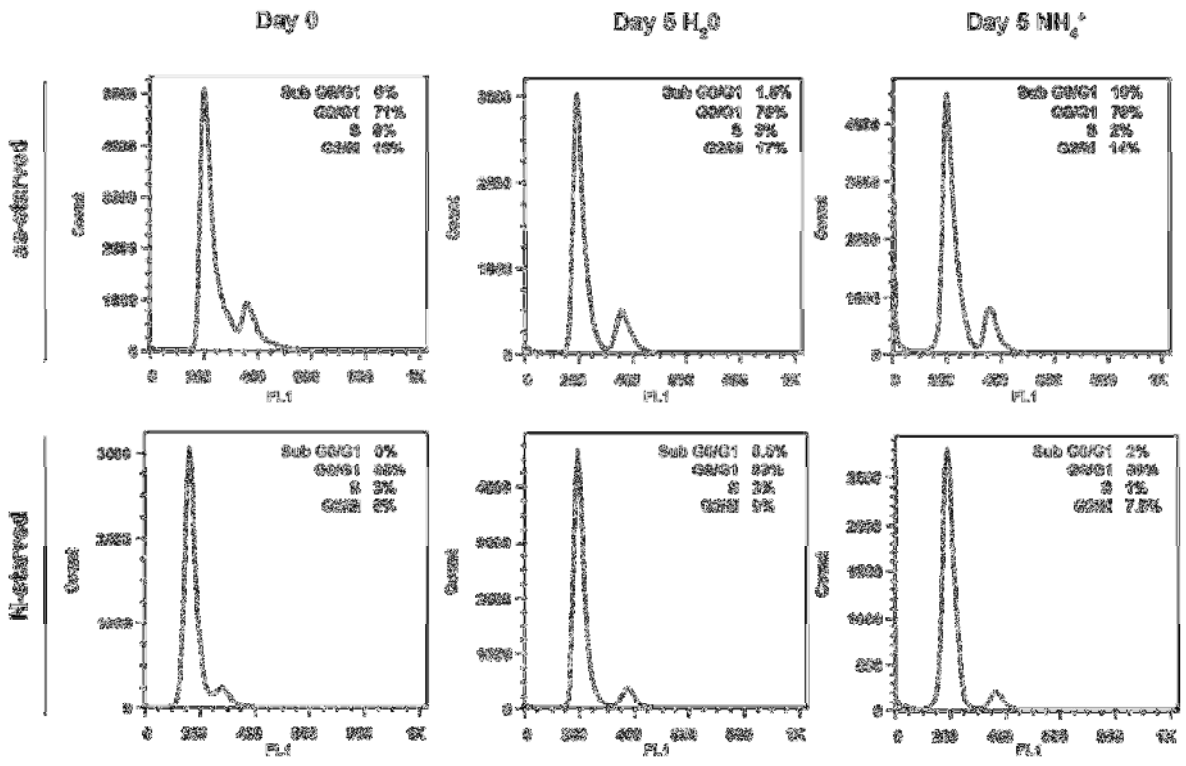

Figure 12. Cell cycle histograms of wild-type *S. cerevisiae* (BY4742) aa-starved and N-starved cells at day 0 and day 5 upon transfer to water or water with 0.5% $(NH_4)_2SO_4$, after a 24 hour period in starvation (aa- and N-) media.

3.1.5. A quantitative measure of the CLS under the different culture conditions

In order to simplify and summarize data analysis of the experiments shown in this section (3.1), we calculated the area under the curve for the survival curves which provides a quantitative measure of the chronological life span [91, 232]. Table 4 summarizes the results obtained under the different culture conditions used.

Overall the results presented in this section suggest that:

(i) NH_4^+ in the culture medium has a substantial concentration-dependent inhibitory effect on CLS indicated by a significant increase in cell survival when the starting NH_4^+ concentration in the medium is reduced;

(ii) The CLS of cells cultured to stationary phase with amino acid restriction or starved for auxotrophy-complementing amino acids and subsequently transferred to water is significantly shortened by the addition of NH_4^+ and,

(iii) acidification of the medium does not promote the observed decrease in cell survival, in contrast to what is observed at the lowest NH_4^+ concentration and in cells grown without amino acid restriction.

Table 4. Values of Area under the survival curve of strain BY4742 cultured in different medium composition.

Aging assay in:	Cell culture or pre-incubation conditions: High-AA		Low-AA				aa-starved	N-starved
	$(NH_4)_2SO_4$ 0.5%	$(NH_4)_2SO_4$ 1%	$(NH_4)_2SO_4$ 0.01%	$(NH_4)_2SO_4$ 0.1%	$(NH_4)_2SO_4$ 0.5%	$(NH_4)_2SO_4$ 1%	$(NH_4)_2SO_4$ 0.5%	
Medium Sc	1205 ± 13	1175 ± 2	805 ± 47	207 ± 12	115 ± 5	71 ± 5	186 ± 7	272 ± 3
Medium Sc pH7	1439 ± 33	1202 ± 5	1214 ± 2	175 ± 5	74 ± 9	74 ± 2	249 ± 3	725 ± 1
H_2O	1375 ± 87	1347 ± 29	1390 ± 5	916 ± 25	745 ± 42	446 ± 44	723 ± 10	679 ± 2
$(NH_4)_2SO_4$ 0.5%	923 ± 77	951 ± 5	706 ± 23	385 ± 20	271 ± 13	133 ± 12	188 ± 10	588 ± 16
$(NH_4)_2SO_4$ 1%	535 ± 2	552 ± 102	n.d.	n.d.	120 ± 10	90 ± 7	n.d.	n.d.

n.d. – not determined. Cells were grown in SC media supplemented with low (Low-AA) or high (High-AA) concentrations of auxotrophy-complementing amino acids and with 0.01%; 0.1%; 0.5% or 1% $(NH_4)_2SO_4$ for 72 hours; or cells were grown in SC media until O.D. 1-1.5, harvested and resuspended in Nitrogen-starvation medium (N-) or in amino acid-starvation medium (aa-) for 24 hours. The aging assays were performed by resuspending cells from the different culture conditions in their respective exhausted medium, exhausted medium (pH 7.0), water (pH 7.0) or in 0.5 and 1% $(NH_4)_2SO_4$, (pH 7.0).

Section 3.2

Characterization of ammonium induced cell death in amino acid starved cells

Section 3.2. Characterization of ammonium induced cell death in amino acid starved cells

As mentioned in Introduction section, programmed cell death (PCD) in eukaryotic cells can be triggered by exogenous and endogenous inducers being coordinated by a complex network of regulators and effectors, which leads to a series of cell structure and functional changes that characterize the death phenotype [233]. Among the different forms of PCD [145], namely apoptosis, autophagic cell death and programmed necrosis, apoptosis is the most common morphological expression of PCD.

The discovery that yeast cells display apoptosis-like characteristics has validated yeast as a model system and led to the emergence of a recent research field that profited from the recognized advantages of yeast for the study of biological processes. Currently, it is consensual that yeast can undergo cell death with typical markers of mammalian apoptosis in response to different stimuli and possess orthologs of mammalian apoptosis regulators, supporting the existence of a primordial apoptotic machinery similar to that present in higher eukaryotic cells (for a revision see [173, 174]). In this context, several cell death markers used in higher eukaryotes are also currently used in yeast. These include externalization of phosphatidylserine to the outer leaflet of the plasma membrane, DNA degradation, chromatin condensation, and the accumulation of reactive oxygen species, all of which can be measured both at a qualitative and/or at a quantitative level [234, 235]. Programmed necrosis has recently been described as an active regulatory mechanism in yeast [177] and consequently new markers for necrosis are emerging such as nuclear release of the yeast HMGB1 (Nhp6Ap) and detection by electron microscopy of plasma membrane rupture and complete disintegration of subcellular structures [176]. In this section we started with the characterization of the cell death induced by ammonium in aa-starved cells and studied the role of key players involved in different cell death scenarios. In the last part of the section we evaluated whether ammonium toxicity required its metabolization.

3.2.1. Ammonium induces apoptosis and necrosis in amino acid starved yeast cells

In order to determine and characterize the cell death scenario occurring in association with the reduction in CLS induced by NH_4^+, several standard markers of cell death were examined in aa-starved cells transferred to water alone or to water containing NH_4^+ with pH adjusted to 7.0 in both cases. We measured the accumulation of reactive oxygen species (ROS) using the fluorescent probe dihydrorhodamine 123 (DHR, which preferentially detects H_2O_2). DHR levels increased over time either in the absence or presence of NH_4^+, but this increase occurred more rapidly in cells incubated with NH_4^+, peaking at day 2 (Figure 13). After day 2, DHR levels started to decrease in the presence of NH_4^+ accompanying the viability decline of these cells (Figure 13). On the contrary, levels of ROS detected using dihydroethidium (DHE, which preferentially detects $O_2^{\cdot-}$), were not significantly different in the absence or presence of NH_4^+ and there was no increase in its levels over time for both conditions.

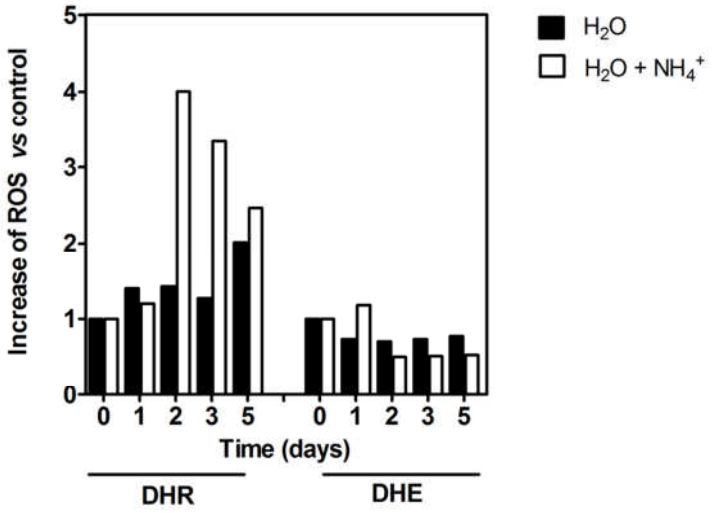

Figure 13. ROS accumulation measured by dihydrorhodamine 123 (DHR, which preferentially detects H_2O_2) and dihydroethidium (DHE, which preferentially detects $O_2^{\cdot-}$) in wild-type *S. cerevisiae* (BY4742) aa-starved cells, upon transfer to: water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). Results are expressed as ratio values estimated by dividing the mean fluorescence intensity of each sample by the mean fluorescence intensity of the control cells (unmarked) for the same time, normalized for T0. $P < 0.001$. Statistical analysis was performed by two-way ANOVA.

Chromatin condensation and nuclear fragmentation is a typical marker of apoptosis and was evaluated using DAPI staining [165]. The results demonstrate (Figure 14A) that the shorter CLS induced by NH_4^+ was accompanied by an increase in the number of cells positively exhibiting this cell death marker and also by the emergence of a population of cells with a sub G0/G1 content of DNA that increased over time, in agreement with nuclear alterations occurring in an apoptotic cell death (Figure 14B). However, assessing apoptotic DNA fragmentation by the TUNEL assay of cells incubated with NH_4^+ only resulted in a relatively small percentage of TUNEL positive cells of the total population (Figure 14C).

Furthermore, staining with annexin V and PI was used to identify apoptotic and necrotic cells (Figure 15), resulting also in a very small percentage of early apoptotic cells [173]. In this double staining approach, annexin V binds phosphatidylserine of the plasma membrane whereas PI, being a membrane-impermeable stain, assesses loss in membrane integrity. Annexin V^+/PI^- staining shows cells with phosphatidylserine exposed on the outer surface of the plasma membrane in the absence of a loss in membrane integrity and therefore cells are considered apoptotic, while PI^+ cells are necrotic. Cells transferred to water containing NH_4^+ exhibited a very small increase in Annexin V staining in the absence of PI staining during the first few days (Figure 15). However, after day 2 these cells exhibited extensive permeabilization of the plasma membrane indicated by PI staining, which indicates they were mostly undergoing necrosis.

We also measured the intracellular calcium concentration as failure of Ca^{2+} homeostasis is associated both with apoptotic and necrotic PCD [160, 174, 236]. In mammalian cells, Ca^{2+} overload induces permeability transition but can also stimulate calpains, which have recently been described as being involved in necrotic cell death execution [160, 176]. The results demonstrate that an increase in the concentration of intracellular Ca^{2+} was observed in cells transferred to water containing NH_4^+ (Figure 14D).

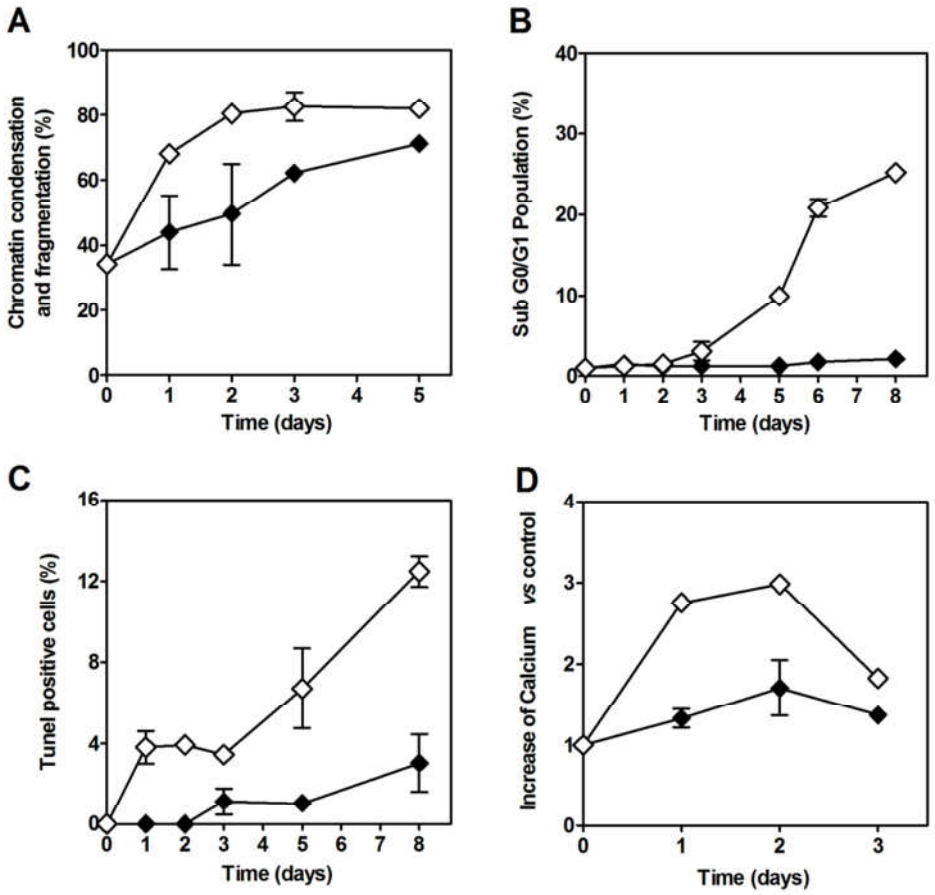

Figure 14. Cell death markers measurements in wild-type *S. cerevisiae* (BY4742) aa-starved cells, upon transfer to: (◆) water (pH 7.0) or (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). (A) Chromatin condensation and fragmentation, (B) Appearance of Sub-G0/G1 peak, (C) TUNEL staining and (D) Calcium accumulation. Values are means ± SEM (n=3). H_2O *vs* 0.5% $(NH_4)_2SO_4$: (B) $P < 0.001$; (C) $P < 0.01$; (D) $P < 0.01$. Statistical analysis was performed by two-way ANOVA.

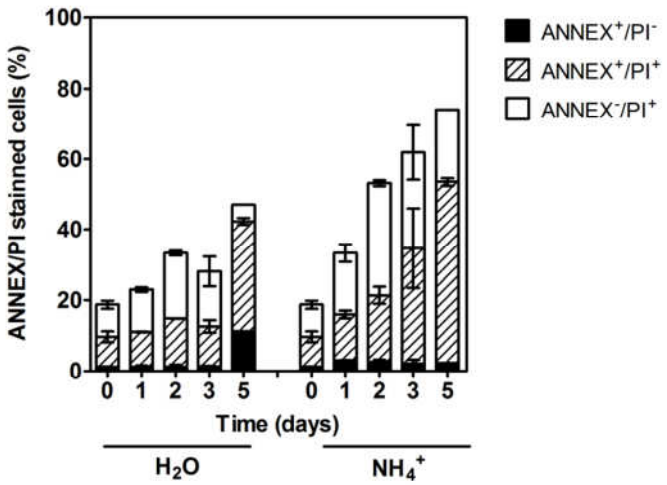

Figure 15. Staining of aa-starved cells of wild-type *S. cerevisiae* (BY4742) with annexin V and propidium iodide (PI), upon transfer to: water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). Values are means ± SEM (n=3). H_2O *vs* 0.5% $(NH_4)_2SO_4$: $P < 0.001$. Statistical analysis was performed by two-way ANOVA.

Taking into account the results from double staining with annexin V and PI and in order to confirm that the cell death scenario induced by NH_4^+ was necrotic we evaluated the nucleus-cytosolic translocation of Nhp6Ap (Figure 16), the yeast homologue of human chromatin bound non-histone protein HMGB1 (high mobility group Box 1) whose nuclear release is considered a marker of necrosis [177]. The translocation from the nucleus to the cytosol of the protein Nhp6Ap tagged with GFP was evident after day1 and continued till day 3 for cells transferred to water containing NH_4^+, whereas for cells transferred to water alone, the tagged protein maintained its nuclear localization (Figure 16) confirming that it was the presence of NH_4^+ that induced the nuclear release of this necrotic marker.

In agreement with necrosis, the evaluation of ATP levels revealed a substantial decrease in ATP content in aa-starved cells transferred to water with NH_4^+ beginning on the first day of assays (Figure 17). ATP depletion could favor a switch from apoptotic to necrotic cell death since apoptotic processes are energy consuming [160]. In fact, ATP depletion is an event described to occur during necrosis in higher eukaryotes and also in plants strengthening the idea of conservation of a necrotic cell death mechanism [160, 162, 237].

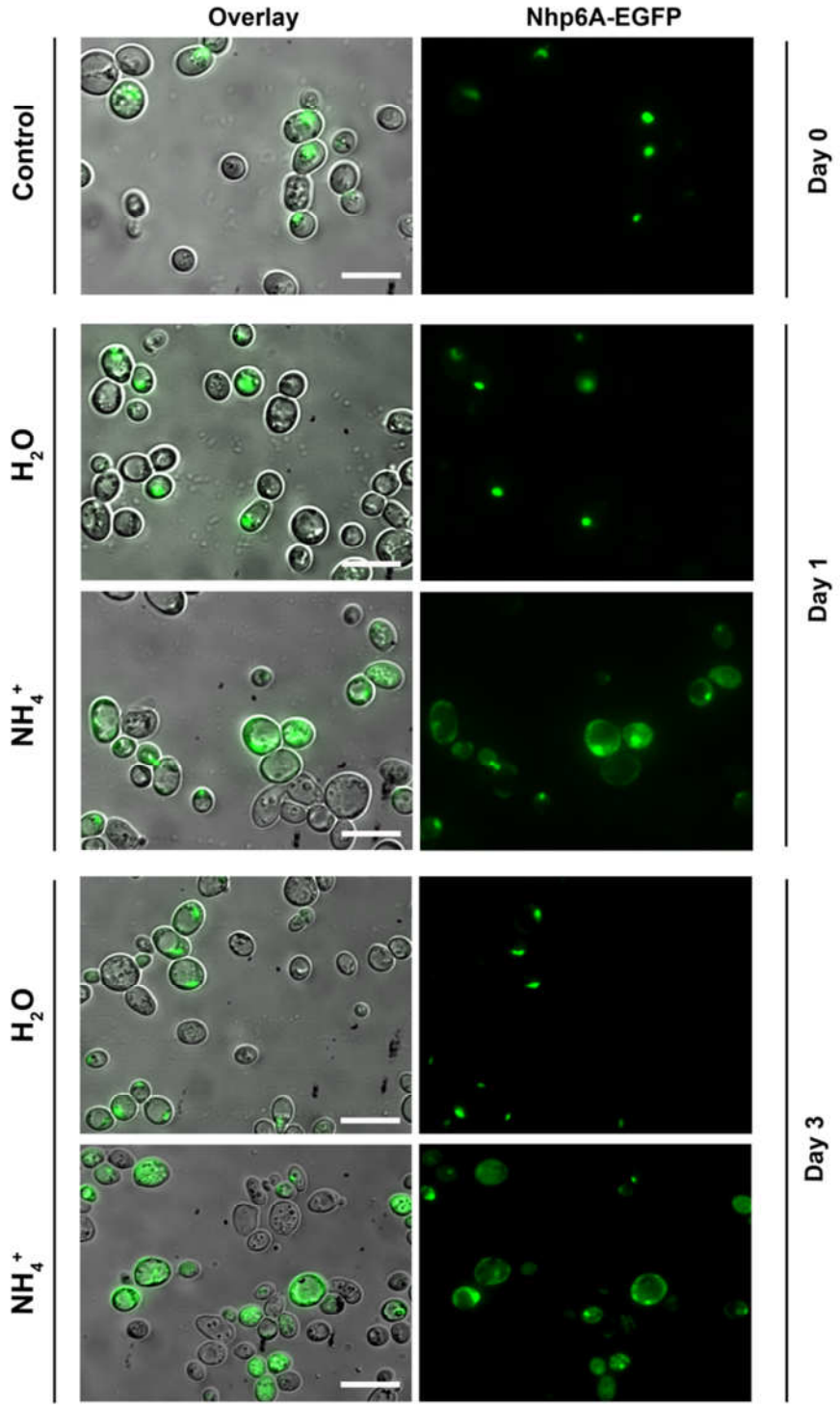

Figure 16. Fluorescence microscopy of wild-type *S. cerevisiae* (BY4742) aa-starved cells (day 0, 1 and 3) expressing Nhp6A–EGFP, upon transfer to water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). Scale bars, 10 μm.

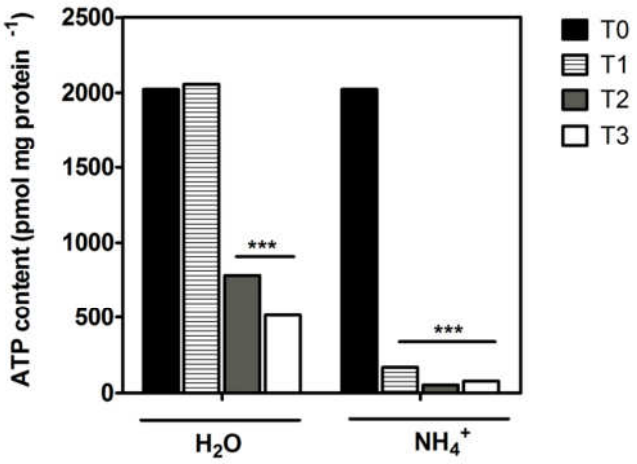

Figure 17. ATP content of wild-type *S. cerevisiae* (BY4742) aa-starved cells (day 0, 1, 2 and 3) upon transfer to water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). Values are means ± SEM (n=3). ***$P < 0.001$ (T0 *vs* T1, 2 and 3 in NH_4^+).

To gain further insight into the cell death induced by NH_4^+ and to clarify the underlying mechanism(s), we employed strains from which genes coding for the yeast metacaspase (Yca1p), apoptosis inducing factor (Aif1p), mitochondrial cyclophylin (Cpr3p) and calpain (Rim13p) had been deleted. Loss of cell viability induced by NH_4^+ in aa-starved cells in water was not altered by deletion of either *YCA1* or *AIF1* (Figure 18A and 19). Therefore, cell death does not depend on Yca1p or Aif1p, which are key factors in several yeast apoptotic processes [172, 184]. In agreement with these results, addition of the caspase inhibitor z-VAD-FMK (benzyloxycarbonyl-VAD-fluoromethylketone) to aa-starved cells after transfer to water and water with NH_4^+ did not cause any significant differences (Figure 18B). These results seem to suggest that caspase-like activity does not play a role in the process, although it cannot be ruled out that activity of other intracellular caspase-like or other aspartic proteases (ASPase) is occurring as it has been previously describe that these proteases are insensitive to Z-VAD-FMK inhibition [238].

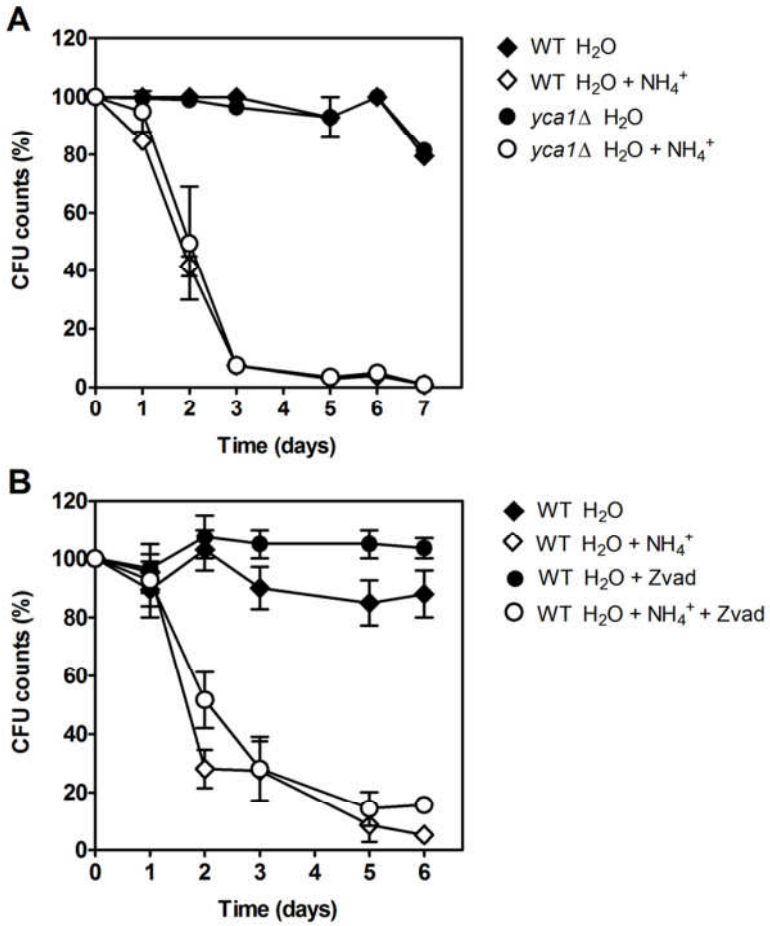

Figure 18. Loss of cell viability induced by NH_4^+ in aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742) and the mutant deleted in the gene coding for the yeast metacaspase (Yca1). (A) Survival of WT and *yca1*Δ aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. (B) Survival of WT aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented or not with) z-VAD-FMK (40 μM). In all the cultures, starting cell density was about 3.8 x 10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). (A) and (B) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

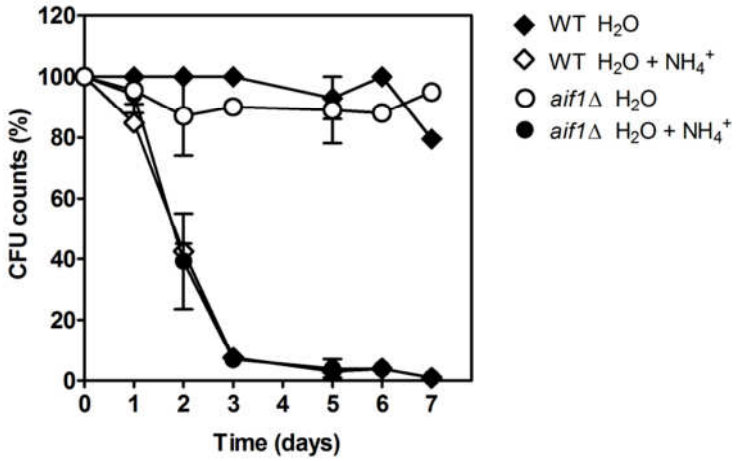

Figure 19. Loss of cell viability induced by NH_4^+ in aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742) and the mutant deleted in the gene coding for the yeast apoptosis inducing factor (Aif1p). Survival of WT and *aif1*Δ aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8 x 10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

aa-starved cells of strains deleted in *RIM13* and *CPR3* coding for the yeast orthologs of mammalian proteins previously associated with necrotic phenotypes [162] displayed loss of cell viability induced by NH_4^+ in water similar to wild type strain (Figure 20A and 21), indicating that those genes are not associated with the NH_4^+ sensitivity phenotype. In agreement with the results obtained with the *cpr3Δ* mutant, loss of cell viability induced by NH_4^+ in aa-starved wild type cells in water was also not altered by simultaneous incubation with cyclosporine, an inhibitor of mitochondrial cyclophylin (Figure 20B). However, it can be observed an increase in death induced by NH_4^+ in the *rim13Δ* mutant, suggesting that instead of mediating cell death, Rim13p, belonging to the calpain family of cysteine protease that are activated by Ca^{2+} [239], may protect against cell death. Consistent with the involvement of calpain activity, is the increase in the intracellular calcium concentration observed in the presence of NH_4^+ (Figure 14D).

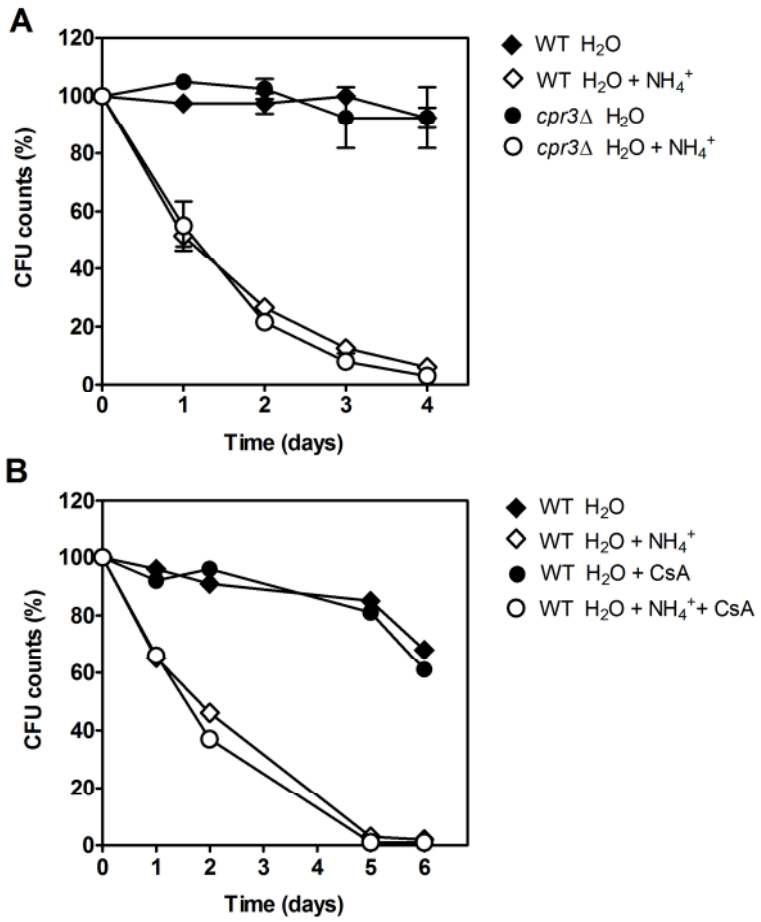

Figure 20. Loss of cell viability induced by NH_4^+ in aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742) and the mutant deleted in the gene coding for the yeast mitochondrial cyclophylin (Cpr3p). (A) Survival of WT and *cpr3Δ* aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. (B) Survival of WT aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented or not with cyclosporine A (CsA) (120 µg/ml). In all the cultures, starting cell density was about 3.8 x 10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). (A) and (B) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

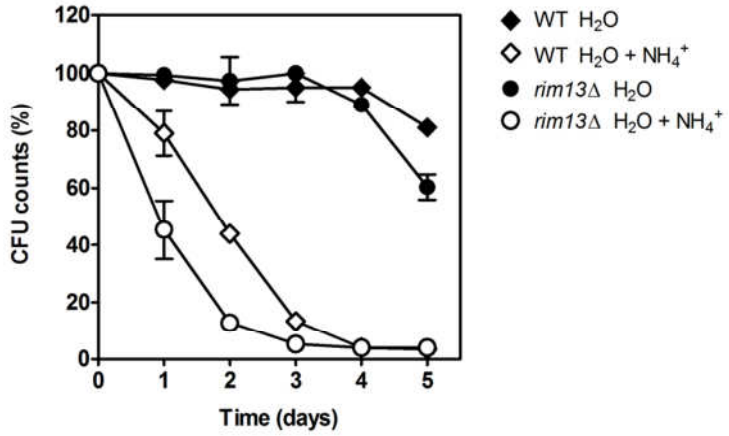

Figure 21. Loss of cell viability induced by NH_4^+ in aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742) and the mutant deleted in the gene coding for the yeast calpain (Rim13p). Survival of WT and *rim13*Δ aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$); $P < 0.001$ (WT 0.5% $(NH_4)_2SO_4$ *vs* *rim13*Δ 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

3.2.2. ROS accumulation and autophagy are not key players in the NH_4^+- induced shortening of CLS

The free radical theory of aging attributes the loss of cell viability/vitality to an increase in ROS accumulation and subsequent oxidative damage [240]. Also, mitochondrial oxidation events have been linked to autophagy regulation in mammalian cells where nutrient starvation stimulated ROS production, namely H_2O_2, which act as a signaling molecule essential for autophagy induction under these conditions [241, 242]. Autophagy is regulated by nitrogen availability through the major nutrient signalling pathways, which also regulate CLS [42, 243]. As described above, NH_4^+ induced a considerable increase in ROS accumulation, particularly H_2O_2 assessed by DHR, in aa-starved cells (Figure 13). In order to assess if ROS accumulation is associated with NH_4^+-induced decrease in CLS, cells were incubated in the presence of several water- or lipid-soluble ROS scavenging agents. The results showed that addition of ascorbic acid, N-acetyl cysteine (Figure 22A), tocopherol, and resvaratrol (Figure 22B) did not reverse the NH_4^+ induced death phenotype; hence ROS accumulation does not appear to be responsible for the loss of cell viability. On the contrary, ascorbic acid and tocopherol induced a shortening of the CLS of cells kept in water without NH_4^+, in agreement with a protective role for ROS under these conditions. This is in agreement with previous

reports showing that calorie restriction or inactivation of catalases extended CLS of *S. cerevisiae* by inducing elevated levels of H_2O_2 [72].

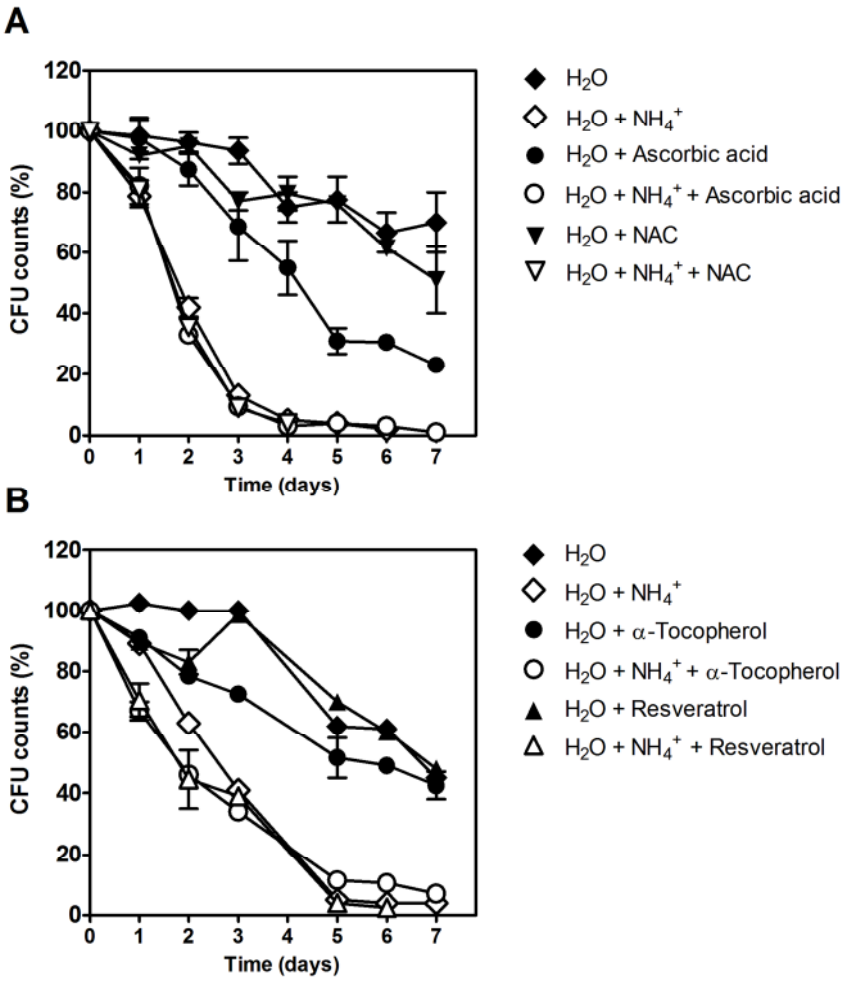

Figure 22. Survival of wild-type (WT) *S. cerevisiae* (BY4742) aa-starved cells, in water or water with 0.5% NH_4^+, supplemented with: (A) hydrophilic antioxidants, Ascorbic acid and N-acetyl cysteine (NAC) or (B) lipophilic antioxidants, α-tocopherol and resveratrol. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3). (A) $P<0.001$ (H_2O *vs* 0.5% NH_4^+); $P<0.01$ (H_2O *vs* H_2O + Ascorbic acid) (B) $P<0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$); $P<0.01$ (H_2O *vs* H_2O + α-Tocopherol).

Following, we asked whether autophagy might be required for the NH_4^+-induced decrease in CLS. *ATG8* codes for a protein essential for autophagosome assembly and its expression is up-regulated by nitrogen starvation shortly after autophagy induction [244]. Thus, we monitored Atg8p levels in cells starved for amino acids (aa- starved cells), before and after transfer to water with or without NH_4^+ (Figure 23A). As expected, autophagy was induced in control cells completely starved for nitrogen (N-starved cells) (Figure 23A) [80, 208]. However, autophagy was not induced in aa-starved cells before they were transferred to water, although autophagy was detected in both aa- and control N-starved cells after transfer to water in the absence of NH_4^+. Importantly, the presence of NH_4^+ in water inhibited the induction of autophagy in aa-starved cells but not in control N-starved cells (Figure 23A).

To evaluate the impact of inhibiting autophagy on cell viability, we used a mutant in the TOR pathway (*tor1*Δ) and its pharmacological inhibitor rapamycin. As referred in introduction (section 1.2.4) Tor1p associates with Tor2p and three other proteins to form the TORC1 complex, which negatively regulates autophagy [104]. The results show that rapamycin did not significantly alter the loss of cell viability induced by NH_4^+ in aa-starved cells (Figure 24). Furthermore, NH_4^+ still prevented autophagy induction in the presence of rapamycin (Figure 23A). In agreement with these results, aa-starved cells othe *tor1*Δ mutant also did not exhibit autophagy either after amino acid starvation or upon transfer to water with NH_4^+ (Figure 23B). However, there was a significant reduction in NH_4^+ toxicity in this mutant (Figure 25), thus excluding inhibition of autophagy as a causal factor in NH_4^+-induced cell death. Although addition of rapamycin had no effect on the survival of cells transferred to water with NH_4^+, surprisingly it could prevent cell death in auxotrophy amino acid-starvation medium (Figure 24). This result enlightens that the cell death processes induced by NH_4^+ and by amino acid starvation are regulated by different pathways. Despite these differences, NH_4^+-induced cell death in aa-starved cells was not prevented by cycloheximide (Figure 26), indicating that death seems to rely on the machinery already present in aa-starved cells before their transfer to water.

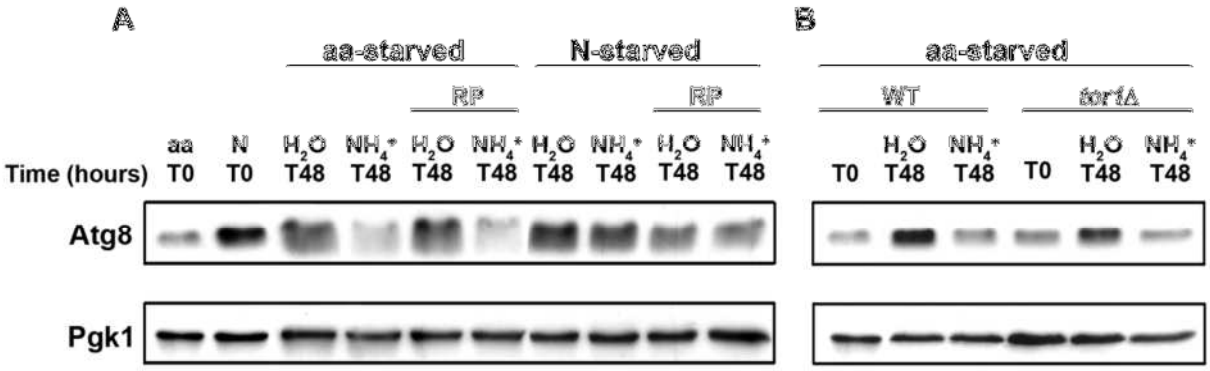

Figure 23. Western-blot analysis of Atg8p levels present in *S. cerevisiae* (BY4742): (A) wild-type (WT) aa-starved or N-starved cells, upon transfer to water or water with 0.5% $(NH_4)_2SO_4$ supplemented or not with rapamycin (RP) (0.2 µg/ml); and in (B) WT and *tor1Δ* aa-starved cells, upon transfer to water or water 0.5% $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0.

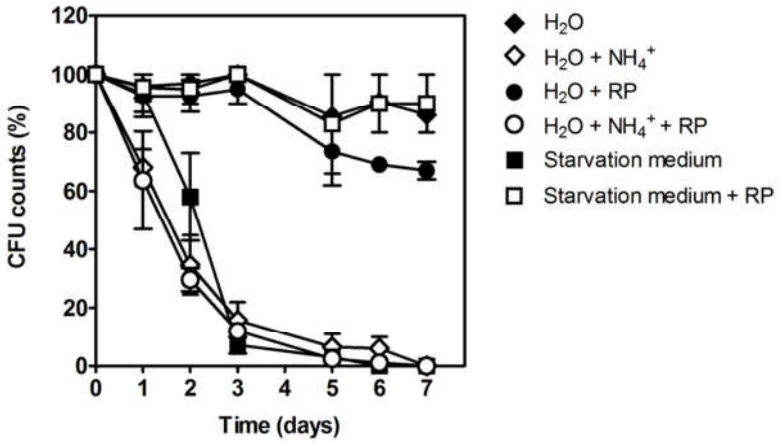

Figure 24. Survival of wild-type (WT) *S. cerevisiae* (BY4742) aa-starved cells upon transfer to water, water with 0.5% $(NH_4)_2SO_4$ or aa-starvation medium supplemented or not with rapamycin (RP) (0.2 µg/ml). In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4).) $P <$ 0.001 (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

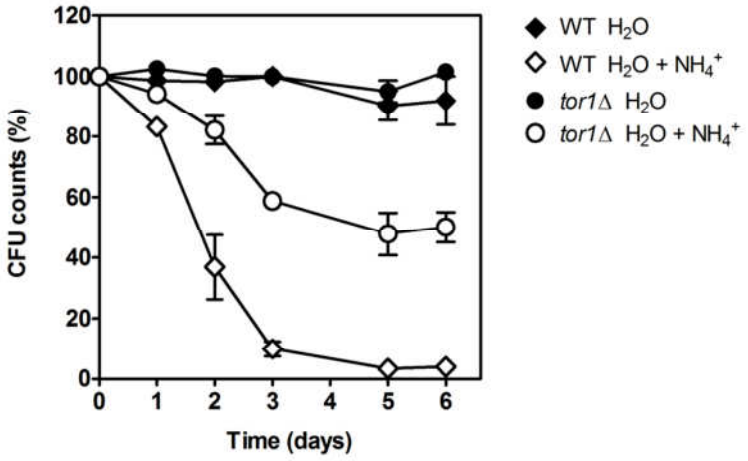

Figure 25. Survival of aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742) and *tor1*Δ strains, upon transfer to water or water with 0.5% $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4).) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$); $P < 0.01$ (t*or1*Δ H_2O *vs* t*or1*Δ 0.5% $(NH_4)_2SO_4$); Statistical analysis was performed by two-way ANOVA.

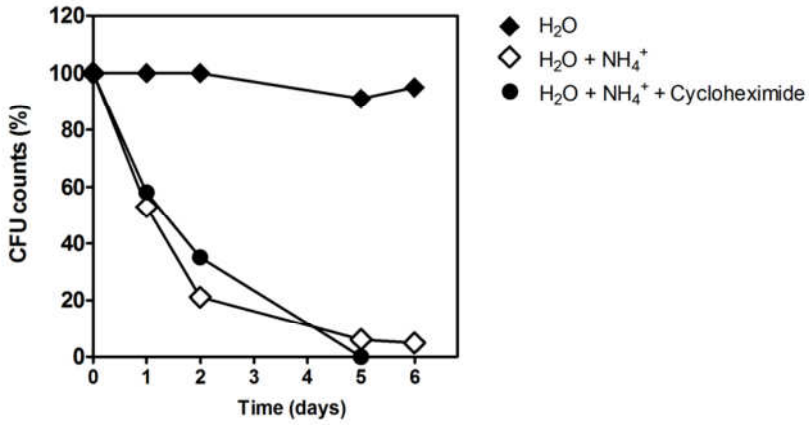

Figure 26. Survival of wild-type (WT) *S. cerevisiae* (BY4742) aa-starved in water or water with 0.5% $(NH_4^+)_2SO_4$, supplemented with cycloheximide (0.01%). Values are means ± SEM (n=3). $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

To further exclude inhibition of autophagy as the causative event of NH_4^+ - induced cell death, we employed wortmannin (Figure 27), an inhibitor of PI3-kinases that blocks autophagy, as well as a mutant deficient for *ATG8* (Figure 28). *atg8*Δ aa-starved cells in water with NH_4^+ displayed loss of cell viability similar to that of wild type (WT) cells (Figure 28A). Addition of wortmannin to aa-

starved WT cells incubated in water with NH_4^+ also had no effect in cell survival (Figure 27). Furthermore, NH_4^+ -induced cell death was not observed in *atg8*Δ N-starved cells (Figure 28B). These results indicate that although NH_4^+ inhibits autophagy, autophagy inhibition is not the cause of the NH_4^+-induced cell death observed in aa-starved cells.

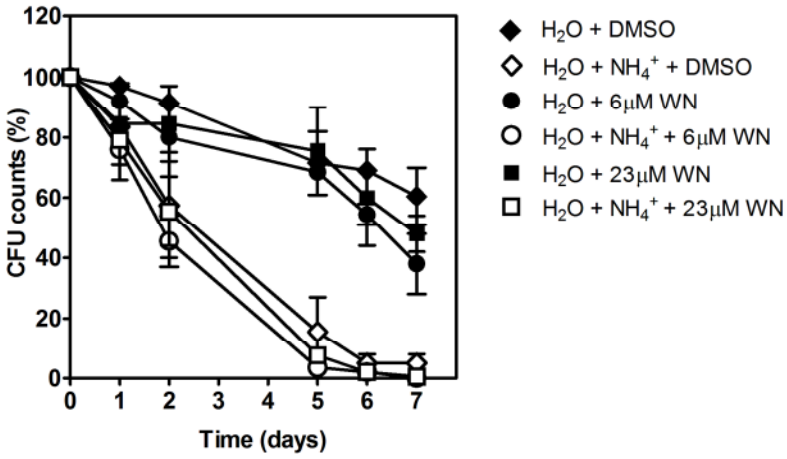

Figure 27. Survival of wild-type *S. cerevisiae* (BY4742) aa-starved cells, in water or water with 0.5% $(NH_4^+)_2SO_4$, supplemented with wortmannin (WN) Values are means ± SEM (n=3). $P < 0.001$ (H_2O + DMSO *vs* 0.5% $(NH_4)_2SO_4$ + DMSO). Statistical analysis was performed by two-way ANOVA.

The importance of autophagy in CLS extension of *S. cerevisiae* has been recently discovered as well as an involvement of autophagy and mitochondrial function in CLS regulation [80, 89]. Also, a role for autophagy in the regulation and maintenance of mitochondrial function by preventing ROS accumulation has been established under conditions of nitrogen starvation, in which maintenance of mitochondrial function is important for cell survival [208]. In the latter study, the failure to respond to starvation with the upregulation of components of the respiratory pathway and ROS scavenging enzymes is the major cause of cell death in *ATG* mutants [208]. However the above presented results demonstrate that neither autophagy nor ROS production seems to be the cause of the NH_4^+-induced cell death. Since glucose, a fermentable carbon source, represses mitochondrial formation, cells were grown and starved for amino acids (aa-starvation) in the presence of a respiratory carbon source that does not exhibit catabolic repression, galactose, in order to increase respiration and assess if this could influence NH_4^+ -induced cell death. The results presented in Figure 29

demonstrate that although with increased respiratory capacity cells are less sensitive to NH_4^+, being able to extend the length of survival, NH_4^+ still induces cell death in this condition, suggesting that ROS do not seem to play an important role in the toxic effects of NH_4^+.

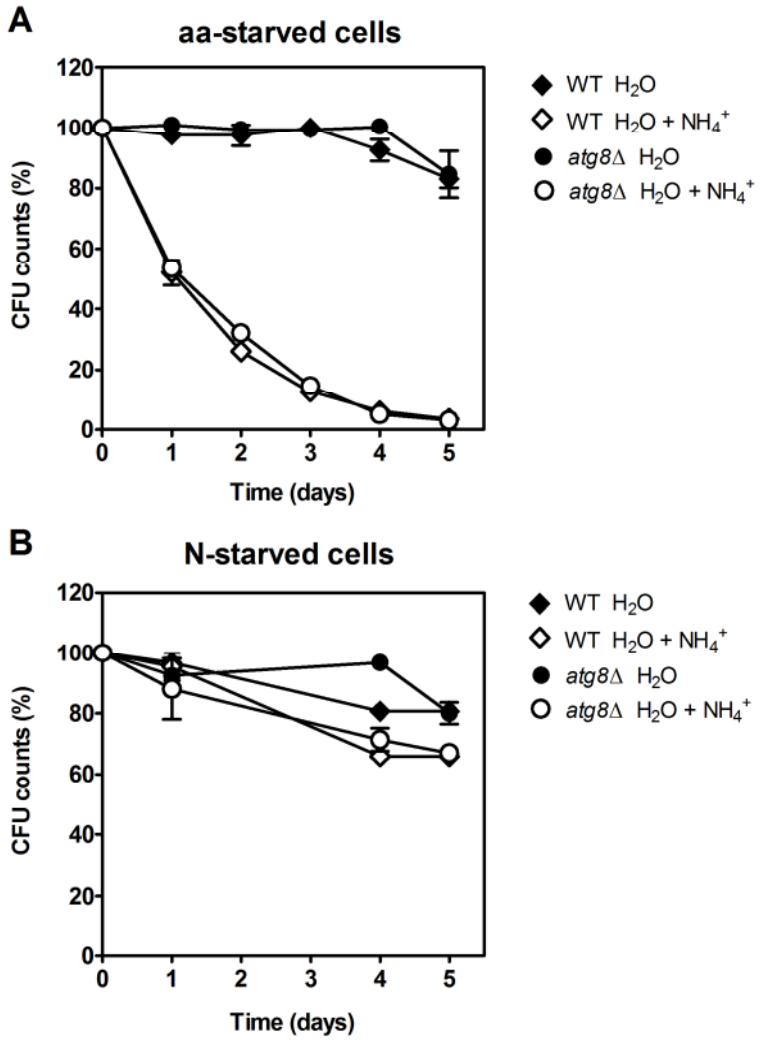

Figure 28. Survival of wild-type (WT) *S. cerevisiae* (BY4742) and *atg8*Δ mutant (A) aa-starved or (B) N-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. Values are means ± SEM (n=3). (A) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

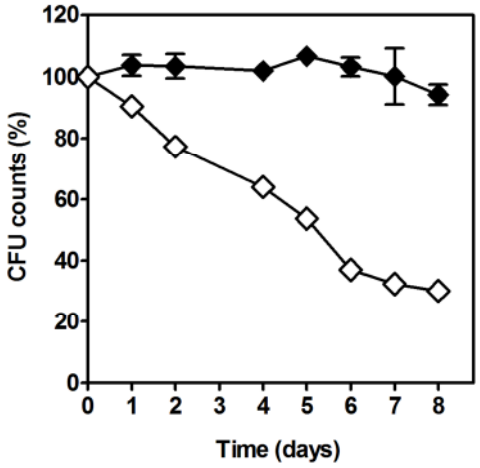

Figure 29. Survival of wild-type *S. cerevisiae* (BY4742) aa-starved cells grown and starved in the presence of galactose, upon transfer to: (◆) water (pH 7.0) or (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). Values are means ± SEM (n=3).

Section 3.3

Metabolism of ammonium is not required for ammonium-induced cell death

Section 3.3. Metabolism of ammonium is not required for ammonium-induced cell death

As referred in introduction (section 1.5), ammonium has a central role in nitrogen metabolism in *S. cerevisiae* being involved in both degradative and biosynthetic pathways [217]. Ammonium enters the cell mostly through plasma membrane transporters, since NH_3, which can enter the cells by passive diffusion, does not occur significantly in the common extracellular acidic-environments, which shift the acid-base balance towards the protonated NH_4^+ form [123].. Ammonium membrane transporters system in yeast belongs to the highly conserved Mep/Amt/Rh superfamily found in all forms of life [211] and comprises three permeases (Mep1, Mep2 and Mep 3) with different kinetics properties [219]. Recent studies have attributed yet another role for Mep1 and Mep2 proteins, demonstrating that they can also function as sensors to activate major nutrient-signaling pathways. This role of Mep2p in signaling PKA activation in response to NH_4^+ in nitrogen starvation medium is not however, dependent on the metabolism of NH_4^+ [135]. Taking these results into consideration, it was assessed whether NH_4^+ toxicity, which leads to a reduction in CLS, was a direct consequence of NH_4^+ signaling or perhaps required its metabolization. In yeasts, the first step of NH_4^+ assimilation is mediated by NADPH-dependent glutamate dehydrogenase, which converts α-ketoglutarate to glutamate, which can be further metabolized to glutamine by glutamine synthetase. Therefore, the activity of these two enzymes was measured. Glutamine synthetase activity was higher in N-starved cells than in aa-starved cells, decreasing for both conditions after transfer to water and water with NH_4^+ (Table 5). This result indicates that the activity of this enzyme is not related to the higher toxicity of NH_4^+.

Table 5. Glutamine synthetase (GS) activity of aa- and N-starved cells of *S. cerevisiae* before (T0) and after transfer to water or water with 0.5% NH_4^+.

Time (Hours)	sp. act. [µmol min^{-1}(mg protein)$^{-1}$]			
	aa-starved cells		N-starved cells	
T0	0.062 ± 0.021		0.137 ± 0.009	
	H_2O	NH_4^+	H_2O	NH_4^+
T2	0.054 ± 0.025	0.040 ± 0.011	0.092 ± 0.016	0.068 ± 0.005
T24	0.030 ± 0.011	0.019 ± 0.021	0.123 ± 0.020	0.056 ± 0.004
T48	0.008 ± 0.001	0.008 ± 0.001	0.089 ± 0.012	0.031 ± 0.010

Data are presented as mean of three independent experiments with SD.

We also tested the effect of NH_4^+ in both aa-starved and N-starved cells in the presence of the glutamine synthetase inhibitor methionine sulfoximine. No significant differences in loss of cell viability were observed (Figure 30A and 30B), further supporting the hypothesis that the toxic effect of NH_4^+ does not require that it be metabolized.

In the assessment of the other enzyme involved in NH_4^+ metabolization, glutamate dehydrogenase, it was observed that enzyme activity at T_0 was higher in N-starved cells than in aa-starved cells, but incubation in water with or without NH_4^+ led to a decrease in its activity (Figure 31). In contrast, glutamate dehydrogenase activity increased approximately 3-fold in aa-starved cells incubated in the presence of NH_4^+.

It was further tested whether α-ketoglutarate depletion or glutamate accumulation, which might result from the higher glutamate dehydrogenase activity, could be the cause of NH_4^+ toxicity. Adding α-ketoglutarate to the medium did not alter the toxic effects of NH_4^+ (Figure 32), whereas adding glutamate resulted in more rapid loss in cell viability, even in the absence of NH_4^+ (Figure 33A). Surprisingly, addition of glutamate to N-starved cells had the opposite effect, resulting in a rescue of cell viability more pronounced in cells incubated in water (Figure 33B). This seems to indicate that starving conditions pre-determine the metabolism functioning which in turn responds in

an opposite manner to the same stimuli. Further supporting that NH_4^+ toxicity does not depend on its metabolization, the non-metabolizable NH_4^+ analogue methylamine also induced cell death in aa- but not N-starved cells (Figure 34). In agreement with these results, the NH_4^+ toxicity observed in SC media cultures (Figure 3) was also not associated with a significant NH_4^+metabolization, as shown from the levels of $(NH_4)_2SO_4$along time (Figure 6B).

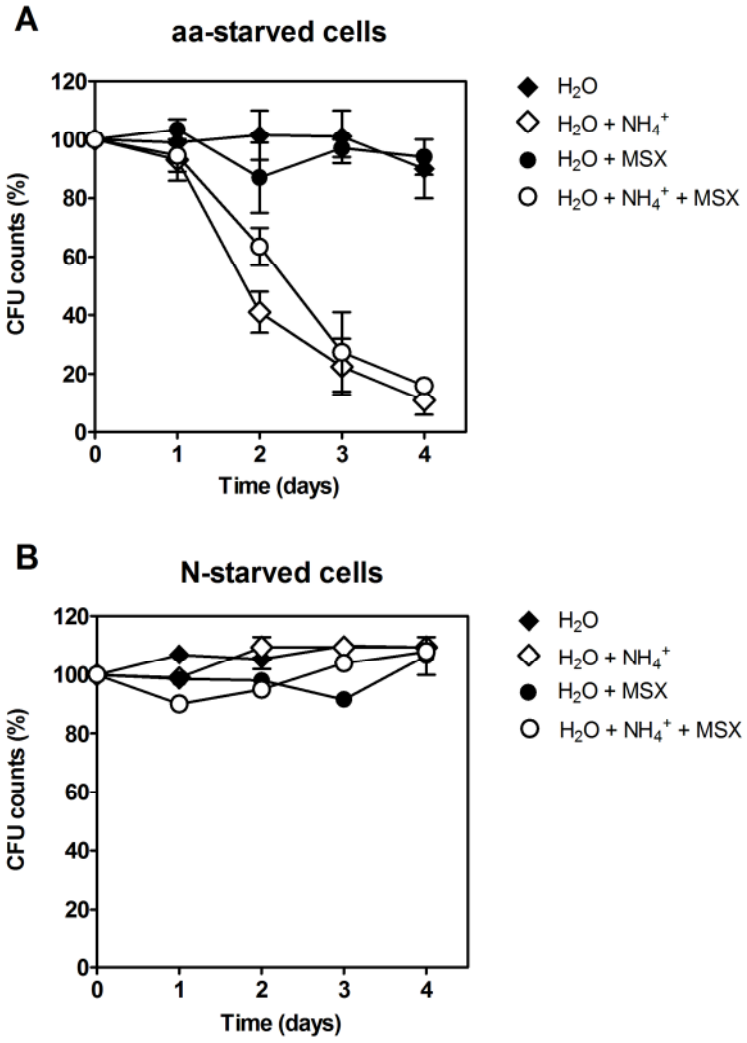

Figure 30. Survival of wild-type (WT) *S. cerevisiae* (BY4742) aa-starved cells (A) or N-starved cells (B), in water or water with 0.5% $(NH_4)_2SO_4$, supplemented or not with methionine sulfoximine (MSX) (1 mM). In all the cultures, starting cell density was about 3.8 x 10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). (A)$P <$ 0.001 (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

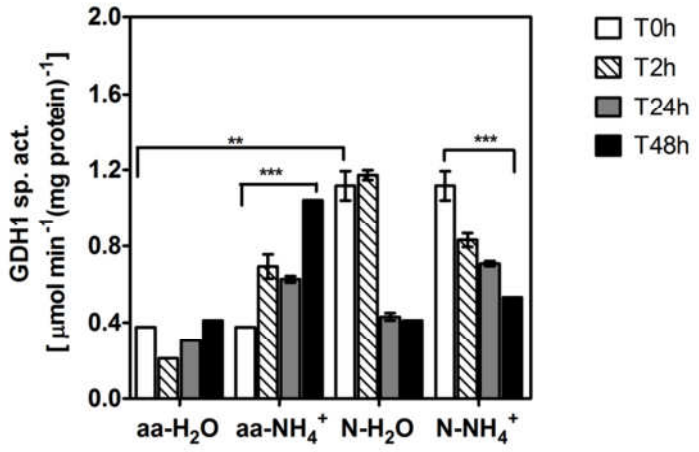

Figure 31. Glutamate dehydrogenase (GDH1) activity of wild-type *S. cerevisiae* (BY4742) aa-starved (aa-H_2O and aa-NH_4^+) and N-starved cells (N-H_2O and N-NH_4^+), before transferred to water (T0h) and after 2, 24 and 48 hours in water or water with 0.5% $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). ***$P < 0.001$; **$P < 0.01$. Statistical analysis was performed by two-way ANOVA.

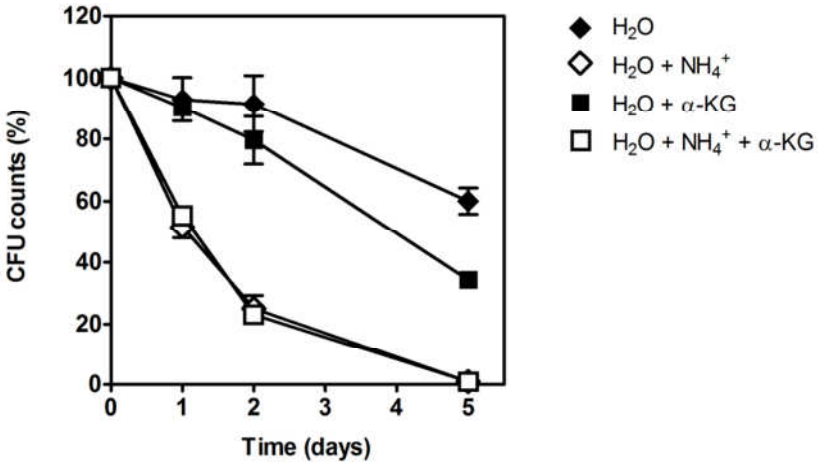

Figure 32. Survival of wild-type (WT) *S. cerevisiae* (BY4742) aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented with α-ketoglutarate (α-KG) (5 mg/ml). In all the cultures, starting cell density was about 3.8 x 10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

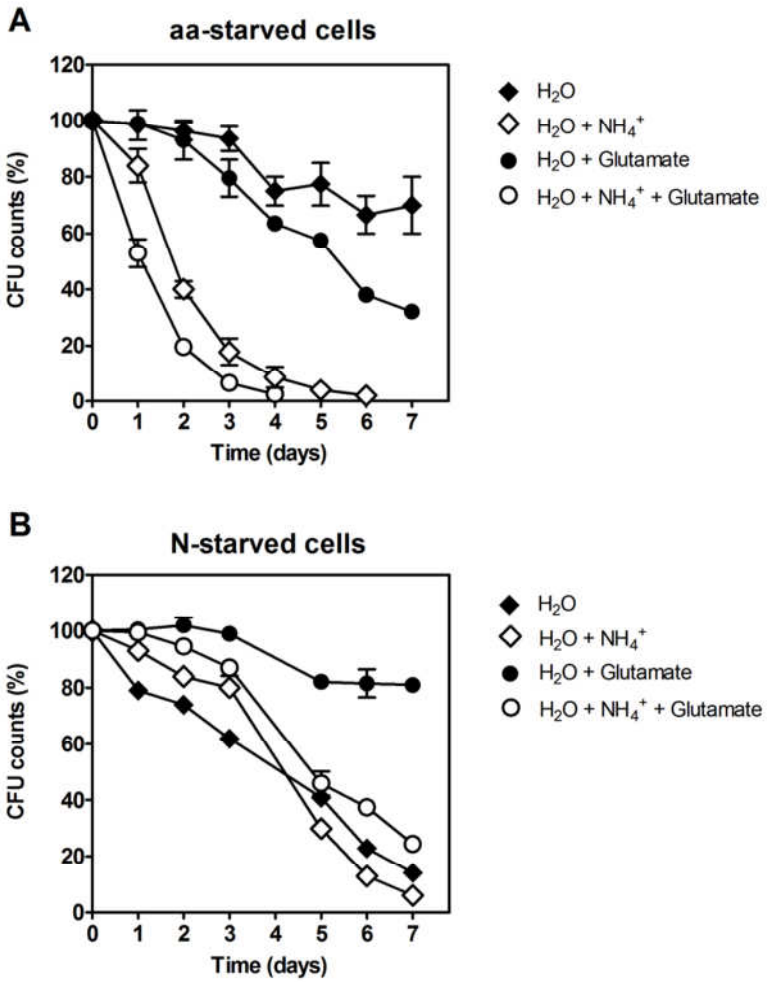

Figure 33. Survival of wild-type (WT) *S. cerevisiae* (BY4742): (A) aa-starved cells and (B) N-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented or not with glutamate (5 mg/ml). In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). (A) $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$); $P < 0.05$ (H_2O *vs* H_2O + Glutamate); (B). $P < 0.01$ (H_2O + Glutamate *vs* 0.5% $(NH_4)_2SO_4$ + Glutamate) Statistical analysis was performed by two-way ANOVA.

Taken together, these results suggest that although glutamate could play a role in NH_4^+-induced cell death to some extent, NH_4^+-induced shortening of CLS does not appear to require that it be metabolized.

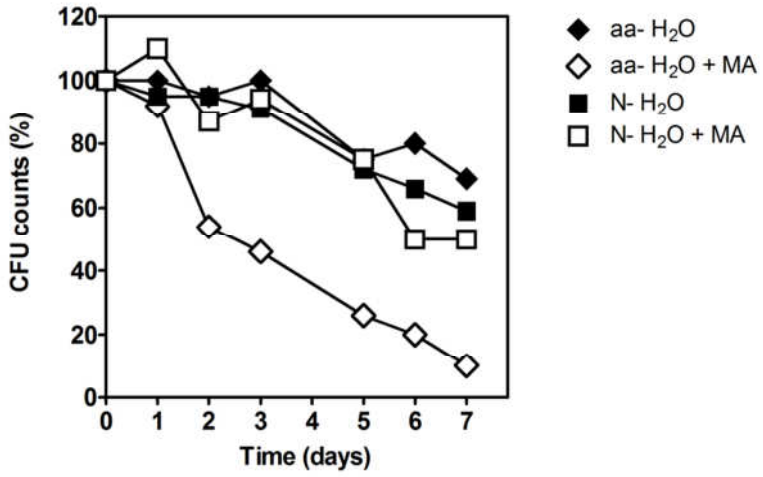

Figure 34. Survival of of wild-type (WT) *S. cerevisiae* (BY4742) aa-starved or N-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented with methylamine (MA) (30 mM). In all the cultures, starting cell density was about 3.8 x 10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). $P < 0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

Section 3.4

PKA and TOR regulate the ammonium induced CLS reduction of amino acid-starved cells

Section 3.4. PKA and TOR regulate the ammonium-induced CLS reduction of amino acid-starved cells

The results presented in the previous section seem to rule out metabolization of NH_4^+ as necessary for cell death induction in amino acid starved cells. Since NH_4^+ is known to activate PKA pathway through Mep2p in a Sch9p dependent manner when added to nitrogen-starvation medium [135], the involvement of nutrient signaling pathways in NH_4^+-induced cell death was assessed. The absence of autophagy inhibition as a causal factor in NH_4^+ -induced cell death led us to hypothesize that NH_4^+ toxicity might be mediated by PKA activation. CLS is under the control of both TOR, Sch9p and PKA signalling pathways [42]. These pathways promote cell division and growth in response to nutrients while inhibiting the general stress response and autophagy.

Trehalase is a target of PKA regulation and its activity has been extensively used to monitor PKA activation [120]. As shown in Figure 35, trehalase activity was much higher in aa-starved cells upon transfer to water with NH_4^+ than in the same cells without NH_4^+ or in N-starved cells (negative control) under both conditions

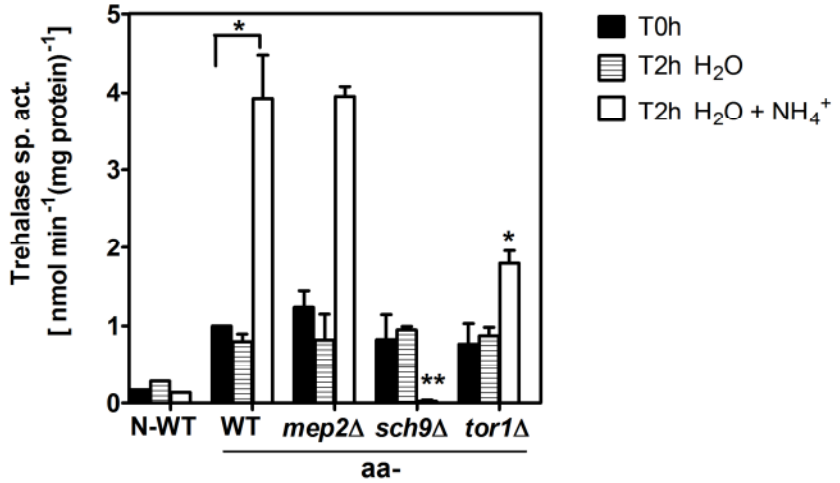

Figure 35. Trehalase activity of wild-type (WT) *S. cerevisiae* (BY4742) N-starved cells and WT and mutant (*mep2Δ*, *sch9Δ* and *tor1*Δ) aa-starved cells, before transferred to water (T0h) and after 2 hours in water (T2h H_2O) or water with 0.5% $(NH_4)_2SO_4$ (T2h H_2O + NH_4^+). In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4).* $P < 0.05$ (WT H_2O *vs* WT 0.5% $(NH_4)_2SO_4$), (WT 0.5% $(NH_4)_2SO_4$ *vs* *tor1*Δ 0.5% $(NH_4)_2SO_4$); ** $P < 0.01$ (WT 0.5% $(NH_4)_2SO_4$ *vs* *sch9*Δ 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

In support of the hypothesis that activation of PKA increases sensitivity to NH_4^+, addition of cAMP increased cell death in the presence of NH_4^+ in N-starved cells and had no effect on aa-starved cells, which display high PKA activity even in the absence of added cAMP (Figure 36A and 36B).

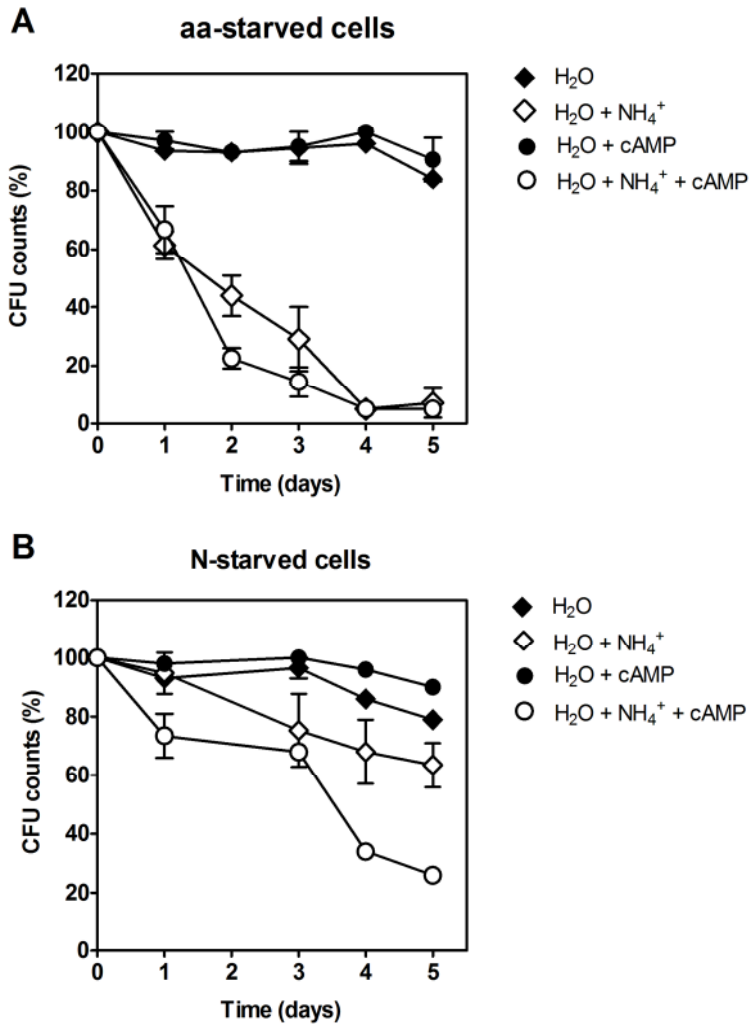

Figure 36. Survival of of wild-type (WT) *S. cerevisiae* (BY4742): (A) aa-starved cells or (B) N-starved cells, after transfer to: (◆) water (pH 7.0); (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (●) water (pH 7.0) supplemented with cAMP (4 mM); (○) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) supplemented with cAMP (4 mM). In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4).

In addition, deletion of *RAS2*, a regulator of PKA activity through the stimulation of cAMP production, caused a partial reversion of the NH_4^+ sensitivity phenotype of aa-starved cells (Figure 37), although it is described that PKA activation by NH_4^+ is cAMP-independent [135]. The NH_4^+ permease Mep2 (and Mep1 to a lesser extent) function as sensors for NH_4^+-induced activation of PKA, whereas Mep3p, the other member of the family of NH_4^+ transporters, does not [135, 219]. As shown in Figure 37, the *mep2*Δ and *mep1*Δ strains exhibited a decrease in NH_4^+-induced death in aa-starved cells, although this decrease was significant only for *mep2*Δ. This is in agreement with the more predominant role of Mep2p in PKA signalling. In order to identify the specificity of the signalling process through PKA, the effect of deleting the genes that code for the three isoforms of the catalytic subunit of this kinase, *TPK1*, *TPK2* and *TPK3*, was also tested. Only deletion of *TPK1* caused a significant reversion of the NH_4^+-induced decrease of the CLS, whereas no differences were detected for strains deficient in *TPK2* and *TPK3* (Figure 38).

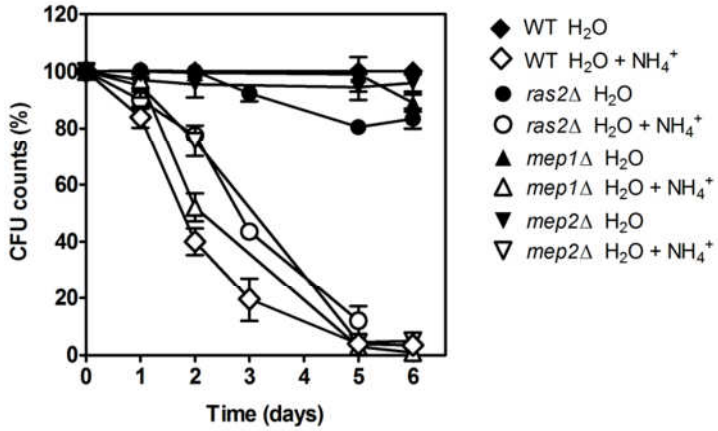

Figure 37. Survival in water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) of aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742), *mep1*Δ, *mep2*Δ and *ras2*Δ strains. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). $P < 0.05$ (WT 0.5% $(NH_4)_2SO_4$ *vs ras2Δ* 0.5% $(NH_4)_2SO_4$); $P < 0.01$ (WT 0.5% $(NH_4)_2SO_4$ *vs mep2Δ* 0.5% $(NH_4)_2SO_4$); $P < 0.05$ (WT 0.5% $(NH_4)_2SO_4$ *vs mep1Δ* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

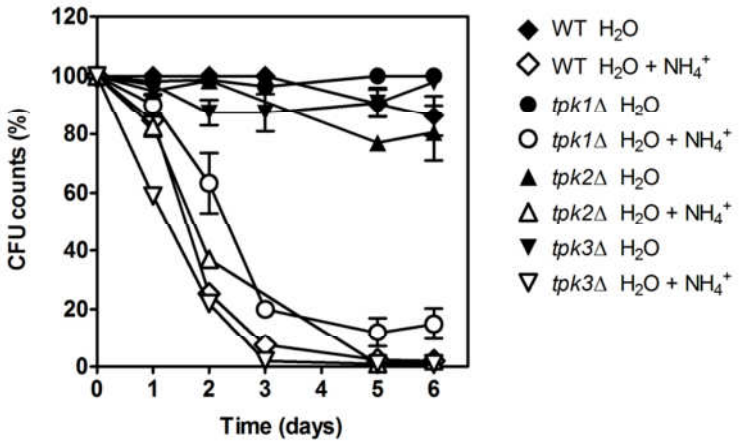

Figure 38. Survival in water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) of aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742) and *tpkΔ* mutants (*tpk1*Δ, *tpk2*Δ or *tpk3*Δ). In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). $P < 0.01$ (WT 0.5% $(NH_4)_2SO_4$ *vs* *tpk1*Δ 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.

Sch9p is a protein kinase with high sequence homology to Tpk1, 2, 3 kinases and regulates cell metabolism in response to several nutritional signals, such as nitrogen and carbon source [61]. It shares many targets with PKA and TORC1, and different interactions between these pathways, either cooperating or antagonizing, have been described [118]. Data from Figure 39 show that *sch9*Δ aa-starved cells underwent increased cell death upon transfer to water plus NH_4^+ and that the lack of Sch9p reduced survival after cells were transferred to water. These results suggest that pathways regulated by Sch9p are important for survival under these conditions.

To evaluate the dependence of PKA activation on Sch9p, Tor1p, and Mep2p trehalase activity was measured in aa-starved cells of the corresponding deletion mutants. Trehalase activity was similar in all strains before or after transfer to water. However, in the presence of NH_4^+, trehalase activity decreased in *tor1*Δ and in *sch9*Δ cells and was almost completely undetectable in the latter strain (Figure 35). These results establish that NH_4^+ signalling to PKA requires Tor1p and Sch9p. However, the opposite cell death phenotypes of *sch9*Δ compared to *tor1*Δ and *tpk1*Δ cells observed in aa-starved cells in the presence of NH_4^+ suggest that the role of Sch9p in the process is essentially independent of the TOR-PKA pathway.

Hog1p is a kinase that regulates and is regulated by Sch9p and mediates stress response

independently of PKA and TOR pathways [142]. To assess whether Hog1p might play a role in resistance to the toxic effects of NH_4^+ mediated by Sch9p, we examined the effects of NH_4^+ in a *hog1*Δ strain (Figure 39). Like *sch9*Δ cells, *hog1*Δ cells were more sensitive to the toxic effects of NH_4^+, which suggests that Sch9p may be signaling Hog1p to mediate increased resistance. This result also reinforces the independent role of Sch9p from TOR-PKA pathway since *hog1*Δ cells presented a sensitive phenotype very similar to *sch9*Δ cells.

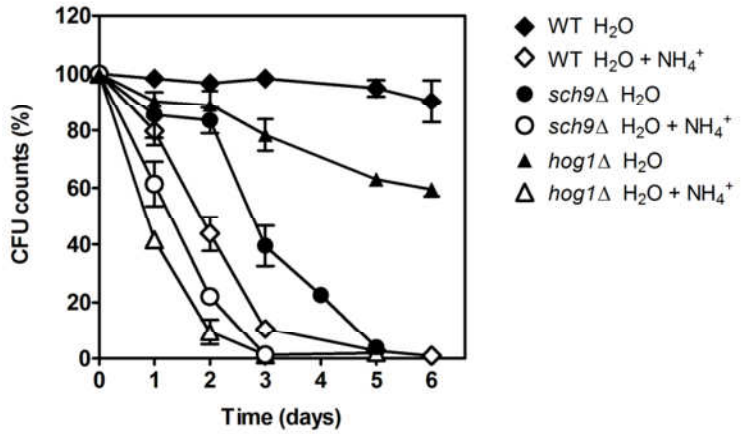

Figure 39. Survival in water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) of aa-starved cells of wild-type (WT) *S. cerevisiae* (BY4742), *sch9*Δ and *hog1*Δ strains. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=3-4). $P < 0.05$ (WT 0.5% $(NH_4)_2SO_4$ *vs hog1*Δ 0.5% $(NH_4)_2SO_4$), $P < 0.001$ (WT H_2O *vs sch9*Δ H_2O). Statistical analysis was performed by two-way ANOVA.

Section 3.5

Influence of auxotrophic amino acid markers and strain background on ammonium toxicity

Section 3.5. Influence of auxotrophic amino acid markers and strain background on ammonium toxicity

3.5.1. Role of starvation for each of the auxotrophic-complementing amino acids of BY4742 strain in ammonium induced CLS shortening

Several studies in the literature report different effects of amino acids on life span regulation and depending on which amino acid is deprived the outcome on CLS regulation differs [76, 77, 80]. In this context, while it is known that starvation of non-essential amino acids (strains without auxotrophys) used as preferred nitrogen sources can extend CLS [26, 52, 81], starvation for auxotrophy-complementing amino acids (essential amino acids) reduces CLS [76, 77]. A recent study reported that when extra supplementation of the auxotrophic-complementing amino acids studied, lysine, histidine and leucine was employed, the latter had a more pronounced effect in CLS extension than the others in both autophagy-competent and autophagy-deficient strains [80]. In the same study, CLS was also extended by supplementation of non-essential amino acids, particularly isoleucine and its precursor threonine and valine [80]. In our studies, as presented in previous sections we have shown that ammonium induces cell death associated with shortening of CLS. This effect was correlated to the concentration of NH_4^+ added to the culture medium and is particularly evident in cells starved for auxotrophy-complementing amino acids.

In order to elucidate if the ammonium effect on yeast survival described in the previous sections is dependent on the specific auxotrophy-complementing amino acid that is deprived in the medium, and thus to evaluate the auxotrophy-complementing amino acids inputs in the observed death scenario, we tested the effect of adding to the standard starvation medium without any of the three auxotrophy-complementing amino acid (aa-starved cells), each amino acid individually or in combination of two of the three auxotrophy-complementing amino acids. For that, following the protocol systematized in Figure 8, cells were deprived in the starvation medium of one amino acid at a time (Figure 40A) or cells were deprived of two amino acids at a time (Figure 40B) and compared to standard starvation of all the three auxotrophy-complementing amino acids (aa-starved cells). The results obtained from the first series of experiments (Figure 40A) revealed that the removal, from the starvation medium, of any of the three auxotrophy-complementing amino acids individually at a time,

induced a decrease of CLS upon transfer of cells to water with NH_4^+, the effect being less accentuated when lysine alone was removed. The most pronounced effect on CLS shortening induced by NH_4^+ was observed for cells starved in the absence of leucine. Additonaly, from the experiments in which two of the amino acids were removed at a time, in different combinations (Figure 40B), we could observe that the presence of leucine during starvation for the other two amino acids increased survival in comparison to starvation for all three amino acids (Figure 40B). The results suggested that, under amino acid starvation, from the three auxotrophy-complementing amino acids tested, leucine had the most positive influence in the survival of cells in the presence of ammonium and induced the most sensitive effect when it was not present In turn, the presence of lysine in the starvation medium without the other two amino acids had the opposite effect of leucine, increasing ammonium induced cell death (Figure 40B). Histidine does not seem to have a major role in regulating CLS in response to ammonium, as its presence or absence in the starvation medium resulted in a cell death profile similar to that exhibited by cells under standard starvation condition.

The results regarding leucine are in accordance with the literature since it has been described a more important role for leucine in CLS extension in auxotrophic strains [76, 80]. In a recent study, supplementation of extra leucine to SC medium or transformation of auxotrophic leucine strain into a prototrophic leucine strain, resulted in CLS extension suggesting that leucine levels in the SC medium were limiting for the BY4742 strain used in the study. Also, these low levels were responsible for the activation of the GAAC pathway in *leu2*Δ strains thus diminishing CLS. The importance of leucine was attributed to the regulation of the branched side chain amino acids synthesis that appears to be misregulated in a *leu2*Δ strain. Deletion of the *LEU3* gene that codes for the primary transcriptional regulator of the superpathway for branched side chain amino acids biosynthesis, severely extended CLS. In agreement, supplemental levels of the branch side amino acids isoleucine, threonine and valine, also extended CLS in a *leu2*Δ strain, suggesting that the misregulation of this biosynthetic pathway likely results from the *leu2*Δ0 mutation in the BY4742 strain [80]. The negative effect observed for lysine in cell survival during ammonium induced cell death can possibly be due to the fact that autophagy is inhibited in the presence of ammonium, and the lack of autophagy might somehow be responsible for this effect since lysine seems to act in an autophagy-dependent manner on the regulation of CLS. Autophagy deficient strains showed no

improvement in CLS extension after regaining LYS prototrophy in contrast to wild-type autophagy competent cells that increased CLS extension with LYS prototrophy [80]. Also, TPK1p has been implicated in the branched chain amino acids biosynthesis pathway [194], which could be correlated with these results showing that leucine seems to have a positive role in NH_4^+-induced decrease of the CLS.

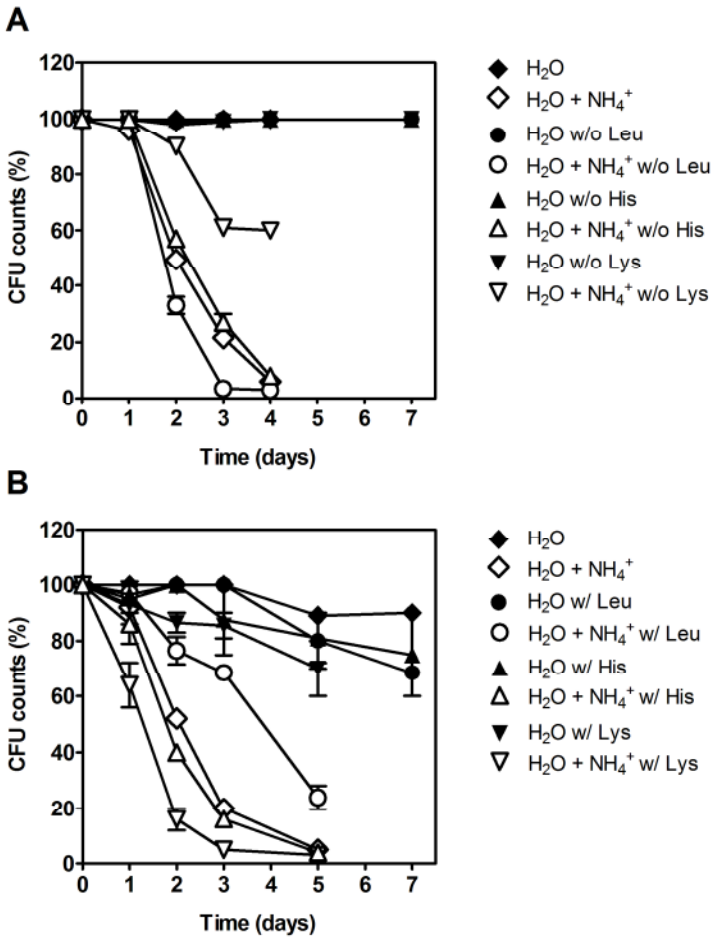

Figure 40. Survival of wild-type (WT) *S. cerevisiae* (BY4742): (A) aa-starved cells for all three amino acids (◆;◇) and cells starved for one specific amino acid at a time (Without; w/o); and (B) aa-starved cells for all three amino acids (◆;◇) and cells complemented with one amino acid at a time (With; w/); upon transfer to water or water with 0.5% $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8 x 10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n=2). $P < 0.01$ (H_2O + NH_4^+ *vs* H_2O + NH_4^+ w/Leu). Statistical analysis was performed by two-way ANOVA.

3.5.2. Influence of strain background on ammonium induced CLS shortening

In order to assess if the strain background could influence ammonium toxicity, four strains with different backgrounds (BY4742, BY4741, W303-1A and CEN-PK2-1C) and/or carrying different amino acids auxotrophys, were tested. We first tested BY4742 and BY4741, two strains with the same background but with different auxotrophic markers, in cells aged in the culture medium. We tested the influence of ammonium in each strain by evaluating CLS of cells grown in SC medium with or without ammonium supplementation. The results presented in Figure 41A show that BY4742 cells grown with low concentrations of auxotrophy-complementing amino acids were very sensitive to the ammonium negative effects on CLS, since in medium without ammonium supplementation a decrease in cell death was not observed. For BY4741 cells, a similar result was obtained for cells grown with low concentrations of auxotrophy-complementing amino acids, although these cells presented a less pronounced shortening of CSL in the presence of ammonium (Figure 41B).

As for BY4741, cells grown in SC medium supplemented with high concentrations of auxotrophy-complementing amino acids, contrary to BY4742 cells, presented an increase in CLS in medium without ammonium supplementation (Figure 41B). The results seem to indicate that auxotrophys within the same genetic background can influence the toxic effects of ammonium, which is in agreement with previous results indicating that each individual auxotrophy has a different effect on CLS upon starvation of the respective amino acid [76, 80].

To evaluate the contribution of the genetic background to the toxics effects of ammonium, we used two strains, W303-1A and CEN.PK2.1C, which have the same amino acid auxotrophys (tryptophan, leucine and histidine). Cells of these two strains were grown in SC media supplemented with low concentrations of auxotrophy-complementing amino acids, condition in which the toxic effects of ammonium on CLS are more evident, with or without ammonium supplementation. As presented in Figure 42, both strains exhibited a decrease in CLS when ammonium is supplemented. Also, strain CEN.PK2-1C (Figure 42B) was more sensitive than strain W303-1A (Figure 42A) to the toxic effects of ammonium. Comparing all three genetic backgrounds, the results indicated that the toxic effects of ammonium were more pronounced in BY strains, followed by CEN.PK2 and W303. These results clearly demonstrate that in amino acid restriction conditions ammonium is toxic and the severity of this toxicity is strain-dependent.

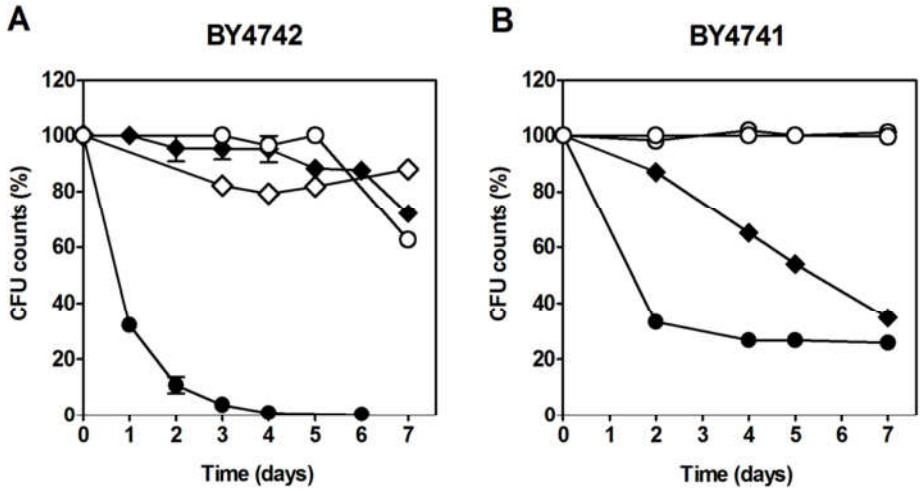

Figure 41. Survival of wild-type *S. cerevisiae* strains (A) BY4742 and (B) BY4741 in SC media supplemented with: (○,●) low and (◇,◆) high concentrations of auxotrophy-complementing amino acids (AA), and with (dark symbols) or without (open symbols) 0.5% $(NH_4)_2SO_4$ supplementation. Values are means ± SEM (n=3). $P < 0.001$ (Low AA with 0.5% $(NH_4)_2SO_4$ *vs* Low AA without 0.5% $(NH_4)_2SO_4$. Statistical analysis was performed by two-way ANOVA.

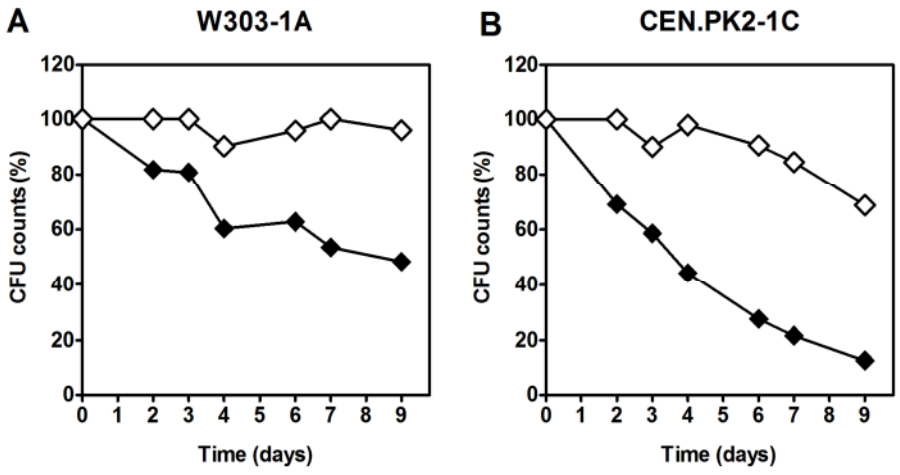

Figure 42. Survival of wild-type *S. cerevisiae* strains: (A) W303-1A and (B) CEN.PK2-1C in SC media supplemented with low concentrations of auxotrophy-complementing amino acids, with (dark symbols) or without (open symbols) 0.5% $(NH_4)_2SO_4$ supplementation. Values are means ± SEM (n=2). (A) $P < 0.01$ (B) $P < 0.001$. Statistical analysis was performed by two-way ANOVA.

As mentioned above, the composition of the culture medium can modulate the chronological life span (CLS) of yeast cells [35]. Culturing cells in different media generates different outcomes in CLS. For example, cells grown in rich YPD medium are known to present an extension of CLS when compared to cells grown in synthetic complete (SC) medium [35, 90].

In order to assess the influence of medium composition during growth, on ammonium toxicity in water in different strain backgrounds, we used minimal K medium [222], which is a define medium that allows us to manipulate all components of the medium. For that, cells were grown in minimal K medium with or without amino acid restriction in the medium (low and high concentrations of the auxotrophy-complementing amino acids). After 72 hours of growth, cells were transferred to water (pH 7.0), water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) and water with 1% $(NH_4)_2SO_4$ (pH 7.0). For strain W303-1A, as shown in Figure 43, there was no shortening of CLS upon transfer to water or water with ammonium of cells grown in K medium supplemented with high (Figure 43A) or low (Figure 43B) concentrations of auxotrophy-complementing amino acids. These results imply that for this strain, grown in minimal K medium, restriction of amino acids did not seem to potentiate ammonium toxicity upon transfer to water with ammonium, as verified for BY4742 strain grown in SC medium (Figure 3). Taking into account that this result could be due to strain background allied to different auxotrophys and different growth medium conditions, we increased the amino acid restriction by lowering one (Figure 43C) or two-fold (Figure 43D) the condition of low concentration of auxotrophy-complementing amino acids defined in this study (see section 2.2 of chapter 2). The results presented in Figure 43C and 43D demonstrate that restricting more severely auxotrophy-complementing amino acids led to shortening of CLS upon transfer to water with ammonium, demonstrating that the ammonium toxicity phenotype is dependent on the severity of the amino acid restriction which in turn seems to vary from strain to strain.

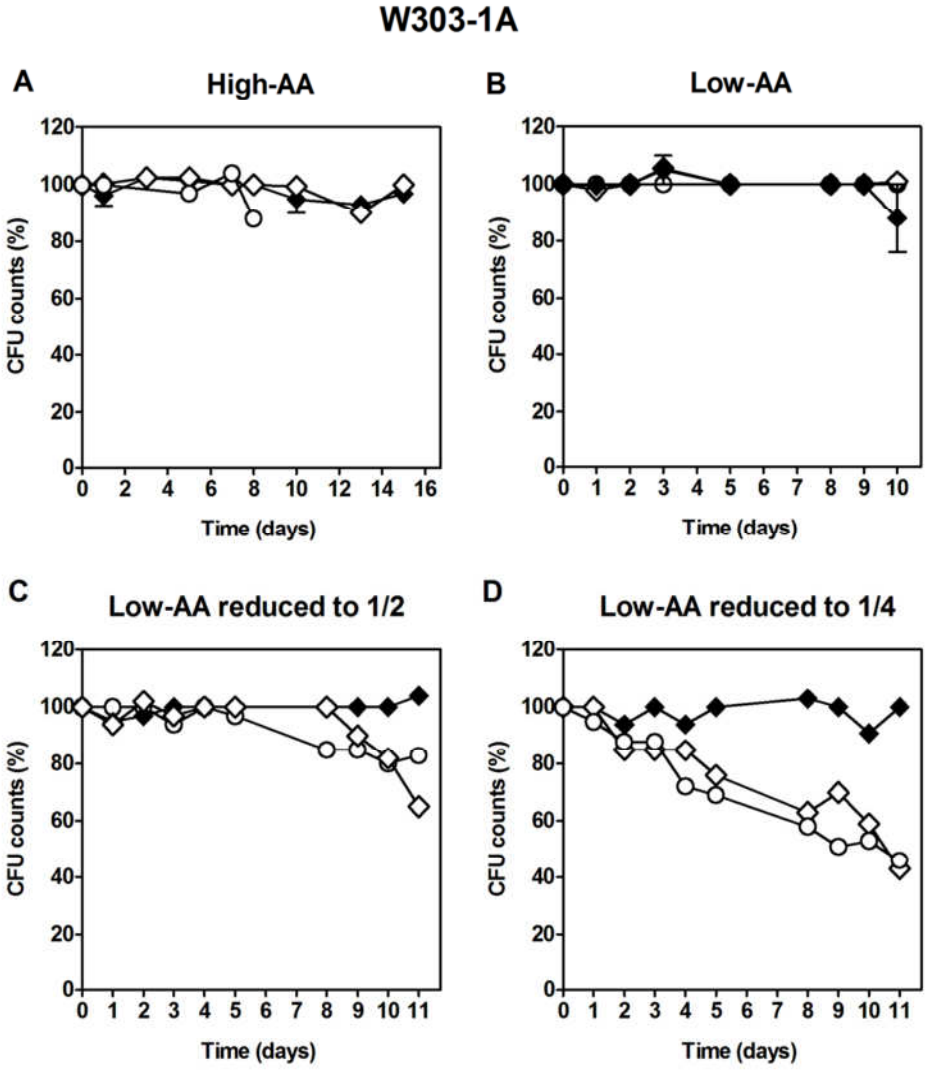

Figure 43. Survival of wild-typw *S. cerevisiae* (W303-1A) grown in minimal K medium supplemented with different concentrations of auxotrophy-complementing amino acids (AA): (A) high (leucine 300 mg/L; histidine 50 mg/L; lysine 50 mg/L); (B) low (leucine 60 mg/L; histidine 10 mg/L; lysine 10 mg/L); (C) low concentration of AA reduced to half (leucine 30 mg/L; histidine 5 mg/L; lysine 5 mg/L) and (D) low concentration of AA reduced to one quarter (leucine 15 mg/L; histidine 2.5 mg/L; lysine 2.5 mg/L). After 72 hours of growth, cells were transferred to: (◆) water (pH 7.0); (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) and (○) water with 1% $(NH_4)_2SO_4$ (pH 7.0). Values are means ± SEM (n=2). (D) $P < 0.01$. Statistical analysis was performed by two-way ANOVA.

To verify if medium composition was in fact contributing to ammonium sensitivity, BY4742 was also tested in minimal K medium. For that, cells were grown in this medium with or without amino acid restriction in the medium (low and high concentrations of the auxotrophy-complementing

amino acids, respectively). After 72 hours of growth, cells were transferred to water (pH 7.0), water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) and water with 1% $(NH_4)_2SO_4$ (pH 7.0). The results presented in Figure 44 confirm that in fact medium composition affected the sensitivity of strains to ammonium, as cells of BY4742 strain previously grown in minimal K medium presented an increase in survival when transferred to water with ammonium (0.5% or 1% $(NH_4)_2SO_4$ (pH 7.0)) when compared to cells of the same strain previously grown in SC medium (Figure 5B). However, when cells previously grown in minimal K medium were transferred to water, they presented a decrease in survival when compared to cells pre-grown in SC medium.

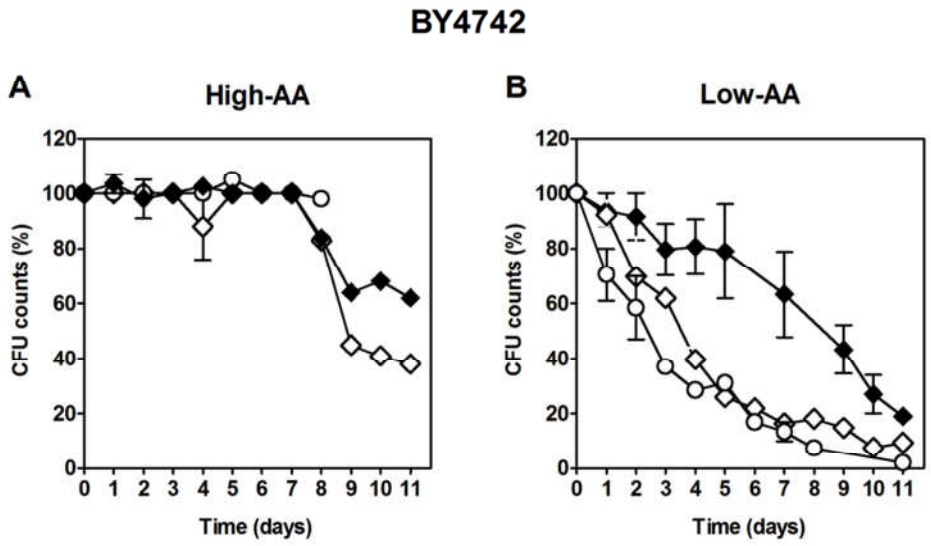

Figure 44. Survival of wild-type *S. cerevisiae* (BY4742) grown in minimal K medium supplemented with (A) high and (B) low (L-AA) concentrations of auxotrophy-complementing amino acids. After 72 hours of growth, cells were transferred to: (◆) water (pH 7.0); (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) and (○) water with 1% $(NH_4)_2SO_4$ (pH 7.0). Values are means ± SEM (n=3). (B) $P < 0.05$. Statistical analysis was performed by two-way ANOVA.

Overall these results demonstrate that several factors contribute to ammonium sensitivity including background of the strain, auxotrophic markers present in the strains and also medium composition. Still, a common denominator is present: the toxicity of ammonium in amino acid restriction conditions. However, these amino acid restriction conditions were tested in strains bearing auxtotrophys in several amino acid biosynthetic pathways and so we questioned if

ammonium toxicity would also apply to prototrophic strains. To evaluate this scenario, a prototrophic strain, CEN.PK113-7D, was tested in different conditions to assess a possible toxic effect of ammonium. For that, firstly cells were grown in minimal K medium without addition of amino acids for 72 hours and subsequently transferred to water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). The results presented in Figure 45, show that also for a prototrophic strain, transferring cells to water with ammonium had a negative effect on cell survival, confirming that ammonium alone was toxic.

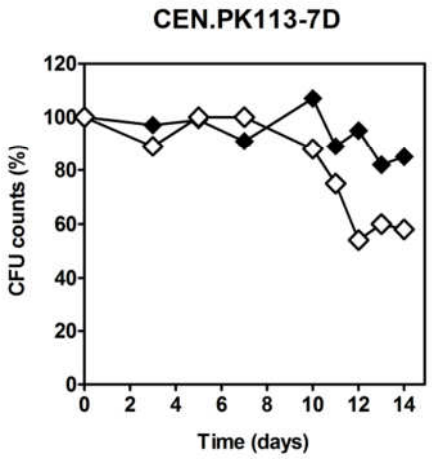

Figure 45. Survival of wild-type *S. cerevisiae* (CEN.PK113-7D) grown in minimal K medium. After 72 hours of growth, cells were transferred to: (◆) water (pH 7.0) and (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). $P < 0.05$. Statistical analysis was performed by two-way ANOVA.

In order to further evaluate ammonium toxicity in the present prototrophic strain, we used a similar methodology to that used for BY4742 starved cells, with the proper alterations due to the lack of auxotrophys. For that, cells were grown to mid exponential phase in SC medium and then transferred for 24 hours to: i) SC medium and ii) SC medium without 0.5% $(NH_4)_2SO_4$ (equivalent to N-starved cells). After 24 hours, cells were transferred to water (pH 7.0), water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) and water with 1% $(NH_4)_2SO_4$ (pH 7.0). The results presented in Figure 46A show that also for cells grown in SC medium, ammonium alone could induce CLS shortening. Also, for cells starved during 24 hours for any nitrogen source, the sensitivity to ammonium was diminished (Figure 46B)

in comparison to cells incubated in complete SC medium. Like for BY4742 N-starved cells (Figure 9B), when prototrophic cells were starved for total nitrogen and subsequently transferred to water, there was a decrease in CLS in this condition (Figure 46B). These results indicate that the presence of ammonium during the 24 hour period of incubation was sufficient to increase sensitivity to ammonium in a prototrophic strain. Furthermore, the results showed that ammonium toxicity is not only a consequence of amino acid restriction, although this condition potentiates toxicity, but it is a more generalized scenario affecting not only auxotrophic but also prototrophic strains.

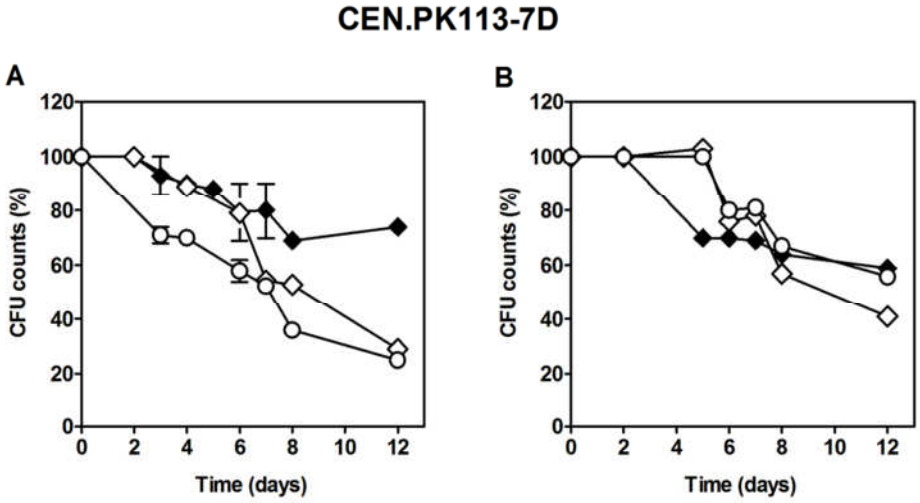

Figure 46. Survival of wild-type *S. cerevisiae* (CEN.PK113-7D) grown in SC medium and incubated during 24 hours in: (A) new SC medium; (B) SC medium with glutamate (5 g/L) and without $(NH_4)_2SO_4$ and (C) SC medium without glutamate and without $(NH_4)_2SO_4$. After 24 hours cells were transferred to: (◆) water (pH 7.0); (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) and (○) water with 1% $(NH_4)_2SO_4$ (pH 7.0). Values are means ± SEM (n=2). (A) $P < 0.05$. Statistical analysis was performed by two-way ANOVA.

CHAPTER 4

General discussion and future perspectives

4. General discussion and future perspectives

4.1 General discussion

The primary molecular mechanisms underlying aging in living organisms are a matter of intense debate, despite decades of study, and with an elderly population increasing in industrialized countries, a healthy aging has become one of the main interests of the general public. In this context, yeast has emerged as one of the most important model organisms to study the environmental and genetic factors affecting longevity, and its exploitation has made huge contributions to the progress in understanding aging. Although some aspects of aging in yeast are specific to this organism, many of the most important features reveled in yeast proved to be evolutionarily conserved in higher eukaryotic organisms [4]. One of this conserved features are the nutrient-signaling pathways and manipulation of these pathways in yeast has uncovered the impact of environmental growth conditions in longevity. During yeast growth, the medium composition is altered suffering a depletion of substrates and is enriched in several compounds resulting from cellular metabolism. Studies using calorie restriction show that reducing glucose concentration of the culture media is sufficient to increase replicative and chronological life span (CLS) [59, 81, 245-247]. Other components of the culture media and factors such as the products of fermentation have also been implicated in the regulation of CLS. Acidification of the culture media mainly due to acetic acid and other organic acids production negatively impacts CLS [91]. Ethanol is another fermentative metabolite capable of inducing CLS reduction in aged cells by yet unknown mechanisms [88].

In the present work, we uncover ammonium as a new extrinsic factor modulating life span in yeast and focus on the mechanisms underlying this modulation. Our studies showed that manipulating the ammonium concentration in the culture medium affects CLS, with high concentrations inducing cell death in association with a reduction in CLS. Also, decreasing the concentration of NH_4^+ in the culture medium increases yeast CLS in both standard amino acid supplemented and amino acid restriction conditions indicating that the toxic effects of NH_4^+ correlate with NH_4^+ concentration in the culture medium. Moreover, after transferring cultured cells to extreme calorie restriction conditions in water, the addition of ammonium drastically decreases the CLS,

indicating that ammonium alone could also induce loss of cell viability as observed in culture media. We also showed that for amino acid restriction conditions, medium acidification, previously reported to decrease survival of chronological aged cells in SC medium [91], did not play a role in survival.

We also reported that the toxic effects of NH_4^+ are particularly detrimental in cells starved for auxotrophy-complementing amino acids aged in water and that starvation for leucine in particular, largely contributes to the susceptibility to NH_4^+ induced cell death. This result regarding leucine is in accordance with the literature since it has been described a more important role for leucine in CLS extension in auxotrophic strains [76, 80]. On the contrary, starvation for lysine positively contributed to cell survival while its presence had a substantial negative effect. This negative effect observed for lysine in cell survival during ammonium induced cell death can possibly be due to the fact that autophagy is inhibited in the presence of ammonium, and the lack of autophagy might somehow be responsible for this effect since lysine seems to act in an autophagy-dependent manner on the regulation of CLS. Autophagy deficient strains showed no improvement in CLS extension after regaining LYS prototrophy in contrast to wild-type autophagy competent cells that increased CLS with LYS prototrophy [80].

Further studies with auxotrophy-complementing amino acids starved cells allowed us to characterize cell death induced by NH_4^+ and establish, that although the toxic effects of NH_4^+ were accompanied by markers for apoptosis, NH_4^+ -induced cell death was predominantly necrotic at later time points. Our data suggest that NH_4^+ causes an initial apoptotic cell death followed by a fast secondary necrosis. Necrosis due to ATP depletion has been reported in other cell death scenarios, namely in tumor cells under metabolic stress [248]. This appears to be the case in NH_4^+-induced necrosis, since ATP depletion was observed in cells incubated in water with NH_4^+ which might block ATP-dependent apoptosis and thus trigger necrosis. The results obtained with the deletion mutant *rim13*Δ point to a protective function of the protease calpain in this cell death process, although Rim13p homologs have been described as players on the execution of the necrotic process in mammals CLS [160]. Recently, it has been suggested that Ras2/PKA signaling may harbour a conserved pro-necrotic function in yeast as observed in mammalians. Actually, PKA and the ras-like Rab protein Rab25 are both required for TNF induced necrosis of mammalian cells [176]. In agreement with this premise our results demonstrate that the necrotic cell death process is

accompanied by high levels of PKA activity, underlying that the Ras2/PKA cascade may in fact comprise a necrotic function.

After characterizing the death process induced by NH_4^+ in aa-starved cells, we further explored the causes underlying the death process. Induction of autophagy is one of the features that occur when cells go into a quiescent state in nutrient exhaustion conditions [99, 123]. Previous studies showed that cells starved for auxotrophic amino acid markers in otherwise complete medium fail to properly arrest in G0 [76]. In accordance, aa-starved cells in our study also do not seem arrested in G0 (indicated by a failure to induce autophagy). It should be noted that this failure to induce autophagy by aa-starved cells was sustained when cells were transferred to water containing NH_4^+ in the absence of other nutrients. This is in contrast with that observed in G0 arrested N-starved cells transferred to water where NH_4^+ could not activate PKA or inhibit autophagy. Treatment with rapamycin under these conditions also could not trigger autophagy induction, possibly due to overactivation of PKA which has been described to abrogate rapamycin-induced effects [249]. Although autophagy was inhibited by NH_4^+ in aa-starved cells, inhibition of autophagy by deletion of *ATG8* did not induce the NH_4^+ sensitivity phenotype in N-starved cells, suggesting that autophagy inhibition is not responsible for the loss of cell viability and shorter CLS induced by NH_4. We also assessed whether activation of PKA could be inducing replication stress, a mechanism responsible for cell aging under different conditions [96]. This could be the case, at least to some extent, since there was a slight increase in the number of budded cells (evaluated by bright field microscopy) for aa-starved (16 %) conditions relative to the control N-starved cells (8%).

In contrast to what has been described for aging cells that reach stationary phase due to carbon limitation [89], we observed that autophagy mutants did not exhibit increased cell death after they were transferred to water, indicating that autophagy is not a key player in cell survival in water when the cells were previously starved for amino acids or nitrogen. It was recently shown that *ATG* genes are important for removing ROS and for maintaining mtDNA and mitochondrial function [208]. This may explain the lack of dependence of cell survival on autophagy in our experimental conditions, as the production of ROS was relatively low. Concordantly, we have observed that the toxic effects of ammonium were not decreased by the presence of ROS scavenging compounds, indication that oxidative stress is not underlying ammonium toxicity. Hence, the cell physiological

state resulting from different culture conditions influences not only life span extension [71], but also the cellular processes essential for its regulation.

In yeasts, the TOR, Sch9p and PKA pathways are key players in the regulation of CLS [42]. Assessing the involvement of nutrient signaling pathways in NH_4^+-induced cell death, showed that activation of PKA correlates with sensitivity to NH_4^+, which is partially suppressed by deletion of *RAS2,* indicating the RAS/Cyr1/PKA pathway is involved in this process. Partial, but not complete, suppression of these effects when *RAS2* is deleted suggests either that the second RAS isoform (*RAS1*) also participates in NH_4^+-induced PKA activation or the existence of two pathways responsible for NH_4^+ toxicity, one that depends on RAS/Cyr1/PKA and one that is independent of this pathway. Previous studies have shown that in nitrogen starvation medium, addition of NH_4^+ directly signals PKA activation through Mep2p and does not depend on its metabolization [135]. Our results show that Mep2p is involved in NH_4^+-induced cell death but does not appear to have a major role in PKA activation. Further experiments using the double mutant (*mep1*Δ*mep2*Δ) will be necessary to assess the involvement of ammonium permeases in PKA activation since Mep1p can also act as a sensor in NH_4^+ signaling to PKA [135, 219]. Still, although glutamate could somewhat mediate the effect of NH_4^+, CLS shortening also seemed to be directly signaled by NH_4^+, as it was not dependent on its metabolization to either glutamate or glutamine.

The deletion of *TPK1,* but not of *TPK2* or *TPK3,* encoding the other two PKA isoforms, significantly reverted the NH_4^+-induced death and shorter CLS. These results suggest that different programmed cell death processes can be regulated by distinct PKA isoforms, since Tpk3p has been reported to regulate apoptosis induced by actin stabilization [194]. Furthermore, it suggests that the postulated pro-necrotic role of Ras/PKA signaling may specifically involve Tpk1p. In addition, our data are also in agreement with previous results showing that CLS extension of glucose-growth limited stationary phase cells depends on PKA inactivation [27]. Our results indicate that PKA inactivation cannot extend cell survival time in the absence of Sch9p, since we observed that *SCH9* deletion abolishes PKA activation in response to NH_4^+, but does not rescue the shortening in CLS induced by NH_4^+. This result is in aggrement with previous studies showing that Sch9 protein kinase is required for nitrogen activation of the PKA pathway in the presence of glucose [62, 123]. Furthermore, the phenotype of aa-starved *sch9Δ* cells in the presence of NH_4^+ was the opposite of that of *tor1*Δ and *tpk1*Δ, suggesting that the role of Sch9p in the process is essentially independent

of the TOR-PKA pathway mediated by a TORC1-Sch9 effector branch. Instead, the two pathways likely regulate their downstream targets that are involved in NH_4^+-induced cell death in an opposing manner. Consistent with this possibility, it was reported that Sch9p positively regulates many stress-response genes and genes involved in mitochondrial function, whereas the same classes of genes are inhibited by the TOR1C pathway [118]. Data suggest that Sch9p may mediate survival in response to NH_4^+ through activation of Hog1p, the yeast closest homolog to p-38 and c-JNK of mammalian cells [250]. Previous reports have shown that *sch9*Δ yeast cells exhibit a longer CLS compared to wild type cells, when aging in SC medium or after transfer from this medium to water [27, 51]. Differences in strain background and/or in culture conditions may account for the discrepancy in results [27, 51, 251, 252]. Supporting this explanation it was also previously reported that *SCH9* deletion shortened the CLS survival of S288c-based strains (as is the case of BY4742 strain used in the present work) pregrown on glycerol [27, 51, 67, 251, 252].

Lastly, we show that severity of NH_4^+ toxicity in amino acid restriction conditions is strain-dependent. This implicates NH_4^+ metabolism regulation as a probable key factor in toxicity. We also show that NH_4^+ toxicity is a generalized effect, not only dependent on amino acid restrictions, but also present in prototrophic strains. This discovery could have major applications in wine fermentations that frequently resort to assimilabe nitrogen supplementations.

Overall the results suggest NH_4^+ is a factor accounting for the loss of cell viability in aging cells and provide, for the first time, a role for ammonium in chronological longevity regulation. Here we have shown that NH_4^+ induced cell death in aging yeast is positively correlated with NH_4^+ concentration in the culture medium. Furthermore, these effects are enhanced in cells starved for auxotrophy-complementing amino acids. Figure 47 summarizes the mechanism involved in NH_4^+ induced cell death in aa-starved cells, showing that NH_4^+ activates PKA through both RAS and TOR/Sch9p signalling cascades and leads to cell death increase with predominant necrotic features. The mediation of NH_4^+ effects seems to involve the NH_4^+ permeases Mep2 and (to a lesser extent) Mep1 as sensors. Sch9p is also mediating survival in response to NH_4^+ possibly through activation of Hog1p. NH_4^+ action on both pathways, resulting in the over-activation of PKA and TOR pathways and inhibition of Sch9p, culminates in the shortening of CLS. Altogether results support that ammonium induces cell death in aging cultures through the regulation of evolutionary conserved pathways.

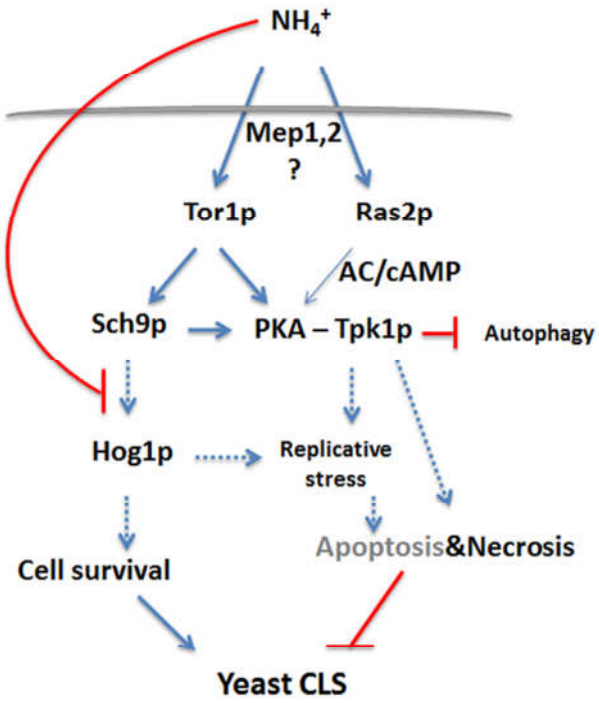

Figure 47. Proposed mechanism for the regulation of cell death associated to CLS shortening induced by ammonium in amino acid-starved yeast cells. NH_4^+ activates PKA through both RAS and TOR/Sch9p and leads to cell death increase with predominant necrotic features associated to ATP depletion. Sch9p is mediating survival in response to NH_4^+ possibly through activation of Hog1p.

Taken together, our results point out, for the first time, a role for ammonium as an extrinsic factor affecting CLS regulation in the culture medium joining other known extrinsic factors such as glucose [12, 35, 57], acetic acid [91, 97, 98] and ethanol [70, 88].

4.2. Future perspectives

As discussed in introduction, NH_4^+ is toxic for mammals, and NH_4^+-induced cell death is involved in different human disorders that are accompanied by hyperammonemia, such as hepatic

encephalopathy [214]. Here we extensively characterized for the first time a cell death process induced by NH_4^+ in yeast cells. This process shares common features with NH_4^+-induced cell death in brain cells. A better understanding of NH_4^+-induced cell death in the yeast cell model can help clarify controversial issues on NH_4^+ toxicity associated to hyperamonemia that are not easy to examine in more complex models. Our results show that the effect of NH_4^+ is not due to different levels of NH_4^+ metabolization, an open question for brain cells, but relies on the over-activation of PKA and the TOR pathway and inhibition of Sch9p (yeast closest homolog of mammalian Akt and S6K). On the other hand, the mitogen activated protein kinase (MAPK) Hog1p was associated with higher cell viability in the presence of NH_4^+ similarly to what was found for its human homolog p38 that mediates endogenous cell protection in response to ammonium in astrocytes [216]. Also, we observed that NH_4^+ toxicity is higher in non-arrested cells, which is consistent with the observation that hyperammonemia presents with much more severe consequences in the developing brain of newborns or infants than in adulthood. Furthermore, our data link NH_4^+ toxicity to amino acid limitation, a situation that can also be present in hyperammonemic patients, who are often on dietary protein restriction [213]. Further experiments will be necessary to establish whether over-activation of TOR and PKA pathways and inhibition of Sch9p is a widely conserved mechanism in NH_4^+ toxicity and induction of cell death. We believe that our model can be useful in the elucidation of conserved mechanisms and pathways of NH_4^+-induced cell death and in identification of therapeutic targets for diseases associated with hyperammonemia. Deprivation of essential amino acids has been employed as a strategy in cancer therapy, but resistance has often been found. Our results establishing that NH_4^+ can stimulate cell death in amino acid-deprived cells and suggests that *S. cerevisiae* might serve as useful model for the identification of signaling pathways for this disease.

Under a biotechnology point of view, our results show that the presence of ammonium in the culture medium can be toxic also for prototrophic strains. We believe that studying the impact of ammonium and amino acids interactions on survival of *S. cerevisiae* during alcoholic fermentation can provide benefitial outcomes. Although wine yeast are usually prototrophs, in the conditions yeast face towards the end of the alcoholic fermentation, the increase of toxic compounds (such as ethanol and acetic acid) can compromise amino acid homeostasis [195] and NCR. Therefore, under such conditions the presence of ammonium and amino acids may affect yeast viability (both positively and negatively) and as a consequence the completion of fermentation. The characterization of the influence of different amino acids and ammonium, present during growth or

added at growth arrest, in CLS regulation can thus be crucial for the optimization of yeast viability at the end of fermentation probably providing important clues to guide management of yeast assimilable nitrogen (YAN) addition. In grape must, YAN can vary in type and amount, being frequently at suboptimal concentrations for the normal developmental of the fermentation process. As a consequence, sluggish or stuck fermentations may occur with great economic impact. To avoid this problem it is a common practice among winemakers to supplement grape juice with YAN (ammonium in the form of diammonium phosphate – DAP and/or amino acids) either at the beginning or some time after the start of the fermentation. Alternatively, nitrogen supplementation can also be applied only when alterations in the normal course of fermentation occur. However, late additions not always result in efficient ammonium consumption [253].

Studies on laboratory strains of *S. cerevisiae* have characterized the selective use of different N-sources by this yeast. Most laboratory strains preferentially use what are known as rich nitrogen sources (ammonium and glutamine, e.g.) in detriment of poor nitrogen sources (threonine and proline, e. g.). For such they repress genes involved in the utilization of the poor nitrogen sources while rich sources are available, a process known as nitrogen catabolic repression (NCR). NCR was found in response to DAP addition at the beginning of fermentation when one industrial wine yeast strain was grown in grape must [254]. However, the efficiency of NCR in response to ammonium is highly strain dependent. Early studies with strains of the ∑1278b genetic background demonstrated ammonium as a preferred nitrogen source whereas studies using S288C genetic background strains, demonstrated that ammonium was not a preferred source, being unable to inhibit the general amino acid permease Gap1p [218]. Also, a recent study, using strains from the Sigma and the TB background, demonstrated that regulation of the NCR differed at the level of the GATA activators Gln3p and Gat1p and the presence or absence of the repressor Gzf3p in the strain background [107]. To further understand the ammonium toxicity, studies aiming to clarify the interconnection in the regulation of ammonium metabolism and amino acids metabolism in CLS, should be carried out.

The results presented in this thesis support that ammonium induces cell death in aging cultures through the regulation of evolutionary conserved pathways. They also show that the study of ammonium toxicity in yeast aging may be a powerful system to understand longevity regulation in multicellular organisms and to give new insights into wine fermentation technics.

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Attachments

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Ammonium Is Toxic for Aging Yeast Cells, Inducing Death and Shortening of the Chronological Lifespan

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Abstract

Here we show that in aging *Saccharomyces cerevisiae* (budding yeast) cells, NH_4^+ induces cell death associated with shortening of chronological life span. This effect is positively correlated with the concentration of NH_4^+ added to the culture medium and is particularly evident when cells are starved for auxotrophy-complementing amino acids. NH_4^+-induced cell death is accompanied by an initial small increase of apoptotic cells followed by extensive necrosis. Autophagy is inhibited by NH_4^+, but this does not cause a decrease in cell viability. We propose that the toxic effects of NH_4^+ are mediated by activation of PKA and TOR and inhibition of Sch9p. Our data show that NH_4^+ induces cell death in aging cultures through the regulation of evolutionary conserved pathways. They may also provide new insights into longevity regulation in multicellular organisms and increase our understanding of human disorders such as hyperammonemia as well as effects of amino acid deprivation employed as a therapeutic strategy.

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Introduction

Starvation of an exponentially growing yeast culture for a given nutrient usually results in the growth arrest of cells in the culture in an unbudded state as they exit the cell cycle. Under extreme starvation conditions such as culturing in water, cells can attain a quiescent state and are able to survive for long periods. The time cells survive in this non-dividing state, known as chronological life span (CLS), is dependent on pre-culture conditions, reaching a maximum for cells grown on respiratory carbon sources and allowed to reach stationary phase [1]. Several nutrient signaling pathways have been implicated in the regulation of yeast CLS, mainly TOR (target of rapamycin), PKA (protein kinase A) and Sch9p [2]. In *Saccharomyces cerevisiae*, TOR signaling responds to nitrogen and possibly to carbon sources. This pathway controls cell growth by activating anabolic processes and inhibiting catabolic processes and mRNA degradation [3–5]. Inactivation of TORC1 (TOR complex 1) or other members of the TOR pathway is accompanied by phenotypic changes characteristic of starved cells, protects against stress, and leads to extension of the longevity of non-dividing yeast [6,7].

The PKA pathway also plays a major role in the control of metabolism, stress resistance, cell cycle, growth, and transcription. It is highly regulated by the nutrient composition of the medium, in particular by the presence of a rapidly fermentable sugar and other essential nutrients such as amino acids and phosphate or ammonium [8,9]. Addition of a rapidly fermentable sugar triggers activation of adenylate cyclase (Cyr1p) and a rapid increase in cAMP levels. This increase boosts the activity of the cAMP-dependent PKA by displacing the regulatory subunit Bcy1p from the catalytic subunits Tpk1p, Tpk2p and Tpk3p. PKA affects several downstream targets, thereby allowing cells to make the necessary adaptations for fermentative growth. These adaptations include upregulation of glycolysis, stimulation of cell growth and cell cycle progression, downregulation of stress resistance and gluconeogenesis, and mobilization of the reserve carbohydrate glycogen and the stress protector trehalose [10,11]. Down-regulation of the PKA pathway by starvation of an essential nutrient causes growth arrest and subsequent entrance into G0. Cells in G0 acquire a variety of characteristics such as accumulation of the carbohydrates trehalose and glycogen, induction of stress-responsive element- and postdiauxic shift-controlled genes, induction of autophagy and increased stress resistance [8,12]. Mutations in components of PKA pathway confer chronological life span extension [13].

The protein kinase Sch9 also plays an important role in nutrient-mediated signaling. It acts in parallel with the PKA pathway and is directly phophorylated by TORC1, mediating many of the TORC1-regulated processes [10,14]. Recent studies revealed that Sch9p also acts independently of TORC1, and can even exert opposite effects to TORC1 in the adaptation to stressful conditions [15].

It has previously been shown that, in the absence of other nutrients, adding glucose to cells suspended in water can cause cells to exit the quiescent state and commit to an apoptotic cell death program that includes production of reactive oxygen species (ROS), RNA and DNA degradation, membrane damage, nucleus fragmentation and cell shrinkage [16]. Chronological aging of

yeast cells in medium also results in a loss of viability with increasing time accompanied by morphological and biochemical characteristics of both apoptosis and necrosis [17,18]. In the present work, we aimed to identify other nutrient signals that could induce cell death of chronological aging yeasts and the signaling pathways involved. Ammonium (NH_4^+) is a nitrogen source commonly used for yeast growth and it is usually not toxic. Production of ammonia in yeast colonies has even been described as a mechanism of protection from cell death during colony development [19]. To our knowledge, only one report in the literature refers to NH_4^+ toxicity in yeast, which was observed in steady-state chemostat cultures limited for potassium [20].

We have found that decreasing the concentration of NH_4^+ in the culture medium increases yeast CLS. Furthermore, we have extensively characterized for the first time a cell death process induced by NH_4^+ in yeast cells. NH_4^+ induced loss of cell viability in aging *S. cerevisiae* cultures either in nutrient-depleted culture medium or upon transfer to water with NH_4^+. This effect was particularly significant for cells starved for auxotrophic-complementing amino acids, but not completely starved for nitrogen. We also determined that activation of PKA stimulated NH_4^+ - induced cell death, consistent with the observation that deficiency in upstream components of the cAMP PKA pathway partially reverted the toxic effect of ammonium. Deletion of *TOR1* also significantly rescued NH_4^+ - induced cell death and decreased PKA activation. In contrast, *SCH9* deletion abolished PKA activation in response to NH_4^+ but did not revert the decrease in cell viability. This indicates that PKA inactivation cannot protect cells from NH_4^+ - induced cell death in the absence of Sch9p, suggesting a potential role of Sch9p in cell survival.

NH_4^+-induced cell death has been implicated in a number of different human disorders that are accompanied by hyperammonemia [21]. However, the precise molecular mechanisms triggering NH_4^+-induced cell death in these disorders are not known. In addition, deprivation of essential amino acids has been employed as a strategy in cancer therapy but resistance has often been found [22]. Our results enhance our understanding of longevity regulation in multicellular organisms. They also suggest that *S. cerevisiae* might serve as a useful model for the identification of signaling pathways and new therapeutic targets for the referred human disorders.

Results

NH_4^+ causes loss of survival in chronologically aged yeast cells

The chronological life span (CLS) of *S. cerevisiae* is strongly affected by the concentration of the auxotrophy-complementing amino acid in the medium. Cells of the auxotrophic *S. cerevisiae* strain BY4742 cultured with an insufficient supply of essential amino acids display reduced lifespan compared with cells grown with increased amino acid supplementation in the medium [23]. In the present work we observed that BY4742 cells grown with insufficient supply of amino acids grow less than those without this restriction, and neither glucose (as previously reported [23]) nor NH_4^+ are completely depleted (Fig. S1A). We first asked whether manipulating the ammonium concentration in the culture medium might affect CLS as previously described for glucose [24]. Reducing the starting concentration of $(NH_4)_2SO_4$ in the medium five- or fifty-fold (from 0.5% to 0.1 and 0.01%, respectively) improved the survival of chronological aging cells cultured with amino acid restriction (Figure 1). In contrast, when the initial $(NH_4)_2SO_4$ concentration in the culture medium, either with or without restriction of amino acids, was increased to 1%, there was

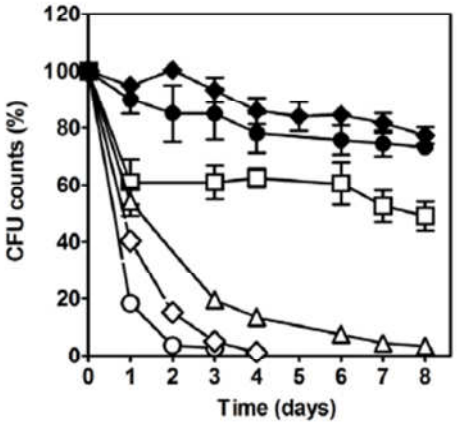

Figure 1. Ammonium stimulates CLS shortening. Survival of *S. cerevisiae* stationary phase cells grown in media supplemented with low (open symbols) and high (dark symbols) concentrations of auxotrophy-complementing amino acid, and supplemented with 0.01% (□); 0.1% (△); 0.5% (◇,◆) or 1% (○,●) ammonium sulphate. In all the cultures, starting cell density was about 3.8×10^7 cells/ml. Values are means ± SEM (n = 3). $P<0.001$. Statistical analysis was performed by two-way ANOVA.
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a decrease in cell survival, although loss of cell viability was much faster for cells grown with amino acid restriction (Figure 1).

We sought additional insights into this phenomena by asking whether increased NH_4^+ could account for the loss of cell viability, as reported in earlier studies [16] which showed that adding glucose to yeast suspensions in water also causes cells to rapidly die. Cells were grown in SC 2% glucose plus 0.5% $(NH_4)_2SO_4$, with or without amino acid restriction in the medium for 72 hours and then transferred to water without NH_4^+ (pH 7.0), water with NH_4^+ (pH 7.0), or to the depleted medium as a control. It has been shown that medium acidification limits survival of yeast cells during chronological aging in SC medium and that the longer survival observed in water can be, at least in part, attributed to the differences in pH [25–27]. To assess whether acidification could play a role in the NH_4^+ -induced loss of cell viability, we measured cell survival in media without adjusting pH (pH 2.6–2.9 due to culture acidification) or adjusted to pH 7.0 (see schematic of methodology in Figure S2). When cells were transferred to water or to depleted medium that was either adjusted or not adjusted to pH 7.0, there was no significant pH variation during the entire experiment. As shown in Figure 2, cells grown with or without amino acid deprivation exhibited a longer CLS after they were transferred to water compared to cells transferred to depleted culture medium that maintained a pH of 2.6–2.9, although loss of cell viability again occurred much faster for cells grown with amino acid restriction. In all cases, addition of NH_4^+ to water reduced cell survival in proportion to its concentration, mimicking its effect in the depleted media. Furthermore, the NH_4^+-induced reduction in CLS observed in water positively correlated with the concentration of NH_4^+ in the growth medium, which indicates that culture conditions pre-determined the cellular response to NH_4^+. Furthermore, transferring cells cultured with insufficient supply of amino acids with 1%, 0.5% or 0.1% $(NH_4)_2SO_4$ to the respective exhausted medium adjusted to pH 7.0 did not lead to a significant difference in CLS relative to the CLS of cells in the exhausted acidic medium (Figure 2C, 2D and 2E). In contrast,

cells cultured under amino acid restriction conditions with the lowest $(NH_4)_2SO_4$ concentration (0.01%) after transfer to the respective exhausted medium adjusted to pH 7.0 exhibited an extended CLS (Figure 2F). Similar results were obtained with cells cultured without amino acid restriction (Figure 2A and 2B), which is consistent with results previously described for similar conditions [26].

To further explore the toxic effects of NH_4^+ during chronological aging under amino acid restriction conditions, a conventional nitrogen starvation protocol [28] was adapted to accommodate the following conditions in SC glucose starvation medium: i) lack of the auxotrophy-complementing amino acids and presence of NH_4^+ (aa-starved cells) or ii) lack of the auxotrophy-complementing amino acids and of NH_4^+ (N-starved cells). Cells were grown to mid exponential phase in SC medium with 2% glucose and then starved for 24 hours in both types of starvation media. Cells were subsequently transferred to water (pH 7.0), with and without NH_4^+ or to the respective 24 hour starvation medium (final pH 2.7–2.9) that was or was not adjusted to pH 7.0 (see scheme of methodology in Figure S2). The initial pH did not significantly change during the assay, except for cells transferred to starvation media at pH 7.0, which reached a final pH around 5.0.

Both aa-starved and N-starved cells survived for a longer period of time in water relative to those in starvation medium (pH 2.7–2.9). Addition of NH_4^+ to water induced a rapid loss of cell viability and shortening of CLS for aa-starved cells (Figure 2G and 2H). Cells cultured in starvation medium that was adjusted to pH of 7.0 also exhibited a rapid decrease in cell viability, indicating that the NH_4^+ effect is not due to the acidification of the medium. In contrast to aa-starved cells, N-starved cells survived for a longer period when transferred to the starvation medium adjusted to pH 7.0. To eliminate the possibility that the reduced survival of aa-starved cells induced by the addition of $(NH_4)_2SO_4$ to water was due to sulphate and not to ammonium, the same experiment was performed in water to which NH_4OH was added instead of $(NH_4)_2SO_4$, and similar results were obtained (Figure S1B).

As discussed in the Introduction, NH_4^+ toxicity was previously described in steady-state chemostat cultures of yeast under limiting potassium concentration [20]. To determine if the ammonium toxicity we observed in our experiments depends on potassium concentration, we repeated our experiments after adding potassium to water at a concentration that according to this earlier study abolished NH_4^+ toxicity. In fact, addition of potassium did not alter the NH_4^+ -induced loss of cell viability (Figure S1C).

Taken together, our results (summarized in Table 1) suggest that (i) NH_4^+ in the culture medium has a substantial concentration-dependent inhibitory effect on CLS indicated by a significant increase in cell survival when the starting NH_4^+ concentration in the medium is reduced; (ii) the CLS of cells cultured to stationary phase with amino acid restriction or starved for auxotrophy-complementing amino acids and subsequently transferred to water is significantly shortened by the addition of NH_4^+ and (iii) acidification of the medium does not promote the observed decrease in cell survival, in contrast to what is observed at the lowest NH_4^+ concentration and in cells grown without amino acid restriction.

Consequently, in subsequent experiments we employed aa-starved cells to address the mechanisms underlying cell death induced by NH_4^+.

NH_4^+ induces apoptosis and necrosis in association with the reduction in CLS in amino acid starved yeast cells

To determine the mechanism by which cell death occurs in association with the reduction in CLS induced by NH_4^+, several standard markers of cell death were examined in aa-starved cells transferred to water alone or to water containing NH_4^+ after the pH was adjusted to 7.0 in both cases. Increased ROS accumulation is a common event in many cell death scenarios, both apoptotic and necrotic [29–31]. We measured the accumulation of reactive oxygen species (ROS) using the fluorescent probe dihydrorhodamine 123 (DHR, which preferentially detects H_2O_2). DHR signals increased with time either in the absence or presence of NH_4^+, but this increase occurred more rapidly in cells incubated with NH_4^+, peaking at day 2 (Figure 3A). In contrast, levels of ROS detected using dihydroethidium (DHE, which preferentially detects O_2^-), were not significantly different in the absence or presence of NH_4^+. The shorter CLS induced by NH_4^+ was also accompanied by an increase in the number of cells exhibiting chromatin condensation and nuclear fragmentation (Figure 3B) and by the emergence of a population of cells with a sub G0/G1 content of DNA that increased over time (Figure 3C and S3A). Incubation with NH_4^+ also resulted in an increase in TUNEL positive cells, although this occurred in a relatively small percentage of the total population (Figure 3D). Furthermore, staining with annexin V and PI was used to identify apoptotic and necrotic cells [30]. In this double staining approach, annexin V binds phosphatidylserine of the plasma membrane whereas PI, being a membrane-impermeable stain, assesses loss in membrane integrity. Annexin V+/PI− staining shows cells with phosphatidylserine exposed on the outer surface of the plasma membrane in the absence of a loss in membrane integrity and therefore cells are considered apoptotic, while PI+ cells are necrotic. Cells transferred to water containing NH_4^+ exhibited a small increase in Annexin V staining in the absence of PI staining during the first few days (Figure 3E). However, after day 2 these cells exhibited extensive permeabilization of the plasma membrane evidenced by PI staining, which indicates they were mostly undergoing necrosis. Necrosis was confirmed by the observation of the nucleus-cytosolic translocation of Nhp6Ap (Figure 3G), the yeast homologue of human chromatin bound non-histone protein HMGB1 (high mobility group Box 1) whose nuclear release is considered a marker of necrosis [17]. Also consistent with necrosis, we observed a significant decrease in ATP content in these cells beginning on the first day of assays (Fig. S3B) which may have limited energy consuming apoptotic processes. Furthermore, NH_4^+-induced cell death was not prevented by cycloheximide (Figure S4D), indicating that death is not dependent on *de novo* protein synthesis. Together these data point to an initial apoptotic cell death induced by NH_4^+ followed by a rapid secondary necrosis.

To clarify the mechanism(s) of cell death induced by NH_4^+, we employed strains from which genes coding for the yeast metacaspase (Yca1p), apoptosis inducing factor (Aif1p), mitochondrial cyclophylin (Cpr3p) and calpain (Rim13p) had been deleted. Loss of cell viability induced by NH_4^+ in aa-starved cells in water was not altered by deletion of either *YCA1* or *AIF1* (Figure S5A and S5B). Therefore, cell death does not depend on Yca1p or Aif1p, which are key factors in several yeast apoptotic processes [32,33]. Strains deleted in *RIM13* and *CPR3* coding for the yeast orthologs of mammalian proteins previously associated with necrotic phenotypes [29] displayed loss of cell viability induced by NH_4^+ in water similar (*cpr3Δ*) or higher (*rim13Δ*), when compared to wild type strain (Figure S5C and S5D), indicating that those genes are not associated with the NH_4^+ sensitivity phenotype. In agreement with the results obtained with the *cpr3Δ* mutant, loss of cell viability induced by NH_4^+ in aa-starved wild type cells in water was also not altered by simultaneous incubation with cyclosporine, an inhibitor of mitochondrial cyclophylin (Figure S5E). We observed an increase in death induced by

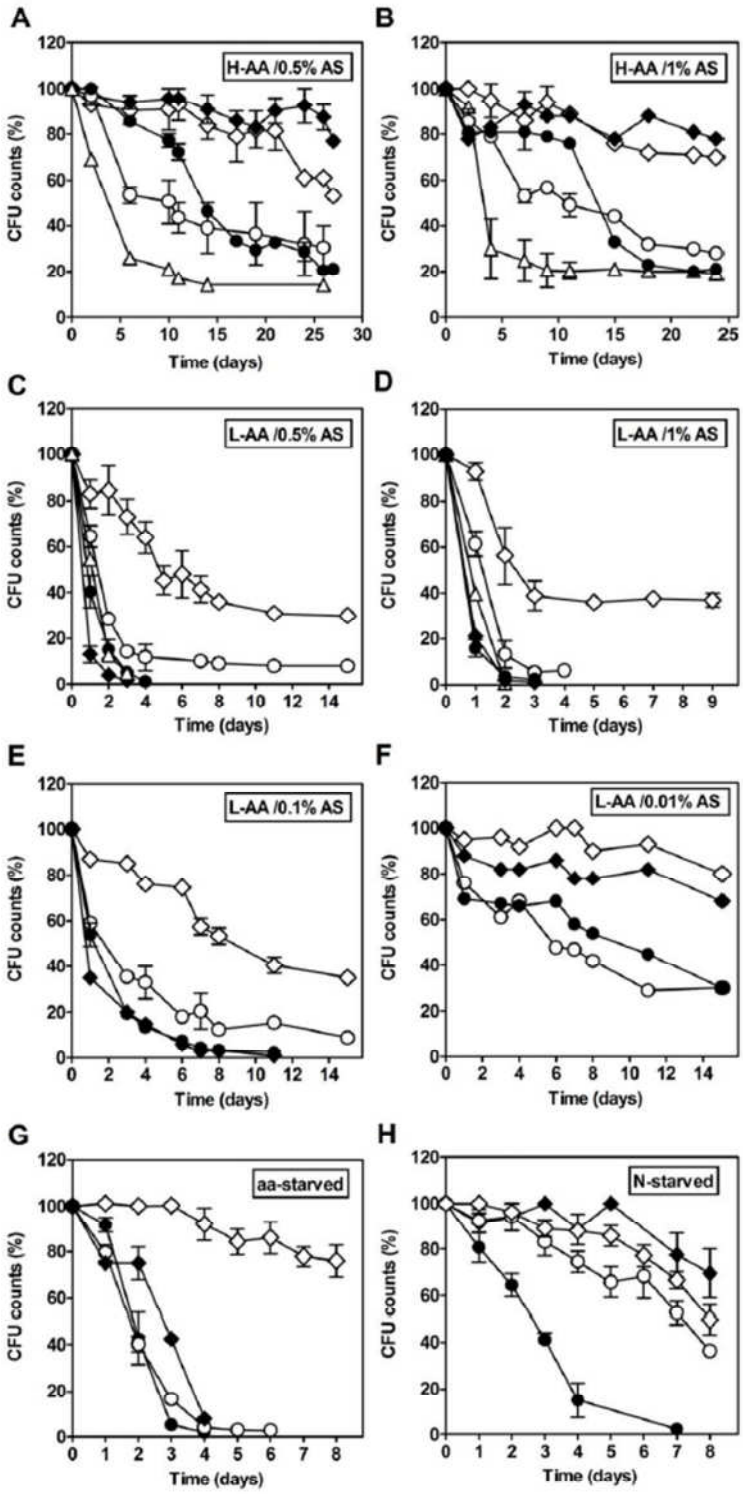
A
H-AA /0.5% AS
B
H-AA /1% AS
C
L-AA /0.5% AS
D
L-AA /1% AS
E
L-AA /0.1% AS
F
L-AA /0.01% AS
G
aa-starved
H
N-starved
CFU counts (%)
Time (days)

Figure 2. Ammonium stimulates cell death of *S. cerevisiae* associated with a shortening of the CLS. A, B, C, D, E and F. Survival of *S. cerevisiae* stationary phase cells grown in media supplemented with low (L-AA) and high (H-AA) concentrations of auxotrophy-complementing amino acid, and supplemented with (F) 0.01%; (E) 0.1%; (A, C) 0.5% or (B, D) 1% ammonium sulphate (AS). After 72 hours of growth, cells were transferred to: (◇) water (pH 7.0); (○) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (△) water with 1% $(NH_4)_2SO_4$ (pH 7.0); (●) exhausted medium; (◆) exhausted medium (pH 7.0). Values are means ± SEM (n = 3–5). **G and H** - Survival of (H) nitrogen starved cells (N-) or (G) amino acid-starved cells (aa-), after transfer to: (◇) water (pH 7.0); (○) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (●) starvation medium; (◆) starvation medium (pH 7.0). In all the cultures, starting cell density was about 3.8×10^7 cells/ml. Values are means ± SEM (n = 8). $P<0.001$ (aa-starved H_2O *vs* aa-starved 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.
doi:10.1371/journal.pone.0037090.g002

NH_4^+ in the *rim13Δ* mutant, suggesting that instead of mediating cell death, Rim13p, belonging to the calpain family of cysteine protease that are activated by Ca^{2+} [34], may protect against cell death. Consistent with the involvement of calpain activity, an increase in the intracellular calcium concentration was observed in the presence of NH_4^+ (Figure 3F).

In summary, although cell death induced by NH_4^+ in aa-starved cells was accompanied by chromatin condensation and DNA fragmentation, both of which suggest an apoptotic process, the subsequent loss of membrane integrity points to accelerated necrosis at later times that may be partially rescued by calpain.

Autophagy is not a key player in NH_4^+-induced cell death

Autophagy is regulated by nitrogen availability via the major nutrient signalling pathways, which also regulate CLS [6,35]. Therefore, we asked whether autophagy might be required for the NH_4^+-induced decrease in CLS. *ATG8* codes for a protein essential for autophagosome assembly and its expression is up-regulated by nitrogen starvation shortly after autophagy induction [36]. Thus, we monitored Atg8p levels in cells starved for amino acids (aa- starved cells), before and after transfer to water with or without NH_4^+ (Figure 4A). As expected, autophagy was induced in control cells completely starved for nitrogen (N-starved cells). Autophagy was not induced, however, in aa-starved cells before they were transferred to water, although autophagy was detected in both aa- and control N-starved cells after transfer to water in the absence of NH_4^+. Importantly, the presence of NH_4^+ in water inhibited induction of autophagy in aa-starved cells but not in control N-starved cells.

To evaluate the impact of inhibiting autophagy on cell viability, we used a mutant in the TOR pathway (*tor1Δ*). Tor1p associates with Tor2p and three other proteins to form the TORC1 complex, which negatively regulates autophagy [10]. The *tor1Δ* mutant also did not exhibit autophagy either after amino acid starvation or upon transfer to water with NH_4^+ (Figure 4B). However, there was a significant reduction in NH_4^+ toxicity in this mutant (Figure 4C), thus excluding inhibition of autophagy as a causal factor in NH_4^+-induced cell death. To address this point further, we employed wortmannin, an inhibitor of PI3-kinases that blocks autophagy, as well as a mutant deficient for *ATG8* (Figure S4). *atg8Δ* aa-starved cells in water with NH_4^+ displayed loss of cell viability similar to that of wild type (*WT*) cells (Figure S4A). Addition of wortmannin to aa-starved *WT* cells incubated in water with NH_4^+ also had no effect in cell survival (Figure S4B). Furthermore, NH_4^+ -induced cell death was not observed in *atg8Δ* N-starved cells (Figure S4C). These results indicate that although NH_4^+ inhibits autophagy, autophagy inhibition is not the cause of the NH_4^+ -induced cell death observed in aa-starved cells.

PKA and TOR regulate the ammonium-induced reduction in the CLS of amino acid-starved yeast cells

CLS is under the control of both TOR, Sch9p and PKA signalling pathways [6]. The absence of autophagy inhibition as a causal factor in NH_4^+ -induced cell death led us to hypothesize that NH_4^+ toxicity might be mediated by PKA activation instead. Trehalase is a target of PKA regulation and its activity has been extensively used to monitor PKA activation [11]. As shown in Figure 5A, trehalase activity was much higher in aa-starved cells upon transfer to water with NH_4^+ than in the same cells without

Table 1. Values of Area under the survival curve (AUC) of strain BY4742 cultured in different medium composition.

		Cell culture or pre-incubation conditions							
		High-AA		Low-AA				aa-starved	N-starved
		$(NH_4)_2SO_4$ 0.5%	$(NH_4)_2SO_4$ 1%	$(NH_4)_2SO_4$ 0.01%	$(NH_4)_2SO_4$ 0.1%	$(NH_4)_2SO_4$ 0.5%	$(NH_4)_2SO_4$ 1%	$(NH_4)_2SO_4$ 0.5%	
Aging assay in:	Medium Sc	1205±13	1175±2	805±47	207±12	115±5	71±5	186±7	272±3
	Medium Sc pH7	1439±33	1202±5	1214±2	175±5	74±9	74±2	249±3	725±1
	H_2O	1375±87	1347±29	1390±5	916±25	745±42	446±44	723±10	679±2
	$(NH_4)_2SO_4$ 0.5%	923±77	951±5	706±23	385±20	271±13	133±12	188±10	588±16
	$(NH_4)_2SO_4$ 1%	535±2	552±102	n.d.	n.d.	120±10	90±7	n.d.	n.d.

n.d. – not determined. Cells were grown in SC media supplemented with low (Low-AA) or high (High-AA) concentrations of auxotrophy-complementing amino acid and with 0.01%; 0.1%; 0.5% or 1% $(NH_4)_2SO_4$ for 72 hours; or cells were grown in SC media until O.D. 1–1.5, harvested and resuspended in Nitrogen-starvation medium (N-) or in amino acid-starvation medium (aa-) for 24 hours. The aging assays were performed by resuspending cells from the different culture conditions in their respective exhausted medium, exhausted medium pH 7, or in 0.5 and 1% $(NH_4)_2SO_4$, pH 7.
doi:10.1371/journal.pone.0037090.t001

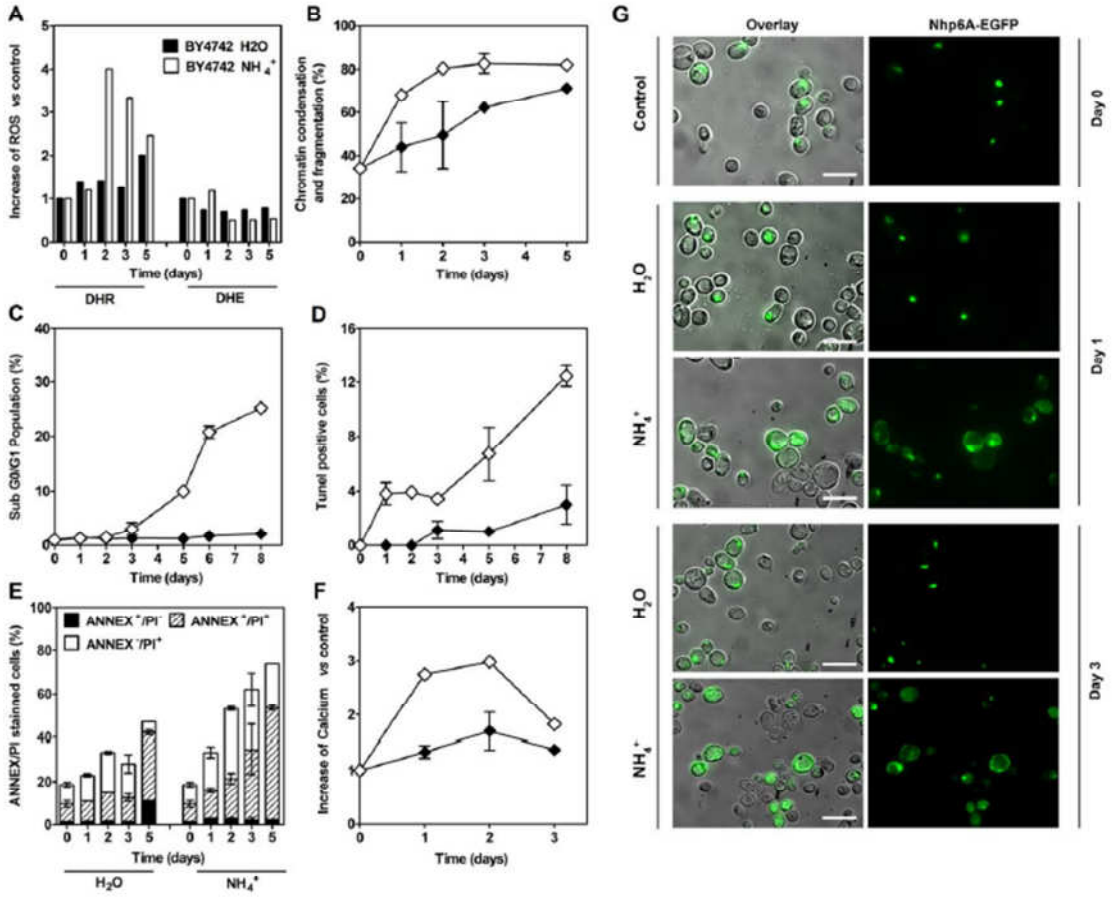

Figure 3. Ammonium-induced cell death was accompanied by an initial small increase of apoptotic cells followed by extensive necrosis. Cell death markers measurements in aa-starved cells of *S. cerevisiae*, upon transfer to: (◆) water (pH 7.0) or (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). (A) ROS accumulation, (B) chromatin condensation and fragmentation, (C) appearance of Sub-G0/G1 peak, (D) TUNEL staining, (E) Annexin/PI positive staining and (F) calcium accumulation. (G) Fluorescence microscopy of aa-starved cells (day 0, 1 and 3) expressing Nhp6A–EGFP, upon transfer to water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0). Scale bars, 10 μm. In all the cultures, starting cell density was about 3.8×10^7 cells/ml. Values are means ± SEM (n = 3). H_2O *vs* 0.5% $(NH_4)_2SO_4$: (A) $P<0.001$; (C) $P<0.001$; (D) $P<0.01$; (E) $P<0.001$; (F) $P<0.01$. Statistical analysis was performed by two-way ANOVA.
doi:10.1371/journal.pone.0037090.g003

NH_4^+ or in N-starved cells (negative control) under both conditions. In support of the hypothesis that activation of PKA increases sensitivity to NH_4^+, addition of cAMP increased cell death in the presence of NH_4^+ in N-starved cells and had no effect on aa-starved cells, which display high PKA activity in the absence of added cAMP (Figure 5B and 5C). In addition, deletion of *RAS2*, a regulator of PKA activity through the stimulation of cAMP production, caused a partial reversion of the NH_4^+ sensitivity phenotype of aa-starved cells (Figure 5E). The NH_4^+ permease Mep2 (and Mep1 to a lesser extent) function as sensors for NH_4^+-induced activation of PKA, whereas Mep3p, the other member of the family of NH_4^+ transporters, does not [28,37]. As shown in Figure 5E, the *mep2Δ* and *mep1Δ* strains exhibited a decrease in NH_4^+-induced death in aa-starved cells, although this decrease was significant only in *mep2Δ*. This is in agreement with the more predominant role of Mep2p in PKA signalling. In order to identify the specificity of the signalling process through PKA, we also tested the effects of deleting the genes that code for the three isoforms of the catalytic subunit of this kinase, *TPK1*, *TPK2* and *TPK3*. Only deletion of *TPK1* caused a significant reversion of the NH_4^+-induced decrease of the CLS, whereas no differences were detected for strains deficient in *TPK2* and *TPK3* (Figure 5F).

Sch9p is a protein kinase with high sequence homology to Tpk1, 2, 3 kinases and regulates cell metabolism in response to several nutritional signals, such as nitrogen and carbon source [38]. Sch9p shares many targets with PKA and TORC1, and different interactions between these pathways, either cooperating or antagonizing, have been described [15]. Data from Figure 5D show that *sch9Δ* aa-starved cells underwent increased cell death upon transfer to water plus NH_4^+ and that the lack of Sch9p reduced survival after cells were transferred to water. These results

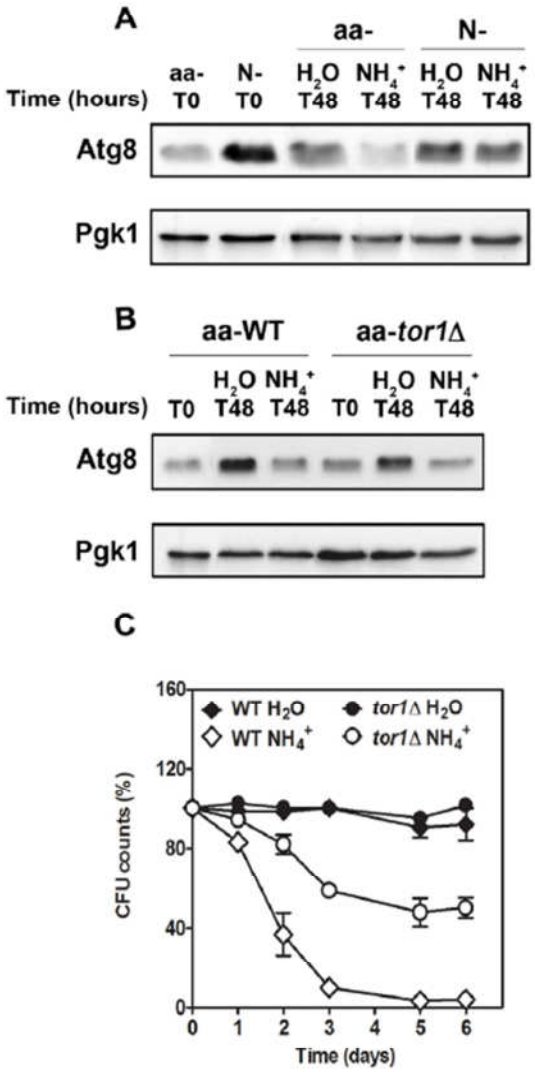

Figure 4. Ammonium inhibits autophagy induction in aa-starved cells of *S. cerevisiae*. Western-blot analysis of Atg8p levels present in: (A) wild-type (WT) aa-starved or N-starved cells, upon transfer to water or water with 0.5% $(NH_4)_2SO_4$; and in (B) WT and *tor1Δ* aa-starved cells, upon transfer to water or water with 0.5% $(NH_4)_2SO_4$. (C) Survival of aa-starved cells of wild-type and *tor1*Δ mutant, upon transfer to water or water with 0.5% $(NH_4)_2SO_4$. In all the cultures, starting cell density was about 3.8×10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n = 3–4). (C) $P<0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.
doi:10.1371/journal.pone.0037090.g004

suggest that pathways regulated by Sch9p are important for survival under these conditions

To evaluate the dependence of PKA activation on Sch9p, Tor1p, and Mep2p, trehalase activity was measured in aa-starved cells of the corresponding deletion mutants. Trehalase activity was similar in all strains before or after transfer to water. However, in the presence of NH_4^+, trehalase activity decreased in *tor1Δ* and in *sch9Δ* cells and was almost completely undetectable in the latter strain (Figure 5A). These results establish that NH_4^+ signalling to PKA requires Tor1p and Sch9p. However, the opposite cell death phenotypes of *sch9Δ* compared to *tor1Δ* and *tpk1Δ* cells observed in aa-starved cells in the presence of NH_4^+ suggest that the role of Sch9p in the process is essentially independent of the TOR-PKA pathway.

Hog1p is a kinase that regulates and is regulated by Sch9p and mediates stress response independently of PKA and TOR pathways [39]. To assess whether Hog1p might play a role in resistance to the toxic effects of NH_4^+ mediated by Sch9p, we examined the effects of NH_4^+ in a *hog1Δ* strain. Like *sch9Δ* cells, *hog1Δ* strains were more sensitive to the toxic effects of NH_4^+, which suggests that Sch9p may be signaling Hog1p to mediate increased resistance.

Metabolism of NH_4^+ is not required for NH_4^+-induced cell death

The role of Mep2p in signaling PKA activation in response to NH_4^+ in nitrogen starvation medium is not dependent on the metabolism of NH_4^+ [28]. Therefore, we next asked whether NH_4^+ toxicity that leads to a reduction in CLS under our experimental conditions might be signaled directly by NH_4^+ or perhaps requires that it be metabolized. In yeasts, the first step of NH_4^+ assimilation is mediated by NADPH-dependent glutamate dehydrogenase, which converts α-ketoglutarate to glutamate, which can be further metabolized to glutamine by glutamine synthetase. Glutamine synthetase activity was higher in N-starved cells than in aa-starved cells, indicating that the activity of this enzyme was not related to the higher toxicity of NH_4^+ (Table S1). We also tested the effect of NH_4^+ in both aa-starved and N-starved cells in the presence of the glutamine synthetase inhibitor methionine sulfoximine. No significant differences in loss of cell viability were observed (Figure S6A and S6B), further supporting the hypothesis that the toxic effect of NH_4^+ does not require that it be metabolized. Glutamate dehydrogenase activity at T_0 was higher in N-starved cells than in aa-starved cells, but incubation in water with or without NH_4^+ led to a decrease in its activity (Figure 6A). In contrast, glutamate dehydrogenase activity increased approximately 3-fold in aa-starved cells incubated in the presence of NH_4^+. We asked whether α-ketoglutarate depletion or glutamate accumulation, which might result from the higher glutamate dehydrogenase activity, could be the cause of NH_4^+ toxicity. Adding α-ketoglutarate to the medium did not alter the toxic effects of NH_4^+ (Figure S6C), whereas adding glutamate resulted in more rapid loss in cell viability, even in the absence of NH_4^+ (Figure 6B). Furthermore, the non-metabolizable NH_4^+ analogue methylamine also induced cell death in aa- but not N-starved cells (Figure S6D). In agreement with these results, the NH_4^+ toxicity observed in SC media cultures (Fig. 1) was also not associated with a significant NH_4^+metabolization, as depicted from the levels of $(NH_4)_2SO_4$ along time (Fig. S1A).

Taken together, these results suggest that although glutamate could play a role in NH_4^+-induced cell death to some extent, NH_4^+-induced shortening of CLS does not appear to require that it be metabolized.

Discussion

Our studies demonstrate that at high concentrations, NH_4^+, which is a commonly employed source of nitrogen in laboratory yeast cultures, induces cell death in association with a reduction in CLS. The toxic effects of NH_4^+ correlate with NH_4^+ concentration

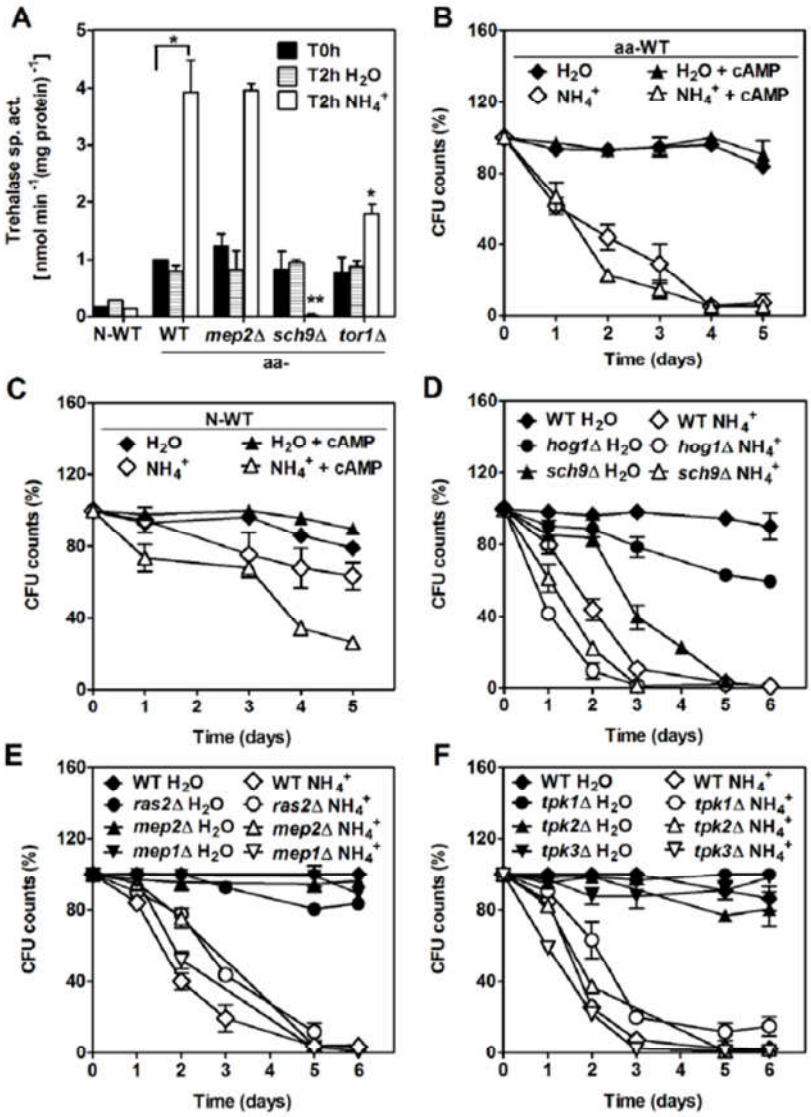

Figure 5. Ammonium reduces CLS of *S. cerevisiae* through the regulation of both PKA and Sch9 activities. (A) Trehalase activity of wild-type (WT) N-starved cells and WT and mutant (*mep2Δ*, *sch9Δ* and *tor1Δ*) aa-starved cells, before transferred to water (T0h) and after 2 hours in water (T2h H_2O) or water with 0.5% $(NH_4)_2SO_4$ (T2h NH_4^+). Survival of (B) wild-type aa-starved cells or (C) N-starved cells, after transfer to: (◆) water (pH 7.0); (◇) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0); (▲) water (pH 7.0) supplemented with cAMP (4 mM); (△) water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) supplemented with cAMP (4 mM). Survival in water (pH 7.0) or water with 0.5% $(NH_4)_2SO_4$ (pH 7.0) of aa-starved cells of: (D) WT, *sch9Δ* and *hog1Δ*; (E) WT, *mep1Δ*, *mep2Δ* and *ras2Δ*; (F) WT, *tpkΔ* mutants (*tpk1Δ*, *tpk2Δ* or *tpk3Δ*). In all the cultures, starting cell density was about 3.8×10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n = 3–4). (A) *$P<0.05$ (WT H_2O *vs* WT 0.5% $(NH_4)_2SO_4$), (WT 0.5% $(NH_4)_2SO_4$ *vs tor1Δ* 0.5% $(NH_4)_2SO_4$); **$P<0.01$ (WT 0.5% $(NH_4)_2SO_4$ *vs sch9Δ* 0.5% $(NH_4)_2SO_4$); (D) $P<0.05$ (WT 0.5% $(NH_4)_2SO_4$ *vs hog1Δ* 0.5% $(NH_4)_2SO_4$), $P<0.001$ (WT H_2O *vs sch9Δ* H_2O); (E) $P<0.05$ (WT 0.5% $(NH_4)_2SO_4$ *vs ras2Δ* 0.5% $(NH_4)_2SO_4$); $P<0.01$ (WT 0.5% $(NH_4)_2SO_4$ *vs mep2Δ* 0.5% $(NH_4)_2SO_4$); $P<0.05$ (WT 0.5% $(NH_4)_2SO_4$ *vs mep1Δ* 0.5% $(NH_4)_2SO_4$); (F) $P<0.01$ (WT 0.5% $(NH_4)_2SO_4$ *vs tpk1Δ* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.
doi:10.1371/journal.pone.0037090.g005

in the culture medium and were enhanced in cells starved for auxotrophy-complementing amino acids. Addition of NH_4^+ to cultures after they were transferred to water reduced cell survival, indicating that NH_4^+ alone could also induce loss of cell viability as observed in culture media. Overall the results suggest NH_4^+ is a factor accounting for the loss of cell viability in aging cells. Although some of the toxic effects of NH_4^+ were accompanied by markers for apoptosis, NH_4^+ -induced cell death was predomi-

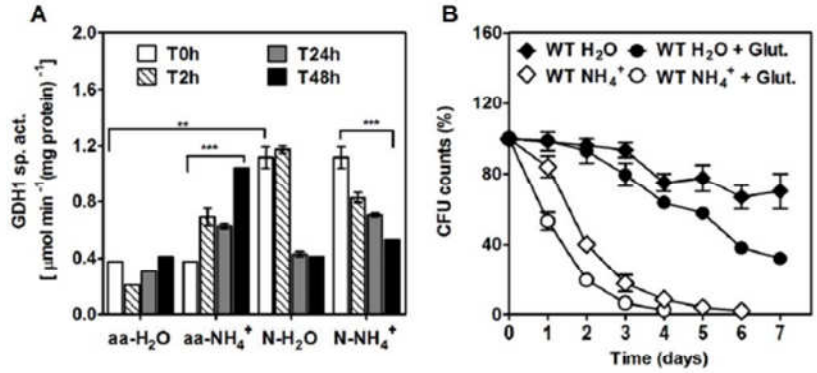

Figure 6. Ammonium-induced loss of cell viability of *S. cerevisiae* does not depend on its metabolization. (A) Glutamate dehydrogenase (GDH1) activity of aa-starved (aa-H_2O and aa-NH_4^+) and N-starved cells (N-H_2O and N-NH_4^+), before transferred to water (T0h) and after 2, 24 and 48 hours in water or water with 0.5% $(NH_4)_2SO_4$. (B) Survival of wild-type aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented or not with glutamate (5 mg/ml). In all the cultures, starting cell density was about 3.8×10^7 cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n = 3–4). (A) ***$P<0.001$; **$P<0.01$ (B) $P<0.05$ (H_2O *vs* H_2O+Glut.). Statistical analysis was performed by two-way ANOVA.
doi:10.1371/journal.pone.0037090.g006

nantly necrotic at later time points. Our data suggest that NH_4^+ causes an initial apoptotic cell death followed by a fast secondary necrosis. Necrosis due to ATP depletion has been reported in other cell death scenarios, namely in tumor cells under metabolic stress [40]. This appears to be the case in NH_4^+-induced necrosis, since ATP depletion was observed in cells incubated in water with NH_4^+ (Figure S3B), which might block ATP-dependent apoptosis and thus trigger necrosis. The results obtained with the deletion mutant *rim13Δ* point to a protective function of the protease calpain in this cell death process.

As discussed in the Introduction, cells in G0 acquire a variety of characteristics including induction of autophagy [8,12]. Previous studies showed that cells starved for auxotrophic amino acid markers in otherwise complete medium fail to properly arrest in G0 [41]. In accordance, aa-starved cells in our study also do not seem arrested in G0 (indicated by a failure to induce autophagy). It should be noted that this failure to induce autophagy by aa-starved cells was sustained when cells were transferred to water containing NH_4^+ in the absence of other nutrients. This is in contrast with that observed in G0 arrested N-starved cells transferred to water where NH_4^+ could not activate PKA or inhibit autophagy. Although autophagy was inhibited by NH_4^+ in aa-starved cells, inhibition of autophagy by deletion of *ATG8* did not induce the NH_4^+ sensitivity phenotype in N-starved cells, suggesting that autophagy inhibition is not responsible for the loss of cell viability and shorter CLS induced by NH_4. We also assessed whether activation of PKA could be inducing replication stress, a mechanism responsible for cell aging under different conditions [42]. This could be the case, at least to some extent, since there was a slight increase in the number of budded cells (evaluated by bright field microscopy) for aa-starved (16%) conditions relative to the control N-starved cells (8%).

In contrast to what has been described for aging cells that reach stationary phase due to carbon limitation [43], we observed that autophagy mutants did not exhibit increased cell death after they were transferred to water, indicating that autophagy is not a key player in cell survival in water when the cells were previously starved for amino acids or nitrogen. It was recently shown that *ATG* genes are important for removing ROS and for maintaining mtDNA and mitochondrial function [44]. This may explain the lack of dependence of cell survival on autophagy in our experimental conditions, as the production of ROS was relatively low. Hence, the cell physiological state resulting from different culture conditions influences not only life span extension [2], but also the cellular processes essential for its regulation.

In yeasts, the TOR, Sch9p and PKA pathways are key players in the regulation of CLS [6]. In our study, activation of PKA correlates with sensitivity to NH_4^+, which is partially suppressed by deletion of *RAS2*, indicating the RAS/Cyr1/PKA pathway is involved in this process. Partial, but not complete, suppression of these effects when *RAS2* is deleted suggests either that the second RAS isoform (*RAS1*) also participates in NH_4^+-induced PKA activation or the existence of two pathways responsible for NH_4^+ toxicity, one that depends on RAS/Cyr1/PKA and one that is independent of this pathway. In nitrogen starvation medium, addition of NH_4^+ directly signals PKA activation through Mep2p and does not depend on its metabolization [28]. Our results show that Mep2p is involved in NH_4^+-induced death but does not appear to have a major role in PKA activation. Still, although glutamate could somewhat mediate the effect of NH_4^+, CLS shortening also seemed to be directly signalled by NH_4^+, as it was not dependent on its metabolization to either glutamate or glutamine.

The deletion of *TPK1*, but not of *TPK2* or *TPK3*, encoding the other two PKA isoforms, significantly reverted the NH_4^+-induced death and shorter CLS. These results suggest that different programmed cell death processes can be regulated by distinct PKA isoforms, since Tpk3p has been reported to regulate apoptosis induced by actin stabilization [45]. Our data are also in agreement with previous results showing that CLS extension of glucose-growth limited stationary phase cells depends on PKA inactivation [46]. Our results indicate that PKA inactivation cannot extend cell survival time in the absence of Sch9p, since we observed that *SCH9* deletion abolishes PKA activation in response to NH_4^+, but does not rescue the shortening in CLS induced by NH_4^+. Furthermore, the phenotype of aa-starved *sch9Δ* cells in the presence of NH_4^+ was the opposite of that of *tor1Δ* and *tpk1Δ*, suggesting that the role of Sch9p in the process is essentially independent of the TOR-PKA pathway mediated by a TORC1-Sch9 effector branch. Instead, the two pathways likely regulate

their downstream targets that are involved in NH_4^+-induced cell death in an opposing manner. Consistent with this possibility, it was reported that Sch9p positively regulates many stress-response genes and genes involved in mitochondrial function, whereas the same classes of genes are inhibited by the TOR1C pathway [15]. Data suggest that Sch9p may mediate survival in response to NH_4^+ through activation of Hog1p, the yeast closest homolog to p-38 and c-JNK of mammalian cells [47]. Previous reports have shown that *sch9Δ* yeast cells exhibit a longer CLS compared to wild type cells, when aging in SC medium or after transfer from this medium to water [46,48]. Differences in strain background and/or in culture conditions may account for the discrepancy in results [46,48–50]. Supporting this explanation it was also previously reported that *SCH9* deletion shortened the CLS survival of S288c-based strains (as is the case of BY4742 strain used in the present work) pregrown on glycerol [1].

In conclusion, here we have shown that NH_4^+ induces cell death in aging yeast in association with a reduction in CLS, both of which are positively correlated with NH_4^+ concentration in the culture medium. Furthermore, these effects are enhanced in cells starved for auxotrophy-complementing amino acids. As for the mechanism involved (Figure 7), the results indicate that in aa-starved cells NH_4^+ activates PKA through both RAS and TOR/Sch9p signalling cascades and leads to cell death increase with predominant necrotic features. The mediation of NH_4^+ effects seems to involve the NH_4^+ permeases Mep2 and (to a lesser extent) Mep1 as sensors. Sch9p is also mediating survival in response to NH_4^+ possibly through activation of Hog1p. NH_4^+ action on both pathways culminates in the shortening of CLS.

As discussed in the introduction, NH_4^+ is toxic for mammals, and NH_4^+-induced cell death is involved in different human disorders that are accompanied by hyperammonemia, such as hepatic encephalopathy [21]. Here we extensively characterized for the first time a cell death process induced by NH_4^+ in yeast cells. This process shares common features with NH_4^+-induced cell death in brain cells. A better understanding of NH_4^+-induced cell death in the yeast cell model can help clarify controversial issues on NH_4^+ toxicity associated to hyperamonemia that are not easy to examine in more complex models. Our results show that the effect of NH_4^+ is not due to different levels of NH_4^+ metabolization, an open question for brain cells, but relies on the over-activation of PKA and the TOR pathway and inhibition of Sch9p (yeast closest homolog of mammalian Akt and S6K). On the other hand, the mitogen activated protein kinase (MAPK) Hog1p was associated with higher cell viability in the presence of NH_4^+ similarly to what was found for its human homolog p38 that mediates endogenous cell protection in response to ammonium in astrocytes [51]. Also, we observed that NH_4^+ toxicity is higher in non-arrested cells, which is consistent with the observation that hyperammonemia presents with much more severe consequences in the developing brain of newborns or infants than in adulthood. Furthermore, our data link NH_4^+ toxicity to amino acid limitation, a situation that can also be present in hyperammonemic patients, who are often on dietary protein restriction [52]. Further experiments will be necessary to establish whether over-activation of TOR and PKA pathways and inhibition of Sch9p is a widely conserved mechanism in NH_4^+ toxicity and induction of cell death. We believe that our model can be useful in the elucidation of conserved mechanisms and pathways of NH_4^+-induced cell death and in identification of therapeutic targets for diseases associated with hyperammonemia. Deprivation of essential amino acids has been employed as a strategy in cancer therapy, but resistance has often been found. Our results establishing that NH_4^+ can stimulate cell death in amino acid-deprived cells and suggests that *S. cerevisiae* might serve as useful model for the identification of signaling pathways for this disease. Furthermore, our finding that NH_4^+ decreases cell survival during aging through the regulation of the evolutionary conserved pathways PKA and TOR also enriches our understanding of longevity regulation in multicellular organisms.

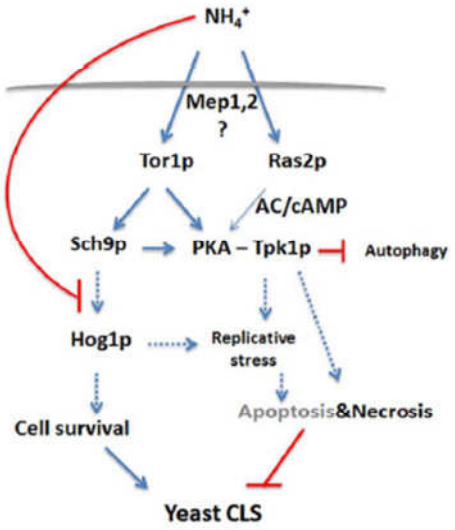

Figure 7. Proposed mechanism for the regulation of cell death associated to CLS shortening induced by ammonium in amino acid-starved yeast cells. NH_4^+ activates PKA through both RAS and TOR/Sch9p and leads to cell death increase with predominant necrotic features associated to ATP depletion. Sch9p is mediating survival in response to NH_4^+ possibly through activation of Hog1p.
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Materials and Methods

Strains and growth conditions

Saccharomyces cerevisiae strain BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (EUROSCARF, Frankfurt, Germany) and the respective knockouts in *YAC1*, *AIF1*, *RIM13*, *RAS2*, *CPR3*, *ATG8*, *SCH9*, *MEP1*, *MEP2*, *TPK1*, *TPK2*, *TPK3* and *TOR1* genes, were used. For experiments with stationary phase cells with or without restriction of auxotrophy-complementing amino acids, cells were cultured at 26°C, 150 rpm, for 72 hours, in defined minimal medium (SC medium) containing 0.17% yeast nitrogen base without amino acids and without ammonium sulphate (Difco, BD), 2% D-glucose; supplemented with 0.01%, 0.1%, 0.5% or 1% ammonium sulphate, and with low (10 mg/l histidine, 10 mg/l lysine, 60 mg/l leucine and 100 mg/l uracil) or high (50 mg/l histidine, 50 mg/l lysine, 300 mg/l leucine and 100 mg/l uracil) concentrations of essential amino acids. After 72 hours, cells were collected by centrifugation and: A) resuspended in growth medium (exhausted medium without adjusting pH - pH 2.9) with a cell density of about 3.8×10^7 cells/ml, B) resuspended in growth medium (exhausted medium) with a cell density of about 3.8×10^7 cells/ml, with pH adjusted to 7.0.; C) resuspended in water (pH 7.0), after being washed three times, at cell density of about 3.8×10^7 cells/ml; D) resuspended in water with ammonium sulphate (0.5% or 1%, pH 7.0.), after being washed three times, at cell density of about 3.8×10^7 cells/ml. Viability of stationary 3 day

old cultures was considered to be 100% of survival and this was considered day 0 of the experiment. pH 7.0 was maintained throughout the experiment in cultures with adjusted pH. For experiments with aa- and N-starved cells, cells were first cultured at 26°C and 150 rpm, in the defined minimal medium described above, supplemented with 0.5% ammonium sulphate, appropriate amino acids and base (50 mg/l histidine, 50 mg/l lysine, 300 mg/l leucine and 100 mg/l uracil) and 2% D-glucose, to exponential phase (OD_{600} = 1.0–1.5). These cells were harvested and resuspended in nitrogen-starvation medium (N-) containing 4% glucose and 0.17% yeast nitrogen base without amino acids and ammonium sulphate, or in amino acid-starvation medium (aa-) containing the same components as N-starvation medium plus 0.5% ammonium sulphate. After 24 hours, cells were collected by centrifugation and: A) resuspended in starvation medium (N- or aa-) with a cell density of about 3.8×10^7 cells/ml, without adjusting pH (pH 2.7); B) resuspended in starvation medium (N- or aa-) with a cell density of about 3.8×10^7 cells/ml, with pH adjusted to 7.0; C) resuspended in water (pH 7.0), after being washed three times, at cell density of about 3.8×10^7 cells/ml; D) resuspended in water with ammonium sulphate (0.5%, pH 7.0.), after being washed three times, at cell density of about 3.8×10^7 cells/ml. Viability of 24 hours starved cultures was considered to be 100% of survival and this was considered day 0 of the experiment. pH 7.0 was maintained throughout the experiment in cultures with adjusted pH. Cell viability was assessed by Colony Forming Units (CFU) at day 0 (100% viability) and in subsequent days, as indicated, of culture aliquots incubated for 2 days at 30°C on YEPD agar plates. BY4742 strain was transformed with plasmids pUG35 and pUG35- *NHP6A-EGFP* [17], kindly provided by Dr. Frank Madeo (University of Gratz, Austria), and was cultured as described above for aa-starved cells, in medium lacking uracil. The methodology of aging experiments with stationary phase cells and with amino acid (aa)- and nitrogen(N)-starved cells is schematically represented in Figure S1.

Ammonium and ATP Determination

Ammonium in the culture media was quantified by Dr. José Coutinho (University of Trás-os-Montes e Alto Douro, Portugal) as previously described [53].

ATP measurements were performed according to [54]. Briefly, cells were collected by centrifugation and the pellet was frozen with liquid nitrogen and stored at −80°C. For the ATP assay, the pellet was mixed with 200 µl of 5% TCA and vortexed for one minute, twice, with one minute interval on ice. This mix was centrifuged for one minute, at 4°C, and 10 µl of the supernatant were added to 990 µl of reaction buffer (25 mM HEPES, 2 mM EDTA, pH 7.75). 100 µl of this mixture was added to 100 µl of Enliten Luciferin/Luciferase Reagent (Promega) and luminescence was measured on a ThermoScientific Fluoroskan Ascent FL.

Measurements of cell death markers

For the detection of chromatin changes, cells were stained with 4,6-diamido-2-phenyl-indole (DAPI, Sigma) according to [55]. DNA strand breaks were assessed by TUNEL with the 'In Situ Cell Death Detection Kit, Fluorescein' (Roche Applied Science) as described previously [55]. In both assays, and also for the nuclear release of the necrotic marker Nhp6Ap–EGFP, cells were visualized by epifluorescence in a Leica Microsystems DM-5000B microscope, at least 300 cells of three independent experiments being evaluated, with appropriate filter settings using a 100×/1.3 oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. To measure DNA content, cells were stained with SYBR Green I as described [56] and staining was assessed by flow cytometry. Plasma membrane integrity was assessed by incubating cells with 5 mg ml^{-1} propidium iodide (PI) (Molecular Probes, Eugene, OR) for 10 minutes at room temperature followed by flow cytometry measurements of PI-stained cells. Intracellular reactive oxygen species were detected by dihydrorhodamine (DHR)-123 staining or dihydroethidium (DHE) (Molecular Probes). For DHR, cells were incubated with 15 mg/mL of DHR-123 for 90 min at 30°C in the dark, washed in PBS and evaluated by flow cytometry. For DHE, cells were incubated with 5 µM and after incubation for 10 min at 30°C cells were washed once with PBS and evaluated by flow cytometry. Phosphatidylserine exposure was detected by FITC Annexin-V (BD Pharmingen) as described previously [55]. Briefly, cell walls were digested with 3% (v/v) glusulase (NEE-154 Glusulase; Perkinelmer) and 7 U/ml lyticase (Sigma) for 40 minutes, at 28°C. For intracellular calcium measurements, cells previously washed with PBS were stained with 10 µM FLuo3 AM (Molecular Probes, Eugene, OR) for 2 hours at 30°C in the dark, subsequently washed in PBS and assessed by flow cytometry. Flow cytometry analysis of the above experiments was performed in an Epics® XL™ (Beckman Coulter) flow cytometer, equipped with an argon ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488-nm blocking filter, a 550-nmlong-pass dichroic and a525-nm bandpass. Red fluorescence was collected through a 488-nm blocking filter, a 590-nmlong-pass dichroic and a620-nm bandpass. Thirty thousand cells per sample were analyzed. Positive controls for apoptosis involved treatment of cells with 160 mM acetic acid for 200 minutes, at pH 3 and 3 mM H_2O_2 at pH 3. For the necrotic marker Nhp6Ap–EGFP, no nuclear release was observed in the presence of 3 mM H_2O_2.

Treatments

Methionine sulfoximine (MSX,Sigma), an irreversible inhibitor of glutamine synthetase, was dissolved in sterile water at a concentration of 100 mM and stored at 4°C. MSX was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 1 mM. Wortmannin (Sigma), a PI3K inhibitor, was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 6 µM or 23 µM. Glutamate was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 5 mg/ml. Adenosine 3′,5′-cyclic monophosphate (cAMP, Sigma) was added to aa-starvation or N-starvation medium or to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 4 mM. α-Ketoglutaric acid potassium salt (Sigma) was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0), at the concentration of 5 mg/ml. Cyclosporin A (Sigma) was added to water (pH 7.0), and water with ammonium sulphate (0.5%, pH 7.0.), at the concentration of 120 µg/ml.

Western Blot analysis

Western blot analysis was performed according to [57]. For Atg8p and Pgk1p detection, rabbit polyclonal anti-Aut7 (1:200; Santa Cruz Biotech) and mouse monoclonal anti-PGK1 (1:5000; Molecular Probes) were used, respectively, followed by Peroxidase-AffiniPure Goat Anti-Rabbit IgG (1:10000; Jackson ImmunoResearch).

Enzyme assays

Glutamine synthetase (Gs) assay was performed according to [58]. Glutamate dehydrogenase activity was determined according

to [59]. Briefly, cell extracts were prepared by adding to the cell pellet a roughly equal volume of 0.5 mm diameter glass beads in the presence of 0.1 M potassium phosphate buffer (pH 6.0), followed by vigorous mixing during 1 minute intervals interspersed with periods of cooling in ice. The NADP-dependent GDH activity was determined by following the disappearance of NADPH at 340 nm. Trehalase activity was determined according to [60]. Briefly, crude enzyme extracts were obtained by ressuspending the cell pellet in ice-cold 50 mM MES/KOH buffer (pH 7.0) containing 50 µM $CaCl_2$, and adding a roughly equal volume of 0.5 mm diameter glass, followed by vigorous mixing during 1 minute intervals interspersed with periods of cooling in ice. The extracts were then dialyzed overnight at 4°C in a dialysis cellulose membrane (Cellu Sep H1, Orange). The dialyzed extract was then used to assess trehalase activity by measuring the liberated glucose with glucose oxidase assay (GOD, Roche).

Supporting Information

Figure S1 Ammonium levels in medium during culture of *S. cerevisiae* with insufficient supply of amino acids, and cell death induced by NH_4OH or by $(NH_4)_2SO_4$ in the presence of increased potassium concentration. (A) Quantification of $(NH_4)_2SO_4$ in SC medium supplemented with low concentrations of auxotrophy-complementing amino acids and 0.5% $(NH_4)_2SO_4$, during culture of wild-type cells; day −3 represents the day of culture inoculation and day zero represents the beginning of aging experiments. (B) Survival of wild-type (WT) aa-starved cells, in water or water with 0.5% NH_4OH. (C) Survival of wild-type (WT) aa-starved cells, in water, water with 0.5% $(NH_4)_2SO_4$ and water with 0.5% $(NH_4)_2SO_4$ supplemented with 13 mM K_2SO_4. Values are means ± SEM (n = 3).
(TIF)

Figure S2 Scheme of the methodology used. (A) experiments with the stationary phase cells and (B) experiments with aa- and N-starved cells.
(TIF)

Figure S3 Effect of ammonium on the cell cycle and ATP content. (A) Cell cycle histograms of aa-starved and N-starved *S. cerevisiae* wild-type cells at day 0 and day 5 upon transfer to water or water with 0.5% $(NH_4)_2SO_4$, after a 24 hour period in starvation (aa- and N-) media. (B) ATP content of aa-starved cells (day 0, 1, 2 and 3) upon transfer to water or water with 0.5% $(NH_4)_2SO_4$. Values are means ± SEM (n = 3). (B) ***$P<0.001$ (T0 *vs* T1,2 and 3).
(TIF)

Figure S4 Effect of *ATG8* deletion and of the inhibitors wortmannin and cycloheximide in NH_4^+ - induced cell death in *S. cerevisiae*. Survival of wild-type (WT) and *atg8*Δ mutant (A) aa-starved or (C) N-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. Survival of WT aa-starved cells, in water or water with 0.5% $(NH_4^+)_2SO_4$, supplemented with (B) wortmannin (WN) or (D) cycloheximide (0.01%). Values are means ± SEM (n = 3). (A); (B) and (D) $P<0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.
(TIF)

Figure S5 Loss of cell viability induced by NH_4^+ in aa-starved cells in water, of *S. cerevisiae* wild-type (WT) and mutants deleted in the genes coding for the yeast metacaspase (Yca1), the apoptosis inducing factor (Aif1), mitochondrial cyclophylin (Cpr3) and calpain (Rim13). Survival of (A) WT and *yca1Δ*, (B) WT and *aif12Δ*, (C) WT and *rim13Δ* and (D) WT and *cpr3Δ* aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$. (E) Survival of WT aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented or not with cyclosporine A (CsA) (120 µg/ml). In all the cultures, starting cell density was about 3.8×10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n = 3–4). (A), (B), (D) and (E) $P<0.001$ (H_2O *vs* 0.5% $(NH_4^+)_2SO_4$); (C) $P<0.001$ (WT 0.5% $(NH_4)_2SO_4$ *vs* *rim13Δ* 0.5% $(NH_4^+)_2SO_4$). Statistical analysis was performed by two-way ANOVA.
(TIF)

Figure S6 Metabolism of NH_4^+ is not required for NH_4^+-induced cell death in *S. cerevisiae*. Survival of wild-type (WT) aa-starved cells (A) or N-starved cells (B), in water or water with 0.5% $(NH_4)_2SO_4$, supplemented with methionine sulfoximine (MSX) (1 mM). (C) Survival of WT aa-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented with α-ketoglutarate (α-KG) (5 mg/ml). (D) Survival of WT aa-starved or N-starved cells, in water or water with 0.5% $(NH_4)_2SO_4$, supplemented with methylamine (MA) (30 mM). In all the cultures, starting cell density was about 3.8×10^7cells/ml and the initial pH was adjusted to 7.0. Values are means ± SEM (n = 3–4). (A), (C) and (D) $P<0.001$ (H_2O *vs* 0.5% $(NH_4)_2SO_4$). Statistical analysis was performed by two-way ANOVA.
(TIF)

Table S1 Glutamine synthetase (GS) activity of aa- and N-starved cells of *S. cerevisiae* before (T0) and after transfer to water or water with 0.5% $(NH_4)_2SO_4$.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CL MJS. Performed the experiments: JS MJS CL. Analyzed the data: JS MJS CL. Wrote the paper: JS MJS CL.

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Review Article

Growth Culture Conditions and Nutrient Signaling Modulating Yeast Chronological Longevity

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The manipulation of nutrient-signaling pathways in yeast has uncovered the impact of environmental growth conditions in longevity. Studies using calorie restriction show that reducing glucose concentration of the culture media is sufficient to increase replicative and chronological lifespan (CLS). Other components of the culture media and factors such as the products of fermentation have also been implicated in the regulation of CLS. Acidification of the culture media mainly due to acetic acid and other organic acids production negatively impacts CLS. Ethanol is another fermentative metabolite capable of inducing CLS reduction in aged cells by yet unknown mechanisms. Recently, ammonium was reported to induce cell death associated with shortening of CLS. This effect is correlated to the concentration of NH_4^+ added to the culture medium and is particularly evident in cells starved for auxotrophy-complementing amino acids. Studies on the nutrient-signaling pathways regulating yeast aging had a significant impact on aging-related research, providing key insights into mechanisms that modulate aging and establishing the yeast as a powerful system to extend knowledge on longevity regulation in multicellular organisms.

1. Cellular Pathways Modulating Aging

Aging is common to most living organisms ranging from bacteria, a unicellular prokaryotic organism, to multicellular eukaryotic organisms like humans. It is a complex biological process that involves accumulation of damage at diverse components of the organism leading ultimately to the loss of function and demise [1–3]. In the aging process, cellular activities compromised are modulated by a network of nutrient and energy sensing signaling pathways that are highly conserved among organisms. These pathways include the insulin/insulin-like growth factor 1 (Ins/IGF-1), the protein kinase/target of rapamycin (TOR), and adenylate cyclase/protein kinase A (AC/PKA) pathways [4]. Pioneering studies using mutations in key genes of these pathways have shown an increase by threefold or more in the lifespan of model organisms like yeast [5, 6], fruit flies [7], worms [8, 9], and mice [10, 11]. Many of these mutations which extend lifespan decrease the activity of the nutrient-signaling pathways mimicking a starvation state during which oxidative stress responses are induced, reducing the levels of reactive oxygen species (ROS) and oxidative damage to macromolecules [12]. Accordingly, it has been shown in different aging models (yeast, flies, worms, fish, rodents, and rhesus monkeys) that reducing growth factors/nutrients intake has profound positive effects in extension of lifespan and also improves overall health by delaying or reducing age-related diseases in mammals including diabetes, cancer and cardiovascular diseases (reviewed in [4]). One of the first models to implicate growth- /nutrient-sensing signaling with longevity was the nematode worm *Caenorhabditis elegans*. In this model it was shown that the recessive mutation in *age-1* coding for phosphatidylinositol 3-Kinase (PI3K) extends lifespan significantly [8]. Also in *C. elegans*, the insulin/IGF-1 pathway was linked to longevity by the discovery that mutating the gene coding for an insulin/IGF-1 receptor ortholog,

DAF-2, doubled its lifespan [9]. This lifespan extension was dependent on the reduction of activity of Daf-2 and consequently of its downstream effector PI3K (encoded by *age-1*), and the subsequent activation of Daf-16, a Forkhead FoxO family transcription factor (FOXO), which regulates several genes involved in stress response, antimicrobial activity, and detoxification of xenobiotics and free radicals [4, 9, 13]. Another pathway involved in longevity regulation in *C. elegans* is the conserved TOR-S6K (Ribosomal S6 protein Kinase) pathway. This pathway interacts with the insulin/IGF-1 pathway converging on the worm ortholog of regulatory associated protein of mTOR, Daf-15, to regulate larval development, metabolism, and longevity [14] and so downregulation of its activity results in extended lifespan [4].

The fruit fly *Drosophila melanogaster* is a more complex model, allowing studies based on sex differences. As in *C. elegans*, reducing the activity of the insulin/IGF-1 pathway mediates cellular protection mechanisms and the extension of lifespan in this organism. Mutations in the insulin-like receptor favour the extension of lifespan yielding dwarf sterile flies with females showing up to 85% extension of adult longevity [15]. Downregulation of the TOR pathway in flies, similarly to *C. elegans*, was shown to increase lifespan when inactivated pharmacologically with rapamycin or with overexpression of dominant-negative forms of S6K or TSC1 or TSC2, which encode negative regulators of TOR [16, 17].

Identical outcomes for genetic or pharmacologic manipulation of insulin/IGF-1 and TOR pathways and for dietary restriction regimes were observed in *D. melanogaster* and *C. elegans*, as well as in yeasts, establishing the evolutionary conserved roles of these pathways in determining lifespan and implicating them as mediators of the protective effects of dietary restriction in different species [4, 18].

In mammals, hormones of the endocrine system, the growth hormone, insulin-like growth factor-1 (IGF-1), and insulin pathways are key players in the hormonal control of aging in association with an increase of antioxidant defenses and increased stress resistance (reviewed in [19]). Deficiency in levels of circulating growth hormone has been shown to enhance antioxidant defenses and stress resistance, reduce tumor burden, and to increase insulin sensitivity (reviewed in [20]). Enhanced insulin sensitivity is a common feature of long-living mutant mice and in humans, studies of centenarian populations strongly correlates this increase in insulin sensitivity with longevity [21, 22]. IGF-1 and insulin also modulate TOR activity through Akt kinase which is a downstream effector of the insulin/IGF-1 pathway [23], and inhibition of mTOR pathway by rapamycin [24] or deletion of its downstream effector S6K, increases mice lifespan [25]. The lifespan extension due to the deletion of S6K was accompanied by slower progression of age-related pathologies and in particular slower loss of insulin sensitivity [25].

Another pathway involved in longevity regulation is the AC/PKA pathway that is conserved from yeast to mammals. Downregulation of the Ras/AC/PKA pathway was first shown in yeast to have a major effect on lifespan extension [4, 6, 26]. Only recently, studies correlating AC/PKA pathway with aging and age-related diseases started to emerge in mammals. Deletion of the mouse AC type 5, which mediates PKA activity by modulating cAMP levels, was reported to significantly increase lifespan, as it does in yeast [27], and improve cardiac stress resistance [28]. Likewise as described for yeast [26], deletions of PKA subunits in mice have recently been shown to increase lifespan while protecting against age-related deleterious changes such as weight gain, hypertrophic liver, and cardiac dysfunction [29]. Although the subunits deleted are the regulatory subunits (RIIβ) and not the catalytic subunits like in yeast (TPK), loss of RIIβ in mice causes a concomitant and compensatory decrease in catalytic subunits showing a mechanistic association between loss of these subunits and lifespan extension [29]. This converging result in such divergent models suggests a highly conserved role for PKA in longevity and opens the possibility for new therapeutic targets for aging and obesity.

In mammals the Ras proteins do not directly signal to PKA through AC [30] as it occurs in yeast [31]; however, a recent study reported that homozygous deletion of Ras-GRF1 promotes both median and maximum longevity in mice [32]. Ras-GRF1 is a guanine nucleotide exchange factor (GEF) responsible for activating Ras by favouring its GTP-bound state [33], suggesting that the cause of longevity extension of the Ras-GRF1 deletion could be the reduction of Ras activity [34]. Therefore, the Ras pathway appears as a conserved pathway in the aging process from yeast to mammals [34].

Yeast has emerged as a highly exploited model to study the environmental and genetic factors affecting longevity. In particular, the genetic tools now available make yeast one of the best established experimental model organisms for screening genes involved in the regulation of fundamental cellular process including the pathways controlling lifespan. In the following sections we focus on the particular case of the lifespan in yeast and its modulation triggered by extrinsic culture medium factors. We start with basic aspects of both replicative and chronological lifespan in yeast. The subsequent sections are dedicated to an overview highlighting the impact of culture medium and products of fermentation on the yeast chronological lifespan.

2. Chronological and Replicative Lifespan in Yeast

Two yeast lifespan models have been characterized: replicative lifespan (RLS) and chronological lifespan (CLS) (reviewed in [35]). RLS is defined as the total number of times a single mother cell can undergo a mitotic event and originate daughter cells before senescence [36]. RLS is accurately measured by moving and counting small daughters away from the mothers via microscopic manipulation and simulates aging of mitotically active mammalian cells [35, 37, 38]. On the other hand, CLS defines the length of time non-dividing yeast cells remain viable [39] thus simulating aging

of the postmitotic mammalian cells [40, 41]. This viability is assessed by cells reentering the cell cycle after transfer from the depleted medium or water to nutrient complete-medium [35]. In CLS, two types of metabolic yeast cells can be studied: postdiauxic or stationary phase cells. Both metabolic state cells are grown in synthetic complete (SDC) medium but while postdiauxic cells are kept in the culture medium, the stationary phase ones are transferred to water (extreme calorie restriction) after 3 days of growth. Some protocols for postdiauxic cells could also use cells grown in YPD (yeast extract, peptone, dextrose) medium [35, 42] instead of SDC medium.

The two paradigms of aging in yeast, CLS and RLS, have become useful tools to compare the aging process in proliferating and nonproliferating cells as well as to serve as models to study the mechanics of the aging process in mitotic and postmitotic cells of multicellular organisms [38].

The yeast *S. cerevisiae* divides by budding and therefore undergoes asymmetrical cell division, with the mother cell retaining more volume than the daughter cell. In this asymmetric division mother cells retain most of the age-associated damage, thus sacrificing individual replicative capacity while daughter cells retain full replicative potential [43, 44]. One of the aging factors affecting RLS is the accumulation of extrachromosomal ribosomal DNA circles (ERCs) [45]. These circular DNA molecules are self-replicating units formed in the nucleus by homologous recombination between adjacent rDNA repeats which segregate asymmetrically to the mother-cell nucleus during cell division. During each division ERCs replicate leading to an exponential accumulation in the mother cell and consequently to cell senescence [44, 45]. This finding in yeasts came in large part from the study of important age-related proteins called sirtuins. Sirtuins are NAD^+-dependent protein deacetylases involved in chromatin silencing and known to mediate longevity in yeast, nematodes, flies, and mammals [40, 44, 46]. Deletion of *SIR2* decreases RLS and its overexpression increases RLS showing that Sir2p mediates RLS in yeast most probably by regulating rDNA recombination and ERCs formation [47]. ERCs appear to be an aging factor specific to yeast, although without relevance in nondividing yeast cells (CLS) and so far without a role in aging of multicellular eukaryotes [46, 48].

Another factor known to decrease longevity both in CLS as RLS is accumulation of oxidative damage due to the production of reactive oxygen species (ROS). Deletion of the yeast antioxidant defense enzymes superoxide dismutases (SOD), reduces significantly CLS [39] and RLS [49]. However, overexpression of cytosolic (*SOD1*) and mitochondrial (*SOD2*) superoxide dismutases increased longevity of nondividing cells [50] while it decreased RLS. Although studies show several similarities but also major differences between CLS and RLS mechanistic regulation, these two models are interconnected as RLS decreases in chronologically aged cells [48]. In addition, both aging models are regulated by the nutrient-signaling kinases, as screenings for long-lived mutants identified the same gene mutations in both paradigms [27, 51–53].

3. Glucose-Signaling Pathways Involved in Yeast Longevity

The most common dietary regimes used to study the interaction between nutrient-signaling pathways and longevity include: dietary restriction (DR) in which the intake of nutrients, but not necessarily calories, is reduced without causing malnutrition; calorie restriction (CR), a regime in which only calories are reduced without compromising other nutrients, for instance amino acids and vitamins [54, 55].

In yeast, when studying both replicative lifespan (RLS) and chronological lifespan (CLS), several results correlating environmental growth conditions and longevity emerged. Many studies, including those using calorie restriction (CR), showed that reducing the glucose or amino acids concentrations of the culture media is sufficient to increase replicative and chronological lifespan [56–60]. The composition of culture media has proven to be an extrinsic factor affecting chronological lifespan but this is still giving rise to different interpretations on longevity regulation.

The manipulation of nutrient-signaling pathways for the study of aging regulating mechanisms, as previously mentioned, can be accomplished by genetic manipulations of key components of these pathways or by dietary (DR) and calorie restriction (CR). In yeast, the vast majority of protocols for CR are based on the decrease of the glucose concentration in the medium from the standard 2% to 0.5 or 0.05%. The latter (0.05% glucose) is considered extreme calorie restriction as well as the one achieved by transferring cells grown in 2% glucose to water [35, 61].

The first studies to report glucose as an agent affecting lifespan in yeast were conducted by Granot and coworkers who showed that addition of glucose to stationary-phase cells previously transferred to water leads to a reduction of CLS [62]. The authors further demonstrated that glucose, in the absence of other complementing nutrients, induces apoptotic cell death accompanied by an increase in ROS production [63]. Further studies in yeast have revealed that the major nutrient-signaling pathways TOR, SCH9, and Ras/AC/PKA are all involved in longevity regulation by glucose [6, 46, 58]. These pathways promote cell division and growth in response to nutrients while inhibiting the general stress response and autophagy. *SCH9* was one of the first genes to be implicated in CLS [27]. Sch9p is the yeast closest homolog of the mammalian AKT/PKB and S6K, and its deletion leads to an increase in both CLS and RLS [27, 53]. Sch9p is a kinase that mediates PKA activation in the fermentable-growth-medium-(FGM)-induced pathway and also mediates many of the TOR complex 1 (TORC1) controlled processes [64–66]. Sch9p was first described as having a partially redundant role with PKA pathway, since deletion of *SCH9* could be compensated by increased activity of PKA and vice versa [67], and later as a direct target of TORC1 regulation [65]. More recently, TORC1 was also identified as a target for regulating longevity in both CLS and RLS [51, 52]. The TOR pathway responds to nitrogen and carbon sources, mainly to control cell growth, through the regulation of processes such as translation initiation, ribosome biogenesis, mRNA and amino acid permeases stability, transcription of

nutrient-regulated genes and stress response genes, and actin cytoskeleton organization and autophagy [68, 69]. Reduction of TORC1-Sch9p signaling was shown to promote longevity by increasing the expression of stress-response genes in a Rim15p-dependent manner [58], as *RIM15* deletion reduced the lifespan extension of the long-lived *sch9*Δ cells [27]. Alternatively, a recent study proposes a Rim15p-independent mechanism for lifespan extension in reduced TORC1-Sch9p signaling [70]. This study shows that in *tor1*Δ cells, CLS is reduced if mitochondrial respiration is uncoupled. The authors suggest that during growth, mitochondrial ROS signaling downregulates both the mitochondrial membrane potential and ROS accumulation of stationary phase cells to promote their longevity [70]. This is in agreement with previous data showing that preadaptation to respiratory growth can also promote extension of CLS [71]. CR also promoted CLS extension by doubling the lifespan of the long-lived *sch9*Δ and *tor1*Δ cells by a Rim15p partially dependent mechanism. In fact, cells with a triple mutation in Rim15p downstream transcription factors (*msn2*Δ *msn4*Δ *gis1*Δ) do not display a reduction of CR promoted CLS extension when compared to the long-lived *sch9*Δ and *tor1*Δ cells suggesting the involvement of additional Rim15p independent transcriptional factors [58].

Another pathway involved in aging is the other major nutrient-signaling pathway Ras/AC/PKA, responsible for the link between glucose availability and the control of growth, proliferation, metabolism, stress resistance, and longevity [6, 27, 50, 72]. Deletion of *RAS2* or a reduced activity of adenylate cyclase (Cyr1p), which is activated by the Ras proteins, causes lifespan extension and stress resistance [50]. Mutation in the *CYR1* gene increases both RLS and CLS while deletion of *RAS2* decreases RLS [53, 73]. Rim15p also mediates *ras2*Δ lifespan extension by enhancing cellular protection against oxidative stress through the activation of *SOD2* [50], indicating that Rim15p is a common denominator of the pathways Ras/AC/PKA, Sch9p, and TOR. In addition, deletion of *MSN2/4* in *ras2*Δ cells leads to lifespan reduction indicating that Msn2p/4p and Gis1p transcription factors controlled by Rim15p are also required for CLS extension. Nevertheless, the Rim15p downstream transcription factors (Msn2p, Msn4p, and Gis1p) appear to have different roles in *sch9*Δ and *ras2*Δ cells given that only the abrogation of *GIS1*, and not of *MSN2/4*, was shown to almost completely abolish the lifespan expansion of *sch9*Δ cells [27, 58]. Therefore, Sch9p and Ras2p seem to differentially modulate the common downstream effectors, which is also corroborated by the higher stress resistance and increased CLS exhibited by *ras2*Δ *sch9*Δ double knockout cells in comparison to the single deletion mutants [58, 74].

More recently, the correlation between glucose signaling, oxidative stress and aging was further addressed in a study showing that increasing glucose from the standard 2% to 10%, promotes a shortening of CLS accompanied by increased levels of intracellular superoxide anion ($O_2^{\bullet-}$), decreased levels of hydrogen peroxide (H_2O_2), reduced efficiency of stationary phase G_0/G_1 arrest, and activation of DNA damage [12]. On the other hand, CR by reducing glucose or by deletion of *SCH9* or *TOR1* extends CLS and diminishes superoxide anion levels promoting at the same time a more efficient G_0/G_1 arrest. These and other results point to superoxide levels as one of the key factors regulating aging [75], which is in agreement with the aforementioned results showing that reduction of signaling pathways leads to the activation of oxidative stress responses mediated by Rim15p [12]. Nevertheless, an alternative activation of oxidative stress responses independent of Rim15p [58] and mediated by H_2O_2 has been also reported [12]. Furthermore, high levels of H_2O_2, which respond to glucose in an inversely dose-dependent manner, promotes activation of SODs, leading to a reduction in superoxide anion levels and therefore to CLS extension [12, 76].

4. Amino Acid Metabolism in the Regulation of the Yeast Chronological Lifespan

In nature, yeast cells enter a resting or quiescent state in the absence of favorable nutritional conditions. When inadequate carbon, nitrogen, sulfur, or phosphorus levels are sensed by yeast cells, growth ceases and cell cycle is arrested as a survival strategy. In natural environments, yeast are prototrophs capable of synthetizing most of their metabolites from simple carbon and nitrogen sources, whereas laboratory strains commonly have auxotrophic markers that confer a nutrient-limiting growth phenotype useful for genetic manipulation. These markers are usually genes involved in the biosynthesis of specific amino acids or nucleotides. Amino acids are important nutrients that can also be recycled by autophagy. This recycling process maintains amino acid homeostasis and is crucial for cell survival under nitrogen starvation leading to rapid loss of viability in autophagy-defective mutants [77] and therefore has been implicated in CLS regulation. Curiously, it was demonstrated that prototrophic and auxotrophic strains display different responses to nutrient starvation [78]. Starvation of "natural" nutrients leads to an arrest in G_0/G_1 cell cycle phase of prototrophics cells, while auxotrophic cells failed to arrest the cell cycle upon starvation of "supplemental" nutrients (auxotrophic nutrients) [78]. It was also observed that auxotrophic cells limited for leucine or uracil consume glucose at a much faster rate, exhausting it from the medium, than prototrophic cells limited for phosphate, sulfate, or ammonium that spare glucose [79]. These findings clearly reveal a failure of auxotrophic cells in regulating nutrient sensing in response to starvation of "supplemental" nutrients [80]. Furthermore, limiting levels of auxotrophy-complementing amino acids, in the growth medium, induce an early arrest in G_2/M phase, negatively affecting chronological longevity and leading to a premature aging phenotype [81]. In accordance, reduction of total amino acid levels, including essential ones, in the medium also decreases CLS [82]. Starvation for leucine in nondividing leucine auxotrophic cells induces a rapid loss of viability [80]. Nevertheless, this phenotype is partially dependent on the carbon source present in the starvation medium but not in that used in the growth medium. For example the presence of ethanol/glycerol or galactose in the starvation medium increases CLS in contrast to starvation

in glucose [80]. However, not all essential amino acids have the same effect on CLS. In fact, methionine starvation of methionine auxotrophic cells has no effect on viability [83]. Another study also reported that from the auxotrophic-complementing amino acids, lysine, histidine, and leucine, the latter has a more pronounced negative effect in CLS in both autophagy-competent and autophagy-deficient strains [84]. The authors pointed out that the enhanced sensitivity of yeast cells to leucine starvation is correlated to the high levels of leucine codon, the most frequent amino acid codon [84]. CLS is extended by the presence of nonessential amino acids, particularly isoleucine, and its precursors threonine and valine, via the general amino acid control (GAAC) pathway. The authors proposed a mechanism for CLS regulation by the branched side chain amino acids (BCAA) leucine, isoleucine and valine, in which low levels of these amino acids induce the GAAC pathway therefore shortening CLS and vice versa [84].

Starvation for nonessential amino acids was reported to extend RLS [56] and starvation for preferred amino acids such as asparagine- or glutamate- induced CLS extension in direct proportion to the nature of the amino acid removed [52].

5. Impact of Products of Fermentation in the Yeast Chronological Lifespan

Ethanol is the main product resulting from alcoholic fermentation and it is used as a carbon source during the diauxic shift and postdiauxic phase. Nevertheless, ethanol is known to negatively affect the metabolic activity of the yeast cells by inhibiting cell growth and fermentation. It is also known to cause among others the damage of cell membranes by increasing membrane fluidity [85, 86] and the inhibition of transport systems across the plasma membrane [87, 88]. The severity of the effects is dependent on the alcohol concentration and at high ethanol levels it results in cell death [89]. Recently, ethanol was described as an apoptotic inducer [90] and has also been implicated as an extrinsic factor in aging, significantly decreasing CLS of severely calorie restricted strains (CR in water), known for their lifespan extension in this condition [91]. In contrast to wild type cells, long-lived *sch9*Δ cells consume all the ethanol from the medium during chronological aging, further supporting ethanol as a modulator of aging [91].

A recent study on the genetic expression profile of long-lived *tor1*Δ, *sch9*Δ, and *ras2*Δ cells revealed an upregulation of genes involved in the metabolism of glycerol. In contrast to wild type cells that accumulate ethanol and rapidly deplete glycerol, those long-lived mutant cells accumulate glycerol whereas ethanol was early depleted. These observations suggest that inhibition of Tor1p/Sch9p mediates a metabolic switch from biosynthesis and release of ethanol to activation of glycerol biosynthesis and its consequent release [74]. Glycerol, unlike glucose and ethanol [58, 61, 91], does not promote aging or cell death and so this metabolic change extends CLS [74, 92].

In calorie restricted cells, ethanol is completely consumed before the beginning of viability decline. Conversely, noncalorie restricted cells were unable to completely consume ethanol before viability decline. The authors suggested a correlation between ethanol accumulation and loss of peroxisome function in noncalorie restricted cells since ethanol suppresses the synthesis of certain proteins localized to peroxisomes [93].

Acetic acid is a byproduct of fermentative metabolism in yeast accumulating in the medium during fermentation of glucose to ethanol and is also one factor described to affect CLS [94, 95]. After sugar is depleted in 2% glucose standard conditions, a shift in metabolism occurs from fermentation to respiration and the metabolization of ethanol also leads to the production and accumulation of acetic acid. Acetic acid is a well-known inducer of apoptotic cell death leading to ROS production [96, 97]. In a recent study, Burtner and coworkers identified acetic acid as an important extracellular factor affecting CLS in SDC medium [94]. The authors showed that cells grown for 48 hours under extreme calorie restriction conditions (0.05% glucose concentration), known to extend CLS, rapidly loss viability if transferred to cell-free supernatants of 2% standard glucose-depleted medium, indicating that cell-extrinsic aging factors were present in the SDC depleted medium [94]. Although several other organic acids also accumulate in the culture medium during chronological aging, only acetic acid was identified as being sufficient to cause chronological aging [94]. In the same study it was also shown that buffering of aging cultures to pH 6 is sufficient to increase CLS, neutralizing the toxic effect of acetic acid. Actually, the acetate anion is not readily taken up from the environment by glucose-grown yeast cells, but the protonated acetic acid can cross the plasma membrane resulting in intracellular acidification [98]. This negative effect of acetic acid in CLS was diminished by mutational inactivation of conserved signaling pathways, namely deletion of *SCH9* and *RAS2*, conferring resistance via unknown mechanisms [94]. *SCH9* and *RAS2* mutant cells are known to have a more frequent growth arrest in G_1 phase when compared to the wild-type, promoted by the reduction in growth signaling in these mutants [99]. In accordance, nutrient-depleted stationary phase cells are continuously subjected to acetic acid-induced growth signals, even in the absence of glucose, that promote cell cycle progression and consequently replication stress due to the lack of favorable conditions [100]. These and other results show that acetic acid, as glucose, activates Sch9p and RAS pathways and seems to mediate cell death by promoting the accumulation of superoxide anion ($O_2^{\bullet-}$) in consequence of downregulation of SODs and other oxidative stress defenses by the activated pathways [12]. The long-lived *ade4*Δ cells (Ade4p is involved in the purine *de novo* biosynthetic pathway) do not accumulate acetic acid in the culture medium when compared to the wild type cells, while the short-lived *atg16*Δ cells (Atg16p is involved in the autophagic process) accumulate acetic acid at higher concentrations than the wild type cells, inversely correlating the amount of acid release from cells and the extension of CLS [101]. Buffering the growth media to pH 6.0 of

the short-lived *atg16*Δ cells and the wild type strain, also dramatically increase CLS to the same levels obtained for the CR growth condition and for the long-lived *ade4*Δ cells, indicating that pH neutralized the toxic effects of acetic acid. Overall the results demonstrate that acetic acid can have an important impact on CLS through a cell extrinsic mechanism that is dependent on media pH.

6. Ammonium Toxicity in Aging Yeast Cells

Ammonium (NH_4^+) toxicity has been well described in animals and plant systems [102]. In yeast, NH_4^+ is commonly used as nitrogen source for growth and it is usually not toxic having a central role in nitrogen metabolism both in degradative and biosynthetic pathways [103]. In yeast, nitrogen sources, prior to their use, need to be converted into glutamate and glutamine. However not all nitrogen sources are equally preferred and yeast can select the nitrogen sources through nitrogen catabolite repression (NCR) mechanism also known as nitrogen discrimination pathway (NDP). This pathway enables yeast to repress genes that code for proteins required for the use of poor nitrogen sources, when in the presence of sufficient quantities of rich nitrogen sources like glutamine [104].

Production of ammonia in yeast colonies has even been described as a mechanism of protection from cell death during colony development [105]. An excess of ammonium was found to be toxic for *S. cerevisiae*, under potassium limitation, resulting in amino acid excretion similar to the detoxifying mechanism found in mammals [106]. The authors described that ammonium toxicity in yeast is related to a "leak current" of ammonium ions that enter the cell through potassium channels, in limiting potassium conditions, and this influx causes an excess of internal ammonium that becomes toxic for the cell. To cope with this ammonium excess, cells excrete amino acids possibly through the Ssy1p-Ptr3p-Ssy5p (SPS)-system of amino acid transporters, which were found to be strongly upregulated in this condition, or by directly excreting ammonium via the Ato (Ammonium Transporter Outward) transporters [106].

Recently, we have reported that ammonium is toxic for aging cells and acts as an extrinsic factor affecting CLS [107]. In this study, it was shown that decreasing the concentration of NH_4^+ in the culture medium increases yeast CLS in amino acid restriction conditions. In contrast, when the initial $(NH_4)_2SO_4$ concentration in the culture medium, either with or without restriction of amino acids, was increased from 0.5% to 1%, there was a decrease in cell survival, demonstrating that the toxic effects of ammonium are correlated with its concentration in the culture medium. Moreover, after transferring cultured cells to extreme calorie restriction conditions in water, the addition of ammonium drastically decreases the CLS, indicating that ammonium alone could also induce loss of cell viability as observed in culture media, and providing, for the first time, a role for ammonium in chronological longevity regulation [107].

Cells starved for auxotrophic-complementing amino acids are particularly sensitive to ammonium-induced cell death [107]. Death induced by ammonium in these cells is mediated through the regulation of the evolutionary conserved pathways PKA, TOR, and SCH9 and is accompanied by an initial apoptotic cell death followed by a fast secondary necrosis. Autophagy, which has been described as essential for cell survival during nitrogen starvation and regulating amino acid homeostasis [84], does not seem to have a role in ammonium-induced cell death.

The ammonium effects were also not dependent on its metabolism as activity of enzymes involved in the metabolism of ammonium showed no correlation with ammonium toxicity and the use of a nonmetabolizable analog produced the same outcome as ammonium [107]. Even though, ammonium signaling is capable of activating the PKA pathway in agreement with previous results showing that the addition of ammonium, in nitrogen starvation medium, directly signals PKA activation through Mep2p [108]. In contrast, in aging yeast, although Mep2p is involved in ammonium-induced death it does not appear to have a major role in PKA activation. Tor1p and Sch9p were shown to be necessary for ammonium-induced PKA activation in amino acid-starvation conditions as deletion of *TOR1* and *SCH9* resulted in a decrease of PKA activation. Ammonium action on both pathways, resulting in the over-activation of PKA and TOR pathways and inhibition of Sch9p, culminates in the shortening of CLS [107].

Altogether results support that ammonium induces cell death in aging cultures through the regulation of evolutionary conserved pathways. They also show that the study of ammonium toxicity in yeast aging may be a powerful system to understand longevity regulation in multicellular organisms.

7. Final Remarks

Yeast has emerged as one of the most important model organisms to study the environmental and genetic factors affecting longevity, and its exploitation has made huge contributions to the progress in understanding aging. Although some aspects of aging in yeast are specific to this organism, many of the most important features reveled in yeast proved to be evolutionarily conserved in higher eukaryotic organisms. The two paradigms of aging in yeast, chronological and replicative lifespans, are useful tools to compare the aging process in proliferating and nonproliferating cells and to study the aging process in mitotic and postmitotic cells. The pathways controlling yeast lifespan occur through complex signaling cascades, allowing cells to stimulate proliferation in optimal conditions and also to induce cell cycle arrest and enter into a quiescent state in nutrient exhaustion conditions. In the present paper, we focused on the particular case of the lifespan in yeast and its modulation triggered by extrinsic culture medium factors. A scheme illustrating the current scenario on the regulation of CLS by different nutrient/energy signalling pathways in yeast described herein is presented in Figure 1. Major advances in this research field have come from dietary regimes that have been shown to increase longevity in organisms ranging

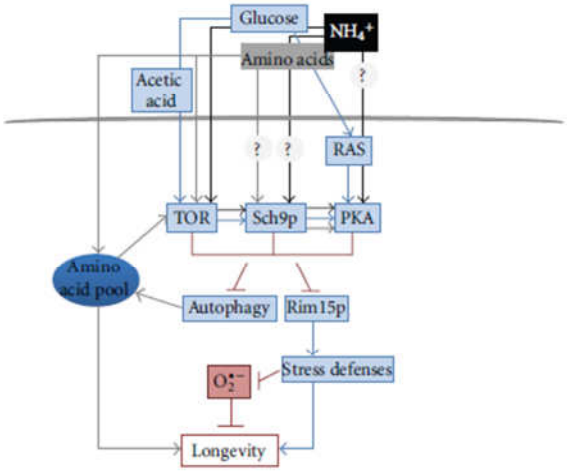

FIGURE 1: Scheme illustrating the current scenario on the regulation of chronological lifespan (CLS) in yeast by the different nutrient/energy signalling pathways described in the present paper (the detailed description and abbreviations are given in the text).

from yeast to mammals. Altogether, data presented clearly establishes that the carbon and the nitrogen sources as well as the products of fermentation, are among the main extrinsic factors modulating yeast chronological longevity. Loss of cell viability induced by these environmental factors in aging cultures is regulated through evolutionary conserved pathways and their study can provide key insights into pathways that modulate aging in mammals, being a powerful system to understand longevity regulation in multicellular organisms.

Authors' Contribution

M. J. Sousa and C. Leão contributed equally to this work.

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