Rómio Júnior Silva Pinto

Protein synthesis and aging: is translation rate a major regulator?

UAlg FMCB
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Rómio Júnior Silva Pinto

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This work was done under the supervision of:
Clévio Nóbrega, PhD

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Faculdade de Medicina e Ciências Biomédicas

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Authorship statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

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(Rómio Júnior Silva Pinto)

30 June 2022

Dedication

I would like to dedicate this dissertation, especially to my late uncle José Lopes, who was a role model in my development and taught me the meaning of life and its significance.

Acknowledgements

First of all, I would like to express my utmost gratitude to my research supervisor, Dr. Clévio Nóbrega, for his constant support, guidance, and advice throughout this project. The completion of this study could not have been possible without his expertise and assistance.

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I would also extend my gratitude to Gary Hosmer for the inspiration and support in this endevour.

Resumo

O envelhecimento é um processo biológico universal caracterizado por uma acumulação de danos e outros efeitos nocivos que levam a uma perda progressiva da integridade fisiológica e funcional que culminam na morte do organismo. As alterações que acompanham o envelhecimento ocorrem nos diversos níveis da organização do ser vivo, ou seja, a nível molecular, dos organelos, celular, dos tecidos, órgãos e sistemas. O envelhecimento constitui um dos fatores principais das patologias humanas, como o cancro, a diabetes, doenças cardiovasculares e doenças neurodegenerativas, nomeadamente, as doenças de Alzheimer e Parkinson. Por esta razão e o aumento progressivo da população idosa, nas últimas décadas têm ocorrido um aumento e progresso na investigação focada no envelhecimento. Notavelmente, ao longo das últimas décadas, tem ocorrido uma mudança gradual no foco da investigação sobre o envelhecimento, ao invés de focar-se nos efeitos e sintomas do envelhecimento, tem-se concentrado nos mecanismos moleculares subjacentes ao processo de envelhecimento. Esta nova abordagem levou à descoberta de que a taxa de envelhecimento é controlada, pelo menos até certo ponto, por vias genéticas e processos bioquímicos conservados na evolução, o que permitiu identificar e categorizar as características celulares e moleculares do envelhecimento, sendo estes definidos como os biomarcadores do envelhecimento. Foram propostos nove biomarcadores do envelhecimento: instabilidade genómica, encurtamento dos telómeros, alterações epigenéticas, perda de homeostasia proteica, desregulação da sensibilidade de nutrientes, disfunção mitocondrial, senescência celular, exaustão das células estaminais, alteração da comunicação intercelular.

De entre os diversos mecanismos moleculares que impactam o envelhecimento, as alterações que afetam o proteoma celular têm um papel fulcral. Quase todos os processos fisiológicos são dependentes de proteínas e, portanto, a preservação da integridade do proteoma é imperativa para a vida do organismo. Vários fatores especializados são dedicados a preservar a integridade do proteoma celular, desde o processo de pré-tradução até o final do ciclo de vida funcional da proteína. A manutenção da homeostase celular correlaciona-se diretamente com a manutenção de um equilíbrio preciso da síntese, degradação e função de cada proteína. Quando esse equilíbrio é perturbado, as proteínas danificadas se acumulam progressivamente, levando a um estado prejudicial e até mesmo à morte celular. O distúrbio deste equilíbrio ocorre naturalmente com o envelhecimento, à medida que a integridade e a eficácia da maquinaria da síntese proteica e dos sistemas de controlo de qualidade das proteínas diminuem gradualmente

devido ao inevitável acúmulo de danos com a idade. Em particular diversos estudos demonstram que a síntese proteica reduz com a idade em diversos organismos, tendo-se observado uma redução de diferentes componentes da maquinaria de tradução, nomeadamente, abundância dos ribossomas e atenuação da atividade e dos níveis dos principais fatores de iniciação e alongamento. Apesar destas descobertas e do crescente número de estudos, vários aspetos da síntese proteica no contexto do envelhecimento permanecem elusivos, especialmente a nível mecanístico.

Neste sentido, o presente estudo tem como objetivo elucidar o papel da síntese proteica e do controle da tradução no envelhecimento e na expectativa de vida, assim como compreender a sua conexão funcional com os vários biomarcadores do envelhecimento. Para isso, analisou-se o perfil da taxa global de tradução em modelos celulares. De modo a estabelecer uma ligação entre o envelhecimento e a síntese proteica focamos na proteína de ligação a poliadenilato 1 (PABP1), uma proteína central no controle da tradução e estabilidade de mRNA. Esta proteína promove a circularização do mRNA, levando à estabilização e estimulação da iniciação da tradução. Por isso, PABP1 poderá desempenhar um papel fulcral na mediação de mudanças na síntese proteica com o envelhecimento.

Para o estabelecimento da relação entre síntese proteica e o envelhecimento, primeiramente analisou-se a variação do PABP1 com o envelhecimento em modelo celular e em morganhos. Seguidamente, o PABP1 foi sobre-expresso no hipotálamo de morganhos envelhecidos e analisou-se diferentes proteínas ligadas ao envelhecimento, nomeadamente: mTOR, relacionado à desregulação da sensibilidade de nutrientes; Ataxina-2, referente à tradução de mRNA; LC3B e P62, alusivo à perda de homeostasia proteica; IL-6 e NF-κB, concernente à alteração da comunicação intercelular, particularmente, inflamação; e por fim PGC-1α, respeitante à disfunção mitocondrial. O hipotálamo foi o foco da sobre-expressão do PABP1 devido o seu envolvimento com diversas funções fisiológicas que diminuem com o envelhecimento, tais como: desenvolvimento, metabolismo, reprodução, ritmo circadiano e homeostase. Por conectar sistema neuroendócrino aos processos fisiológicos, supõe-se que o hipotálamo seja um regulador chave no envelhecimento sistemático.

Foi possível observar que de uma forma geral a taxa de síntese proteica sofre realmente alterações com o envelhecimento. Os resultados obtidos apontam para um papel fundamental da PABP1 no envelhecimento ao induzir a expressão de P62. A expressão de P62 induzida por PABP1 poderá ter, potencialmente, um papel na inflamação, pois podem afetar os níveis de citocinas cruciais, levando à sua redução. Observou-se que

danos celulares que geram instabilidade genómica ou condições de estresse podem afetar a taxa geral de síntese proteica, provavelmente, afetando a longevidade.

Em conclusão, há uma corelação complexa entre o envelhecimento e síntese proteica. A síntese proteica pode ser alterada por danos celulares que afetam os componentes da maquinaria de tradução, como mutações que causam instabilidade genómica ou estresse oxidativo, impactando subsequentemente a longevidade. PABP1, um componente central na maquinaria de tradução, poderá ter um papel no contexto do envelhecimento por induzir a expressão de P62, que está envolvida em vários processos celulares que implicam o envelhecimento. A PABP1 também, potencialmente, poderá diminuir a inflamação no hipotálamo, indiretamente induzindo a diminuição do IL-6 através do P62, o que permite mitigar várias patologias associadas à inflamação com o envelhecimento. No entanto, devido à complexidade da relação da síntese proteica com o envelhecimento, mais estudos futuros são necessários para elucidar o papel da síntese proteica no envelhecimento.

Palavras-chave: Envelhecimento; Síntese proteica; PABP1; Hipotálamo; Tradução de mRNA; Biomarcadores do envelhecimento

Abstract

Aging is a universal biological process characterized by an accumulation of damage and other deleterious changes that lead to a progressive loss of physiological integrity, functionality, and fitness, ultimately resulting in death. It constitutes the primary risk factor for major human pathologies such as cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases. It was proposed nine hallmarks of aging (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication) that are considered to contribute to the aging process and cooperatively determine the aging phenotypes. Among these multiple molecular mechanisms underlying aging, alterations that affect protein synthesis seem to play a central role. Accumulating evidence suggests that protein synthesis and translation control could significantly influence lifespan. Therefore, this study aims to elucidate the role of protein synthesis and translation control in aging and lifespan and understand their functional connection with several hallmarks of aging. To this end, the translation rate profile was analyzed in different cellular models. Furthermore, to establish a link between aging and protein synthesis, we focused on polyadenylate binding protein 1 (PABP1), a central protein in the control of mRNA translation and stability. PABP1 was overexpressed in old mice's hypothalamus, and key proteins tightly linked with the hallmarks of aging were analyzed.

We found that the overall rate of protein synthesis is altered with aging. Moreover, we observed that cellular damage generating genomic instability or stress conditions could impact the overall rate of protein synthesis, possibly affecting lifespan. In addition, our data point to a key role of PABP1 in aging by inducing the expression of p62. PABP1-induced expression of p62 could potentially have a role in inflammaging, as it could reduce the levels of crucial cytokines such as IL-6. Altogether, the results revealed a complex relationship between protein synthesis and aging; therefore, more studies are necessary to elucidate the role of protein synthesis in aging.

Keywords: Aging; Protein Synthesis; PABP1; Hypothalamus; mRNA translation; Hallmarks of aging

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List of Abbreviations

4E-BP	the eukaryotic initiation factor eIF4E binding protein
4E-BP1	4E-binding protein 1
aa-tRNAs	aminoacyl transfer RNAs
AAV	adeno-associated virus
ADP	Adenosine diphosphate
ADSC	adipose-derived stem
AgRP	agouti-related peptide
Akt	protein kinase B
ALP	Autophagy/lysosome pathway
AMPK	Adenosine monophosphate-activated protein kinase
Arc	arcuate nucleus
ATF6	Activating transcription factor 6
Atg	Autophagy-related genes
ATG7	Autophagy Related 7
AUF1	adenylate/uridylate (AU)-rich RNA-binding factor 1
AVP	arginine vasopressin
BiP	Binding immunoglobulin protein
BMAL1	Brain and Muscle ARNT-Like 1
BRASTO	Brain-specific SIRT1-overexpressing
cDNA	Complementary DNA
CHIP	carboxyl-terminus of HSP70-interacting protein
CMA	chaperone-mediated autophagy
Ct	cycle threshold
DMEM	Dulbecco's modified Eagle's medium
DMH	Dorsomedial hypothalamic nucleus
DNA	Deoxyribonucleic acid
dPABP	drosophila PABP
eEF	eukaryotic translation elongation factor
eEF1A	eukaryotic translation elongation factor 1 alfa
eEF2	eukaryotic translation elongation factor 2
eIF	eukaryotic translation initiation factor
eIF2	eukaryotic translation initiation factor 2
eIF2α	eukaryotic translation initiation factor 2 alpha
ePABP	Embryonic Poly(A)-Binding Protein
ER	Endoplasmic reticulum
eRF	The eukaryotic release factor
ERKn	extracellular signal-regulated kinases number - eg.: ERK1, ERK2
FBS	fetal bovine serum
FOXO	Forkhead box O
FSH	follicle stimulation hormone
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRHR	GHRH receptor
GnRH	Gonadotropin-releasing hormone
H2O2	Hydrogen peroxide
HDAC6	histone deacetylase 6

HEK293T	human embryonic kidney 293 cells containing SV40 T-antigen
HGPS	Hutchinson-Gilford progeria syndrome
HnKn	Histone Hnumber lysine Knumber – e.g.: H3K4, H3K27, H4K20
HP1α	Heterochromatin protein 1α
HPG	hypothalamus-pituitary–gonad
HSC	Hematopoietic stem cells
HSC70	Heat shock cognate 71 kDa protein
HSF-1	Heat shock factor 1
HSP	heat-shock proteins
htNSC	hypothalamic neural stem cells
IGF-1	Insulin-like growth factor 1
IGF1R	insulin-like growth factor receptor
IIS	Insulin/insulin-like growth factor 1 signaling
IKK-β	IκB kinase-β
IL-1β	interleukin-1 beta
IL-6	Interleukin 6
INK4/ARF	INK4 (family of cyclin-dependent kinase inhibitors) family and ARF (ADP-ribosylation factor)
INK4a	Cyclin-Dependent Kinase 4 Inhibitor A
INK4b	Cyclin-Dependent Kinase 4 Inhibitor B
IRE-1	inositol-requiring transmembrane kinase/endonuclease 1 alpha
KNDy	Kisspeptin/neurokinin B/dynorphin
LAMP2a	Lysosomal associated membrane protein-2a
LAMP-2A	lysosomal-associated membrane protein type 2A
LC3	microtubule-associated protein light chain 3
LC3B	Microtubule-associated protein 1A/1B-light chain 3
LH	luteinizing hormone
LHA	lateral hypothalamic area
LV	lentiviral vectors
MAPK	mitogen-activated protein kinase
MBH	Mediobasal Hypothalamus
MEK/ERK	Mitogen-activated protein kinase (MEK) / extracellular-signal-regulated kinase (ERK)
Met-tRNA	methionyl-tRNA
Met-tRNAiMet	amino-acyl tRNA
miRNA	microRNA
Mnk1	mitogen-activated protein kinase-interacting kinase
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mTORCn	mTOR complex number – e.g.: mTORC1, mTORC2
NAC	nascent chain-associated complex
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGPS	Nestor-Guillermo progeria syndrome
N-Htt	N-terminal Huntingtin fragments
NKX2-1	NK2 homeobox 1
NLRP3	nucleotide-binding and oligomerization domain (NOD)-, leucine-rich repeat (LRR)- and pyrin domain containing protein 3

NPY	neuropeptide Y
Nrf2	nuclear factor erythroid 2–related factor 2
NuRD	Nucleosome Remodeling and Deacetylase
Ox2r	orexin type 2 receptor
p62/SQSTM1	sequestosome 1
PAB	Poly(A)-Binding Protein in C. elegans/C. briggsae, mainly PAB1 and PAB2
PABP	poly(A)-binding protein
PABP1	poly(A)-binding protein 1 - also called PABPC1 (Poly(A) Binding Protein Cytoplasmic 1)
PABPCn	Poly(A) Binding Protein Cytoplasmic number - eg.: PABPC1, PABPC4, PABPC5
PABPN	nuclear PABPs
PAIP1 and PAIP2	PABP interacting proteins 1 and 2
PAM	PABP interacting motif
PAN3	poly(A) nuclease 3
PCR	polymerase chain reaction
PD	population doublings
PDI	protein disulfide isomerase
PEI	polyethylenimine
PERK	protein kinase RNA-like ER kinase
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α
PGC-1β	Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1β
PGK	phosphoglycerate kinase 1
PI3K	Phosphoinositide 3-kinases
PI3K/Akt	phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)
PKA	protein kinase A
PN	Proteostasis Network
POMC	proopiomelanocortin
PPARϒ	Peroxisome proliferator activated receptor-ϒ
PtdIns3P	Phosphatidylinositol 3-phosphate
PTEN	Phosphatase and tensin homolog
PVN	paraventricular nucleus
RAC	ribosome-associated complex
rDNA	Ribosomal DNA
RLE-1	regulation of longevity by E3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRM	RNA-recognition motif
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcription polymerase chain reaction
S6K	ribosomal protein S6 kinase
S6k1	Ribosomal protein S6 kinase B1
SCN	Suprachiasmatic nucleus
SIRTn	Sirtuin number – e.g.: SIRT1, SIRT2
SST	somatostatin
TERT	Telomerase reverse transcriptase
TNF-α	tumor necrosis factor-alpha
tPABP	testis PABP - normally PABP2 and PABP3
TPR	tetratricopeptide repeat

Ub	Ubiquitin
ULKn	Unc-51 Like Autophagy Activating Kinase number - eg.: ULK1, ULK2
UPR	unfolded protein response
UPS	Ubiquitin/proteasome system
VIP	vasoactive intestinal peptide
VMN	ventromedial nucleus
WT	wild-type
α-MSH	α-melanocyte-stimulating hormone

Chapter 1 – Introduction

1 - Hallmarks of Aging

Aging is a universal process characterized by an accumulation of damage and other deleterious changes that lead to a gradual loss of physiological integrity, functionality, and fitness, ultimately resulting in death (Anisimova et al., 2018; López-otín et al., 2013; Mohamad Kamal et al., 2020). It constitutes the primary risk factor for major human pathologies such as cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases (Kennedy et al., 2014; López-otín et al., 2013; Mohamad Kamal et al., 2020). For this reason and the ever-growing global elderly population, aging research has experienced an unprecedented advance over the last decades, and it has become one of the most exciting areas of scientific study. Notably, over the years, the main focus of aging research has been progressively shifting from studying the effects and symptoms of aging to searching for the underlying mechanisms of the aging process (Bartke, 2021b). This shift led to the discovery that the rate of aging is controlled, at least to some extent, by genetic pathways and biochemical processes conserved in evolution (López-otín et al., 2013). Elucidation of these molecular mechanisms will immediately impact the design of novel interventions that could reduce or delay age-related deterioration in humans (Troulinaki & Tavernarakis, 2005).

Further progress in this field has gone beyond searching for "the mechanism" of aging and formulating the corresponding "theories of aging" to appreciating that aging represents a net result of multiple physiological changes and their intricate interactions (Bartke, 2021b). This new conceptualization paved to way for an attempt to identify and categorize cellular and molecular hallmarks of aging. Similar to the hallmarks of cancer (Hanahan & Weinberg, 2011), the hallmarks of aging try to capture the essence of aging and its underlying mechanisms by tackling several critical questions: the physiological origin of aging-causing damage; the compensatory responses that try to restore homeostasis and their interconnection between the different types of damage; and the possibilities to intervene exogenously to delay aging.

It was proposed nine hallmarks of aging (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication, as illustrated in Figure 1) that are considered to contribute to the aging process and cooperatively determine the aging phenotypes. These hallmarks should ideally fulfill three criteria: (1) manifest during normal aging; its experimental aggravation should accelerate aging; and (3)

its experimental amelioration should retard the normal aging process and hence increase healthy lifespan. However, the proposed hallmarks meet these ideal requisites to varying degrees, with the last criteria being the most difficult to achieve. Thus, not all of the hallmarks are fully supported yet by interventions that succeed in ameliorating aging (López-otín et al., 2013).

Figure 1 - The nine hallmarks of aging
genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. (Adapted from López-otín et al., 2013)

1.1 – Genomic Stability

The accumulation of genetic damage is a common characteristic of aging and occurs progressively and naturally throughout life. During an organism's lifespan, its DNA is in constant exposure to a variety of endogenous agents (physical, chemical, biological, UV/IR radiation) and endogenous threats (reactive oxygen species (ROS), replication errors, hydrolytic reactions, etc.), which continuously challenges the genomic integrity and stability (Figure 2). The DNA lesions resulting from the extrinsic or intrinsic damages are diverse and comprise point mutations and deletions, translocations, chromosomal aneuploidies, telomere shortening, and gene disruption caused by the integration of viruses or transposons. These

genetic lesions may affect essential genes and transcriptional pathways capable of disrupting tissue and organismal homeostasis. It is particularly impactful when it compromises the functional capabilities of stem cells, affecting tissue regeneration. However, organisms have developed a diverse array of advanced genomic stability systems capable of mitigating most of the genetic damage. DNA repair mechanisms are fundamental that even their artificial reinforcement could delay aging, and their dysfunction underlies many human progeroid syndromes such as trichothiodystrophy, Cockayne syndrome, Seckel syndrome, Werner syndrome, Bloom syndrome, and xeroderma pigmentosum (Hoeijmakers, 2009; López-otín et al., 2013).

The genetic damage not only occurs on nuclear DNA but also on mitochondrial DNA (mtDNA), where the subsequent mutations and deletions also contribute to aging. Genetic stability systems include specific mechanisms able to ensure the integrity of mtDNA, albeit less effective than those of nuclear DNA. On the one hand, the limited efficiency of mtDNA repair mechanisms, the oxidative microenvironment, and the lack of protective histones make mtDNA particularly susceptible to aging-associated somatic mutations. On the other hand, the implication of mtDNA mutation in aging is somewhat controversial due to the heteroplasmic character of the mitochondrial genome, in which mutant and wild-type genomes coexist within the same cell (López-otín et al., 2013). However, the mutational load increases with aging and becomes significant, shifting to a homoplasmy state in which one mutant genome is dominant. The mutation load becomes severe and may cause respiratory chain dysfunction and consequently disrupt cells' metabolism and energy production. Surprisingly, studies found that most mtDNA mutations are caused by replications error early in life that underwent polyclonal expansions rather than oxidative damage, thus exposing the limitations and importance of the mtDNA repair mechanisms (Khrapko et al., 1999; López-otín et al., 2013).

Mutations affecting elements of the nuclear lamina can also compromise genome stability. The nuclear lamina is an integral component of the nuclear architecture and is essential for the maintenance and regulation of genome stability by providing a scaffold for tethering chromatin and protein complexes that regulate genome activity. It is involved in most nuclear activities, and due to its crucial role, mutations in genes encoding its protein components or factors affecting its maturation and dynamics can cause accelerated aging syndromes, namely Hutchinson-Gilford progeria syndrome (HGPS) and Nestor-Guillermo progeria syndrome (NGPS) (López-otín et al., 2013).

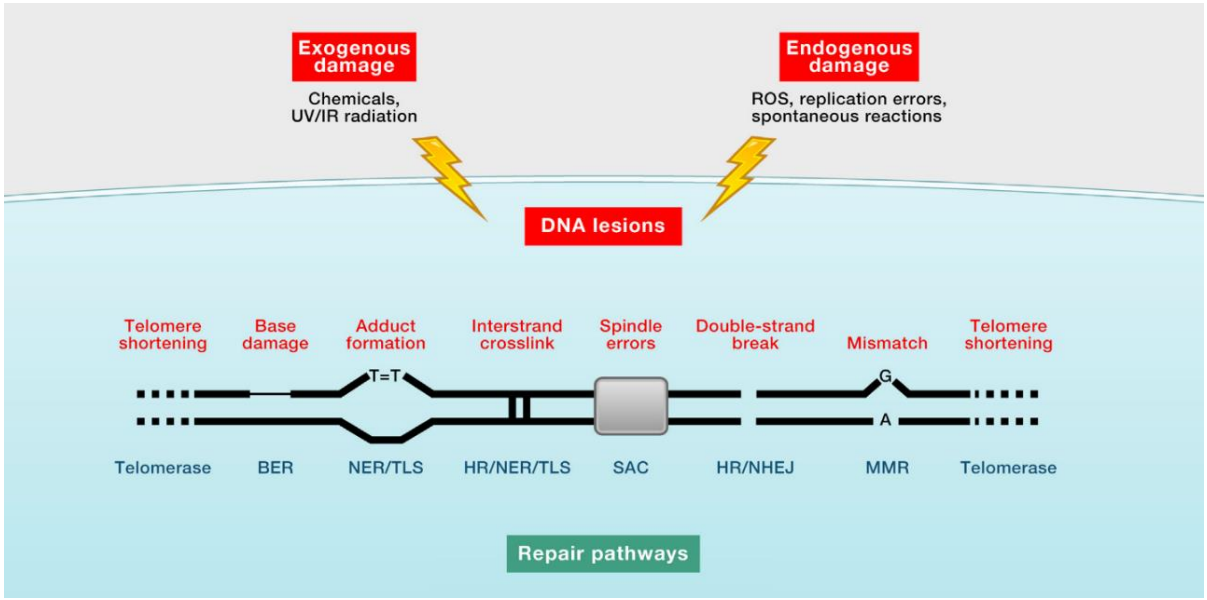

Figure 2 – Genomic instability and telomere attrition
Endogenous or exogenous damage can cause a variety of DNA lesions (schematic representation on a single chromosome). There are several mechanisms involved in the repair of these lesions: BER (base excision repair); HR (homologous recombination); NER (nucleotide excision repair); NHEJ (non-homologous end-joining); MMR (mismatch repair); ROS (reactive oxygen species); TLS (translesion synthesis); SAC (spindle assembly checkpoint). (Adapted from López-otín et al., 2013)

1.2 - Telomere Attrition

Telomeres are repetitive DNA sequences at chromosome ends that are particularly susceptible to age-related deterioration (Bernardes de Jesus & Blasco, 2013; López-otín et al., 2013). Specifically, the attrition or shortening of these regions occurs with aging, common to most adult somatic cells of numerous organisms. Telomeres shorten with each cell division cycle due to, in part, DNA polymerase's inability to completely replicate the terminal ends of linear DNA molecules and the silencing of telomerase in most adult tissues, the only reverse transcriptase capable of such feature (Bernardes de Jesus & Blasco, 2013; López-otín et al., 2013). After subsequent cell division cycles, telomeres length becomes critically short, and cells lose the proliferative ability, reaching a terminal condition known as replicative senescence or Hayflic Limit, a phenomenon first observed in cultures of embryonic fibroblasts (Hayflick & Moorhead, 1961). The correlation between the length of telomeres and aging was further illustrated in genetically modified mice, with shortened or lengthened telomeres corresponding to decreased or increased lifespan, respectively (López-otín et al., 2013). Similarly, telomerase activation strategies have been employed to prevent shortening associated with aging, delay organismal aging, and increase lifespan (Pal & Tyler, 2016; Saul & Kosinsky, 2021).

Some adult stem cells maintain the ability to activate telomerase when they become transit-amplifying cells. However, the level of telomerase activity on stem cells is low, and telomere shortening still occurs during replicative aging, but at a slower rate than that in normal somatic cells (Hiyama & Hiyama, 2007). Still, telomerase activation is crucial for the mobilization of stem cells and the subsequent regenerative capacity of different tissues. Telomerase deficiency in stem cells can lead to the development of premature diseases, such as pulmonary fibrosis, dyskeratosis congenita, and aplastic anemia (Pal & Tyler, 2016; Saul & Kosinsky, 2021).

1.3 - Epigenetic Alternations

Epigenetic alteration is a crucial mechanism behind the age-related deterioration of cellular functions throughout life (Figure 3). Epigenetics refers to the reversible heritable mechanisms that occur without altering the underlying DNA sequence (Pal & Tyler, 2016; Saul & Kosinsky, 2021). It includes alterations in DNA methylation patterns, post-translation modification of histones, and chromatin remodeling (López-otín et al., 2013; Pal & Tyler, 2016; Saul & Kosinsky, 2021). These different types of epigenetic information respond to exogenous and endogenous factors during aging, and thus, they are functionally significant for the process of aging (Pal & Tyler, 2016).

The relationship between DNA methylation and aging is complex, and studies have controversial results. Early reports show an age-associated global hypomethylation. However, later analyses revealed that several loci become hypermethylated with age. Therefore, there is no direct experimental demonstration that altering DNA methylation patterns can extend the organismal lifespan (López-otín et al., 2013; Maegawa et al., 2010).

Regarding histone modification, the elimination of components of histone methylation complexes extends longevity in invertebrates (for H3K4 and H3K27 in nematodes and flies, respectively). Consistently, inhibition of histone demethylases (for H3K27) in worms may extend lifespan by targeting components of key longevity routes such as the insulin/insulin-like growth factor 1 signaling pathway (IIS) (Jin et al., 2011; López-otín et al., 2013). Nonetheless, members of the sirtuin family of NAD-dependent protein deacetylases and ADP ribosyltransferases extensively studied as potential anti-aging factors have controversial results in invertebrates. On the other hand, several of the seven mammalian sirtuin paralogs can improve several aspects of aging in mice. Three members of the sirtuin family, SIRT1, SIRT3, and SIRT6, contribute to healthy aging. The transgenic overexpression of SIRT1 improves aspects of health during aging, including improved genomic stability and enhanced

metabolic efficiency, but does not extend longevity. As for SIRT6, evidence shows a sirtuin-mediated pro-longevity role, which regulates genomic stability, NF-κB signaling, and glucose homeostasis through histone H3K9 deacetylation. SIRT6 deficiency in mutant mice results in accelerated aging while overexpressing it on transgenic mice extends lifespan, associated with reduced serum IGF-1 (Insulin-like growth factor 1) and other indicators of IGF-1 signaling. Likewise, overexpression of SIRT3 has been reported to improve the regenerative capacity of aged hematopoietic stem cells (López-otín et al., 2013).

In addition, protein complexes implicated in chromatin remodeling, such as the heterochromatin protein 1α (HP1α), and chromatin remodeling factors, such as Polycomb group proteins of the NuRD complex, are diminished in both normal and pathologically aged cells (López-otín et al., 2013; Pegoraro et al., 2009). Alterations in these epigenetic factors affect chromatin architecture, leading to a global heterochromatin loss and redistribution, constituting characteristic aging features. This notion is supported by the evidence that flies with loss-of-function mutations in HP1α have a shortened lifespan, while overexpression of this heterochromatin protein extends longevity in flies and delays the muscular deterioration characteristic of old age (Larson et al., 2012; López-otín et al., 2013). Moreover, there is an interesting connection between chromatin formation at repeated DNA domains and chromosomal stability. There is a tendency for heterochromatin assembly at pericentric and telomeric regions, conferring chromosomal stability to these regions. Heterochromatin assembly involves trimethylation of histones H3K9 and H4K20, as well as HP1α binding. Thus, epigenetic alterations can directly exert their control over telomere length, illustrating the interconnection between different hallmarks of aging (López-otín et al., 2013).

Another relevant aging-associated change mediated by epigenetic alterations is the increase in transcriptional noise and an aberrant production and maturation of many mRNAs. Microarray-based comparisons of young and old tissues from different species have identified age-related transcriptional changes in genes encoding crucial components of inflammatory, mitochondrial, and lysosomal degradation pathways (de Magalhães et al., 2009; López-otín et al., 2013). These aging-associated transcriptional signatures also affect noncoding RNAs, including a specialized class of miRNAs (gero-miRs) that is associated with the aging process by targeting components of longevity networks or by regulating stem cell behavior (López-otín et al., 2013).

Theoretically, unlike DNA mutations, epigenetic alterations are reversible, offering opportunities to design novel anti-aging treatments. Thus, manipulating the epigenome is a potential approach for improving age-related pathologies and extending healthy lifespan (López-otín et al., 2013).

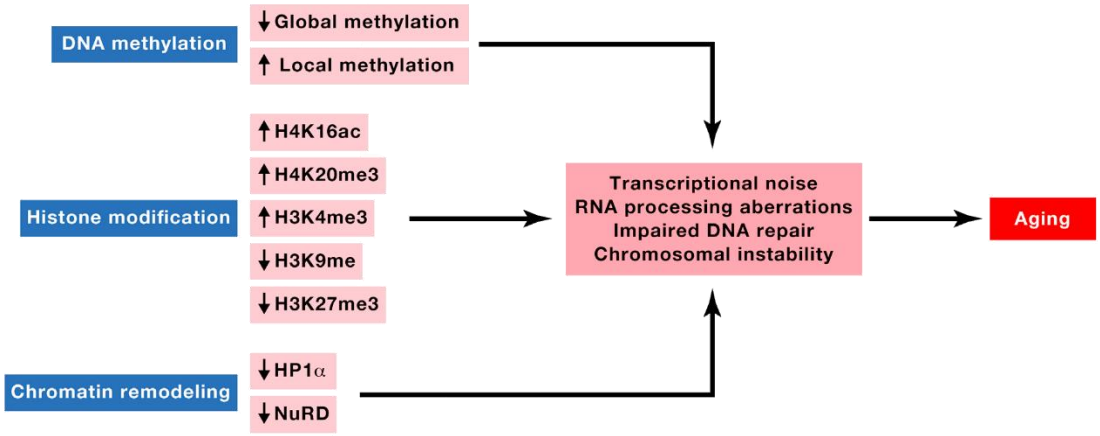

Figure 3 – Epigenetic alterations
Alterations in the methylation of DNA, histone modification (acetylation and methylation), and changes in chromatin-associated proteins can induce epigenetic changes that contribute to the aging process. (Adapted from López-otín et al., 2013)

1.4 - Loss of Proteostasis

Aging and some aging-related diseases, such as Alzheimer's, Parkinson and Huntington's diseases, are associated with the loss of protein homeostasis (Figure 4). Cells have at their disposal quality control mechanisms to preserve the conformational stability and functionality of their proteome. These mechanisms include the chaperones or heat shock proteins to stabilize and maintain the protein's native states (or folded) and proteolytic systems, such as ubiquitin/proteasome system (UPS) and the autophagy/lysosome pathway (ALP) to degrade damaged proteins. These systems function in a coordinated fashion to restore the functional conformation of misfolded proteins or remove and degrade them, thus preventing the accumulation of damaged products and assuring proteome integrity (López-otín et al., 2013).

With aging, there is a deterioration of protein quality systems resulting in a chronic accumulation of misfolded and aggregated proteins that compromise cells' normal functionality and contribute to the development of age-related diseases (López-otín et al., 2013). Stress-induced synthesis of cytosolic and organelle-specific chaperones is significantly impaired in aging (Klaips et al., 2018). Studies support a causative impact of chaperone decline on longevity. Transgenic worms and flies overexpressing chaperones have increased lifespan. Conversely, transgenic mice deficient in a chaperone of the heat shock family presented accelerated aging phenotypes, whereas long-lived strains have an increased expression of the same cochaperone (López-otín et al., 2013; Min et al., 2008; Walker & Lithgow, 2003).

An important reason for the failure to upregulate chaperone transcription with age can be traced to the inability of the master regulator of the heat shock response, the transcription factor HSF-1, to bind to the chaperone gene promoter (Koga et al., 2011). Overexpression of HSF-1 leads to an increase in longevity and thermotolerance in nematodes. Different factors related to longevity can also modulate HSF-1, such as SIRT1, illustrating the integrative nature of the hallmark of aging. SIRT1 was shown to activate HSF-1, whereas SIRT1 downregulation reduces the heat shock response (López-otín et al., 2013). Approaches to maintaining or enhancing proteostasis generally consist in stimulating chaperone activity for protein folding and stability by pharmacological induction or by small molecules employed as pharmacological chaperones (Calamini et al., 2012; López-otín et al., 2013).

The UPS and ALP also decline with age, reinforcing the idea that collapsing proteostasis is a common feature of old age. Increasing the expression or enhancing the activity of various components of the UPS extends the longevity of different model organisms, such as yeast, nematodes, and human cultured cells. Similarly, enhancing the activity of its different components, such as the chaperone-mediated autophagy receptor (LAMP2a), can improve cellular homeostasis and improve the ability to respond to cell damage in old mice livers (Koga et al., 2011).

Furthermore, inducing macroautophagy (the main variant of autophagy) can increase lifespan. Interventions using chemical inducers, such as the mTOR inhibitor rapamycin or spermidine, can prolong longevity in several model animals. The mTOR kinase is a well-known inhibitor of autophagy and is the central regulator of anabolism; therefore, regulation with an mTOR inhibitor is a valid strategy to extend lifespan, not only by improving autophagy but also by affecting other cellular functions, such as mRNA translation, ribosome biogenesis, angiogenesis, mitochondrial metabolism, growth, and others (Koga et al., 2011). Caloric restriction is another intervention for macroautophagy-mediated lifespan extension in worms (López-otín et al., 2013).

Thus aging is associated with a loss of proteostasis as different components of the protein quality system deteriorate with age. Genetic manipulation or pharmacological treatment aims to improve the proteostasis network that can delay aging in various organisms (Koga et al., 2011; López-otín et al., 2013).

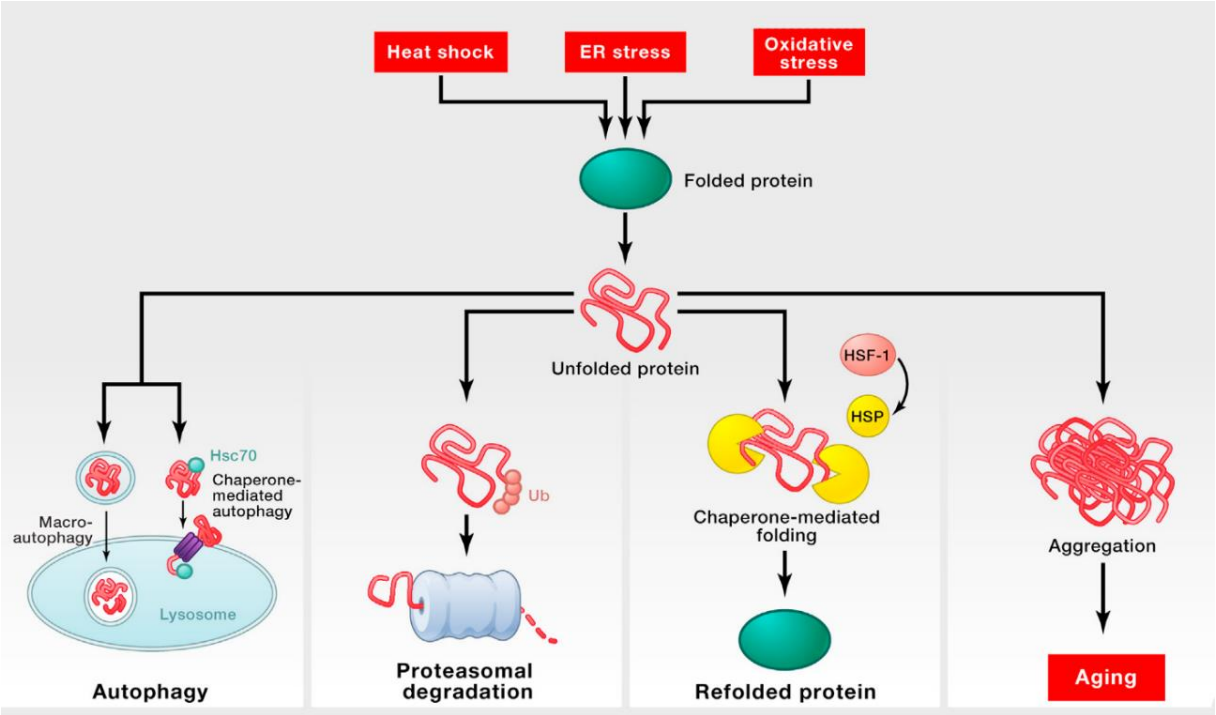

Figure 4 – Loss of Proteostasis
Several forms of cellular stress can cause the unfolding or misfolding of proteins. These proteins are usually refolded by chaperones or targeted to degradation by the ubiquitin-proteasome or lysosomal pathways. If these protein quality systems are unable to refold or degrade, it could lead to their accumulation and aggregation, resulting in proteotoxic effects, which contribute to aging. **Figure abbreviations:** ER stress - Endoplasmic reticulum stress; HSP - heat-shock proteins; HSF-1 – heat-shock factor 1; Ub – Ubiquitin; Hsc70 - Heat shock cognate 71 kDa protein. (Adapted from López-otín et al., 2013)

1.5 - Deregulated Nutrient sensing

The insulin/insulin-like growth factor 1 signaling (IIS) pathway was the first defined genetic pathway demonstrated to regulate aging and age-related disease in model organisms(Z. Ke et al., 2017). It is one of the most conserved age-related evolutionary pathways, with several downstream effectors linked with aging, such as the FOXO family of transcription factors and the mTOR complexes, thus illustrating its significant impact on organismal aging. Attenuating the IIS pathway at different levels via genetic manipulation or dietary restriction improves lifespan in several model organisms. By reducing IIS signaling, pro-longevity FOXO transcription factor homologs (DAF-16) are activated, enhancing longevity in worms and flies by increasing the expression of several genes involved in stress resistance, immune function, and metabolism (López-otín et al., 2013; Pan & Finkel, 2017). Similarly, tumor-suppressor PTEN overexpression in mice and subsequent inhibition of PI3K increases longevity by improving mitochondrial oxidative metabolism and enhancing brown adipose tissue activity (López-otín et al., 2013).

Interestingly, IIS declines naturally in both normal aging and accelerated aging. The reasoning behind this decrease is that it represents a defensive mechanism to minimize cellular damage by lowering cell growth and metabolism. Therefore, physiological or pathological aged organisms decrease IIS signaling to extend their lifespan. However, excessive IIS pathway attenuation may be detrimental, inducing harmful consequences that could be lethal (López-otín et al., 2013).

Similarly, alterations in the IIS pathway affect the mechanistic target of rapamycin (mTOR) signaling, which also significantly influences lifespan. The mTOR kinase is a Serine/Threonine protein kinase from the phosphoinositide 3-kinase-related family, highly conserved among eukaryotes. It is the main target of the immunosuppressive drug rapamycin. mTOR is part of two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), regulating almost all aspects of anabolic metabolism (López-otín et al., 2013). mTORC1, is a metabolic sensor for nutrients, energy, and stress. Its downstream effectors are involved in several processes, such as regulation of ribosomal biogenesis, autophagy, protein translation, lipid synthesis, mitochondrial metabolism, pyrimidine synthesis and modulation of the senescence-associated secretory phenotype (Pan & Finkel, 2017). mTORC1 inhibition significantly extends the lifespan of several model organisms. Its specific inhibitor rapamycin greatly extends longevity in mice, and it is considered the most robust chemical intervention to increase lifespan in mammals. Even with unaffected levels of mTORC2, downregulation of mTORC1 extends lifespan, and mice deficient in ribosomal protein S6 kinase B1 (S6k1, one of the mTORC1 primary substrates) are also long-lived. Therefore, the downregulation of mTORC1/S6K1 appears to be a key mediator of mammalian longevity. Moreover, mTOR activity increases during aging in mouse hypothalamic neurons, contributing to age-related obesity, which is reversed by direct infusion of rapamycin to the hypothalamus. Thus, the intense trophic and anabolic activity signaled through the IIS or the mTORC1 pathways are significant aging accelerators (López-otín et al., 2013).

Conversely, the adenosine monophosphate-activated protein kinase (AMPK) and sirtuins, two other important nutrient sensors, act opposite to IIS and mTOR, signaling nutrient scarcity and catabolism instead of nutrient abundance and anabolism. AMPK and SIRT1 sense low-energy states by detecting high AMP and NAD+ levels, respectively. Their upregulation favors healthy aging. AMPK activation has multiple effects on metabolism and even inhibits mTORC1. SIRT1 deacetylates and activates the PPARγ coactivator 1α (PGC-1α), which then orchestrates a complex metabolic response that includes mitochondriogenesis, enhanced antioxidant defenses, and improved fatty acid oxidation. Furthermore, SIRT1 and AMPK can engage in a positive feedback loop, connecting both sensors of low-energy states into a unified response (López-otín et al., 2013)

1.6 - Mitochondrial dysfunction

As mentioned in genomic instability, mtDNA accumulates point mutations and deletions that hinder the functionality of the mitochondria. A decrease in ATP production accompanies aging due to diminished efficacy of the respiratory chain and the subsequent electron leakage. The most apparent underlying mechanism is increasing reactive oxygen species (ROS) production, which results from mitochondrial dysfunction that occurs with aging and causes further mitochondrial deterioration and cellular damage. However, recent contradictory data have shown that ROS production positively affects aging, forcing a reevaluation of the mitochondrial free radical theory. Studies in yeast and *C.elegans* showed that increased ROS might prolong lifespan (Durieux et al., 2011; López-otín et al., 2013). In mice, genetic manipulations that increase ROS and oxidative damage do not accelerate aging, and increased antioxidants do not affect lifespan (López-otín et al., 2013; Pérez et al., 2009; Y. Zhang et al., 2009). Impairing mitochondrial function without ROS increase accelerates aging in mice. All these data indicate that ROS could have a role in triggering proliferation and survival as a response to physiological and stress conditions. ROS is a stress-elicited survival signal in this framework that increases with age to counter the age deterioration effects and promote survivability. Still, increasing ROS levels beyond a certain threshold could be detrimental and eventually aggravate age-associated damage (López-otín et al., 2013).

ROS independent mitochondrial dysfunction could also contribute to aging via several mechanisms. For example, stress and DNA damage promote permeabilization of mitochondria, which in turn release proapoptotic factors from the intermembrane space, such as cytochrome c, which induces apoptosis via the caspase pathway (Kroemer et al., 2007). Mitochondria permeabilization could also trigger inflammatory reactions by favoring the activation of inflammasomes. In addition, mitochondria dysfunction may directly impact cellular signaling and intraorganellar crosstalk by affecting the interface between the outer mitochondrial membrane and the endoplasmic reticulum (López-otín et al., 2013).

Aging also contributes to the reduced mitochondrial bioenergetics through multiple converging mechanisms. Notably, reduced biogenesis of mitochondria resulting from, among other reasons, telomere attrition and sirtuins age-related dysfunction. Telomere attrition results in subsequent p53-mediated repression of the transcriptional cofactors PGC-1α and PGC-1β, subsequently reducing mitochondriogenesis (López-otín et al., 2013; Sahin & de Pinho, 2012). Telomerase activation can partially reverse the age-related mitochondrial decline in wild-type mice (Bernardes De Jesus et al., 2012). Likewise, SIRT1 modulates mitochondrial biogenesis through PGC-1α and removes damaged mitochondria by autophagy, whereas SIRT3, the main mitochondrial deacetylase, targets many enzymes involved in energy

metabolism, including components of the respiratory chain, tricarboxylic acid cycle, ketogenesis, and fatty acid β-oxidation. Hence, telomeres and sirtuins may control mitochondrial function and thus play a protective role against age-associated diseases (López-otín et al., 2013).

The other mechanisms that underlie dysfunctional mitochondrial bioenergetics are: the accumulation of mutations and deletions in mtDNA oxidation of mitochondrial proteins; destabilization of the macromolecular organization of respiratory chain (super)complexes; changes in the lipid composition of mitochondrial membranes; alterations in mitochondrial dynamics resulting from an imbalance of fission and fusion events; and defective quality control by mitophagy (mitochondrial specific macroautophagy). Thus, increased damage and reduced turnover in mitochondria may contribute to the aging process due to lower biogenesis and reduced clearance (López-otín et al., 2013).

1.7 - Cellular senescence

Cellular senescence is defined as a stable cell cycle arrest coupled with phenotypic changes. Hayflic and Moorhead (1961) first described this phenomenon in cultures of human fibroblast serially passage. However, the senescent effect observed in this study was caused mainly by telomere shortening (Hayflick & Moorhead, 1961). Nowadays, we know that other aging-associated stimuli can trigger senescence independently of the telomeric process. Senescence can be stimulated by the non-telomeric DNA damage and de-repression of the cyclin-dependent kinase inhibitors family and ADP-ribosylation factor (INK4/ARF) locus that gradually occurs with chronological aging (López-otín et al., 2013).

INK4/ARF is the best documented gene locus that controls aging and aging-associated pathologies. This locus encodes three related genes (ARF [also known as $p19^{ARF}$ and $p14^{ARF}$], $p15^{INK4b}$, and $p16^{INK4a}$) that, in turn, encode distinct tumor suppressor proteins (W. Y. Kim & Sharpless, 2006). $p16^{INK4a}$ and $p19^{ARF}$ are particularly important in the induction of cell senescence, as their expression correlates with chronological age. Indeed, levels of $p16^{INK4a}$, and to a lesser extent $p19^{ARF}$, have been reported to increase with aging in humans and mice (W. Y. Kim & Sharpless, 2006; López-otín et al., 2013). The age-dependent increase of these proteins coupled with the observed accumulation of senescent cells in old tissues suggests that $p16^{INK4a}$- and $p19^{ARF}$-induced senescence contribute to physiological aging. This hypothesis is supported by studies in mutant mice with premature aging due to extensive and persistent damage and subsequent dramatic levels of senescence, having their progeroid phenotypes ameliorated by eliminating $p16^{INK4a}$ or p53 (Baker et al., 2011; López-otín et al., 2013).

However, the notion that senescence contributes to aging undervalues what is conceivable as its primary purpose: to prevent the propagation of damaged and stimulate their demise by the immune system. Paradoxically, mice with a mild and systemic increase in $p16^{INK4a,}$ $p19^{ARF}$, or p53 tumor suppressors exhibit extended longevity. Furthermore, the elimination of p53 aggravates the phenotypes of some progeroid mutant mice (López-otín et al., 2013; Matheu et al., 2009).

Therefore, the activation of INK4a/ARF and subsequently p53 can also be regarded as a beneficial compensatory response to avoid the propagation of damaged cells and its consequences on aging and cancer. However, when damage is widespread, the regenerative capacity of tissues can be depleted, and under these extreme conditions, the p53 and INK4a/ARF responses can become deleterious and accelerate aging (López-otín et al., 2013).

1.8 - Stem Cell Exhaustion

One of the most apparent aging traits is the decline of regenerative capacity. For instance, the age-related decline in hematopoiesis reduces adaptive immune cells (immunosenescence) and increases the risk of anemia and myeloid diseases (López-otín et al., 2013; Shaw et al., 2010). Indeed, a functional decline of stem cells has been observed in almost all adult stem cell compartments, including the mouse forebrain, bone, and muscle fibers. The mechanisms underlying the depletion of stem cell reserves with age are not fully understood yet. However, some of the age-associated damages affecting somatic cells could also affect stem cells, leading to reduced cell-cycle activity that correlates with stem cell exhaustion. Indeed, studies with hematopoietic stem cells (HSC) on aged mice revealed an overall reduction of cell-cycle activity, with old HSCs undergoing fewer cell divisions than younger HSC, suggesting an accumulation of damage and overexpression of cell-cycle inhibitory proteins . Accordingly, old HSC depleted of $p16^{INK4a}$ have better engraftment competence than old wild type HSC, due to increased cell-cycle activity. Thus, stem cell exhaustion unfolds as an integrative consequence of multiple types of aging-associated damages that most likely underlies tissue and organismal aging (López-otín et al., 2013).

On the one hand, a deficient proliferation of stem cells could adversely affect the long-term maintenance of the organism. On the other hand, excessive proliferation could also be deleterious by accelerating the depletion of stem cell niches. Therefore, induction of INK4a during aging and decreased serum IGF-1 may reflect an attempt to preserve the quiescence of stem cells. This notion has been demonstrated in *Drosophila* and p21 null mice studies, where

excessive proliferation leads to premature exhaustion of stem cells and accelerated aging (López-otín et al., 2013).

Stem cell rejuvenation is a promising approach to reversing the aging phenotype. Transplantation of stem cells could provide a therapeutic benefit deriving from systemic effects mediated by donor stem cells' secreted factors. Transplantation of muscle-derived cells from young mice to progeroid mice extends lifespan and improves degenerative alterations, even in tissues where donor cells are not detected (Lavasani et al., 2011; López-otín et al., 2013). Likewise, improving stem cell function through pharmacological interventions is another potential approach to extending lifespan. In particular mTORC1 inhibition by rapamycin, which can postpone aging by affecting several mechanisms, may also improve stem cell function in different tissues. Although stem cell exhaustion is likely the ultimate culprit of tissue and organismal aging, stem cell rejuvenation opens the door for promising interventions that could retard and reverse the aging phenotype (López-otín et al., 2013).

1.9 - Altered intercellular communication

Aging is associated with alterations in intercellular communication, either endocrine, neuroendocrine, or neuronal. Several neurohormonal signaling such as renin-angiotensin, adrenergic, and insulin-IGF1 tend to be deregulated in aging. However, the most notable aging-associated change in intercellular communication is the systemic chronic inflammatory status, the so-called "inflammaging". Several reasons underlie inflammaging, such as the accumulation of proinflammatory tissue damage; a progressively more incompetent immune system to eliminate pathogens effectively and dysfunctional host cells; the tendency of senescent cells to secret proinflammatory cytokines; the overactivation of NF-κB transcription factor; or the occurrence of a dysfunctional autophagy response. All these alterations lead to increased activation of the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome and other proinflammatory pathways, resulting in increased production of IL-1β (interleukin-1 beta), tumor necrosis factor, and interferons (López-otín et al., 2013).

The enhanced activation of NF-κB is a transcriptional signature of aging that stresses the relevance of inflammatory pathways in aging. Genetic or pharmacological inhibition of NF-kB signaling prevents age-associated features in various mouse models of accelerated aging, and it can even lead to phenotypic rejuvenation of aged skin in transgenic mice (Adler et al., 2007; López-otín et al., 2013). Moreover, inflammatory and stress responses activate NF-κB in the hypothalamus, resulting in reduced production of gonadotropin-releasing hormone (GnRH).

The decline of this hormone contributes to numerous aging-related changes such as bone fragility, muscle weakness, skin atrophy, and reduced neurogenesis. GnRH treatment prevents aging-impaired neurogenesis and decelerates aging development in mice (López-otín et al., 2013; G. Zhang et al., 2013). These findings emphasize the relevance of inflammatory pathways in aging and suggest that the hypothalamus modulates systemic aging by integrating NF-kB-driven inflammatory responses with GnRH-mediated neuroendocrine effects. This notion also reinforces the role of "inflammaging" in the pathogenesis of several aging-associated diseases such as obesity, type 2 diabetes, and atherosclerosis (López-otín et al., 2013).

Further evidence connecting inflammation and aging is provided by the mRNA decay factor AUF1 (adenylate/uridylate (AU)-rich RNA-binding factor 1), implicated in the halting of the inflammatory response by mediating cytokine mRNA degradation. Studies with AUF1-deficient mice exhibit cellular senescence and premature aging phenotype that is ameliorated by re-expression of this RNA binding factor (López-otín et al., 2013; Pont et al., 2012). Furthermore, AUF1 also contributes to maintaining telomere length by activating the expression of the telomerase catalytic subunit TERT (telomerase reverse transcriptase), reinforcing the intricate relationship between the different hallmarks. \Likewise, sirtuins may also play a role in the inflammatory response. SIRT1 deacetylates components of the inflammatory signaling such as NF-κB to downregulate inflammation-related genes. Hence, reducing SIRT1 levels correlates with the development and progression of many inflammatory diseases, while pharmacological activation of SIRT1 may prevent them (López-otín et al., 2013).

Inflammaging is also associated with a decline of the adaptive immune system (immunosenescence), which aggravates the aging phenotype at a systemic level due to failure to effectively clear infectious agents and host cells. Immunosenescence also correlates with the accumulation of senescent cells since one of the roles of the adaptive immune system is eliminating them. Moreover, inflammaging also inhibits epidermal stem cell function (López-otín et al., 2013).

Finally, to restore the age-related dysfunctional intercellular communication, several interventions may be used to improve cell-cell communication properties lost with aging, such as genetic, nutritional, or pharmacological. These approaches take advantage of the bystander effect (aging-related changes in one tissue can lead to aging-specific deterioration of other tissues) to facilitate the retardation of the aging process significantly by targeting one tissue to affect neighboring tissues (López-otín et al., 2013).

2 - Hypothalamus

The hypothalamus is a small, central region of the human brain located under the thalamus, separated by the hypothalamic sulcus of Monro (Figure 5) (Gabriela Pop et al., 2018). It is composed of nerve fibers and a conglomerate of nuclear bodies that receive many external and internal signals from virtually all organs in the form of hypothalamus-end-organ axes (Chen et al., 2015; Gabriela Pop et al., 2018). These neurosecretory cells interpret and integrate all the information and respond accordingly to the body's needs while maintaining the overall homeostasis (Chen et al., 2015). The hypothalamus is involved in many physiological functions through its neuronal networks, such as thermoregulation, the circadian rhythm, reproduction, development, hormonal regulation, and energy and water homeostasis (Chen et al., 2015; Gabriela Pop et al., 2018; K. Kim & Choe, 2019).

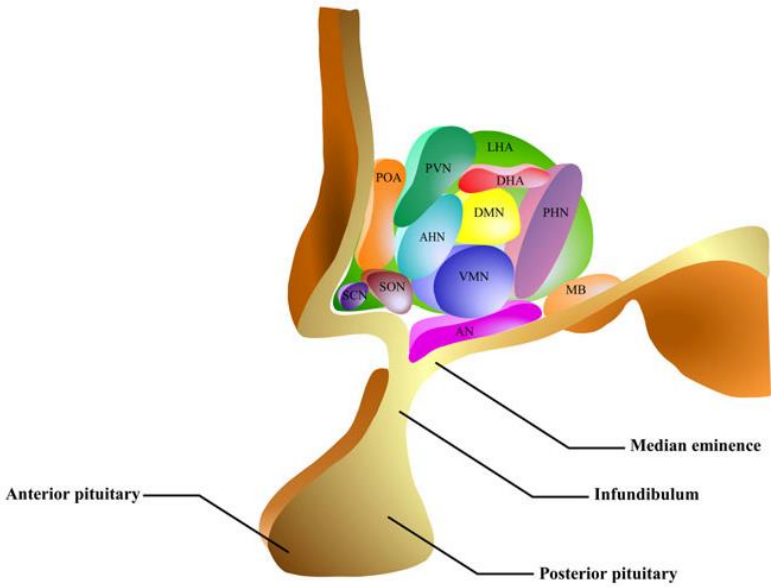

Figure 5 - Schematic representation of hypothalamic nuclei (sagittal section)
AN or Arc: arcuate nucleus; VMN: ventromedial nucleus; DMN: dorsomedial nucleus; PVN: periventricular nucleus; DHA: dorsal hypothalamic area; PFA: perifornical area; LHA: lateral hypothalamic area; SCN: suprachiasmatic nucleus; SON: supraoptic nucleus; POA: preoptic area; MB: mammillary bodies; ME: median eminence. (adapted from Elizondo-Vega et al., 2015)

2.1 - Hypothalamus: a master regulator of systemic aging

Hypothalamus controls most physiological functions that decline with aging (Figure 6), such as development, metabolism, reproduction, circadian rhythm, and homeostasis. Furthermore, It connects the neuroendocrine system to physiological processes, allowing environmental factors influencing aging to be transmitted to peripheral organs, thus affecting systemic aging. Therefore, the hypothalamus is hypothesized to be the master regulator for systematic aging. Understanding the underlying cellular mechanisms (Figure 6) is crucial for developing anti-aging and aging-control technologies (K. Kim & Choe, 2019).

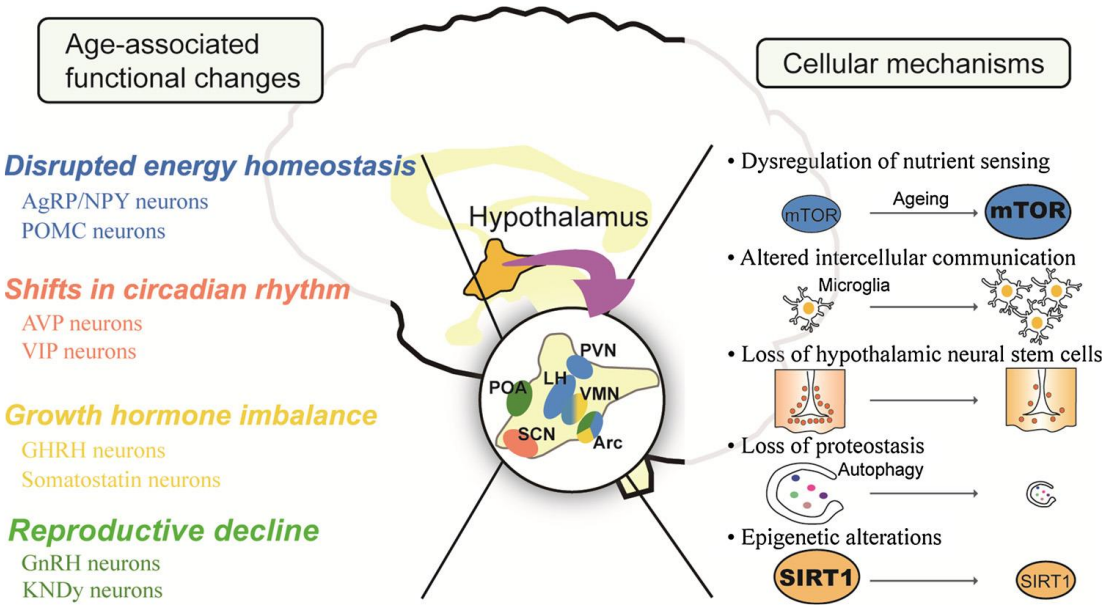

Figure 6 - The hypothalamus as a regulator of systemic aging
The Hypothalamus controls several aspects of systemic aging. The age-associated functional changes include disruption of energy homeostasis, shifts in the circadian rhythm, imbalance in GH levels, and decline in reproduction, which is mediated through age-associated changes in the master regulatory neurons, such as the AgRP/NPY,POMC, AVP, VIP, GHRH, SST, GnRH, and KNDy neurons. In addition, the hypothalamus is where most of the signaling pathways implicated in the aging process are altered with aging, such as nutrient sensing, inflammation, neural stem cell, proteostasis, and epigenetic regulation. **Abbreviations:** GH – Growth hormone; AgRP/NPY - agouti-related peptide/neuropeptide Y ; POMC - pro-opiomelanocortin ; AVP - Arginine vasopressin; VIP - vasoactive intestinal peptide; GHRH - growth hormone-releasing hormone; SST - somatostatin ; GnRH - Gonadotropin-releasing hormone; KNDy - Kisspeptin/neurokinin B/dynorphin; mTOR - Mammalian target of rapamycin; SIRT1 -sirtuin 1; PVN - periventricular nucleus; POA - preoptic area; SCN - suprachiasmatic nucleus; LH - lateral hypothalamic area; VMN - ventromedial nucleus; Arc - arcuate nucleus. (adapted from K. Kim & Choe, 2019)

2.2 - Age-associated functional changes

2.2.1 - Disrupted energy homeostasis

The hypothalamus regulates energy homeostasis by effectively and efficiently controlling the neuroendocrine machinery that integrates metabolic feedback (Jais & Brüning, 2017). With aging, the effectiveness and efficiency of the neuroendocrine machinery decline, leading to a shift in metabolic processes and energy balance. The increasing prevalence of metabolic syndromes in aged people illustrates this age-related disruption of energy homeostasis. One such syndrome resulting from the variation in metabolic activity with aging is sarcopenic obesity, characterized by increased body fat and a progressive and generalized loss of skeletal muscle mass, strength, and physical function (Gonzalez et al., 2021; K. Kim & Choe, 2019). Curiously, aging is also accompanied by a general loss of appetite, contrasting with the observed age-induced obesity, which indicates that the imbalance between feeding behavior and energy expenditure stems from age-related changes affecting hypothalamic regulator neurons of energy metabolism. These are neurons located in the arcuate nucleus (Arc), neurons expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY), and neurons expressing proopiomelanocortin (POMC). The antagonistic interaction between AgRP/NPY and POMC neurons constitutes the central metabolic controlling axis. The AgRP/NPY neurons control orexigenic behaviors via their respective peptides and energy expenditure by inhibiting the thermogenesis of the brown adipose tissue. In contrast, the POMC neurons exert an anorexigenic effect by releasing α-melanocyte-stimulating hormone (α-MSH) (K. Kim & Choe, 2019).

Several studies supported that changes in AgRP/NPY and POMC neurons underlie the disruption of energy homeostasis with aging. Studies in aged mice showed a significantly decreased activity of anorexigenic POMC neurons (Yang et al., 2012). In addition, increasing POMC expression via adeno-associated virus (AAV) infection in the basomedial hypothalamus of aged rats reverted the age-related changes in food intake, weight gain, and peripheral fat mass. As for the orexigenic neurons, studies in aged rats revealed a reduction in NPY peptide and receptor levels. AgRP neurons seem to have their activity affected depending on the feeding status. Evidence suggests that AgRP RNA level does not change in regularly fed rats. However, AgRP RNA levels drastically reduced in response to fasting (K. Kim & Choe, 2019).

The reported data correlates with the observed age-dependent obesity and loss of appetite. The decreased activity of POMC neurons seems to be crucial for age-related obesity. The central application of α-MSH can induce more bodyweight loss and extended periods of reduced food intake in aged rats than in young rats. As for the loss of appetite, the global age-related changes

in AgRP/NPY neurons suggest that they differentially affect energy metabolism dependent upon feeding status (K. Kim & Choe, 2019).

2.2.2 - Growth hormone imbalance

Among the many functions of the hypothalamus, growth and development control is one of the most important. Hypothalamus achieves this by timely controlling the production and release of growth hormone-releasing hormone (GHRH) and somatostatin (SST) into the portal vessel to regulate the production and secretion of the growth hormone (GH) from the pituitary gland. The GHRH neurons in the Arc (arcuate nucleus) and SST neurons in the ventromedial nucleus (VMN) project their axonal terminal into the median eminence to access the portal vessel and release their respective hormones, characterized by their antagonistic functions. GHRH stimulates GH production and releases by binding to the GHRH receptor (GHRHR) on somatotropes cells in the anterior pituitary. At the same time, SST inhibits GH secretion in the somatotropes cells via GH negative feedback loop (K. Kim & Choe, 2019).

As its name suggests, the growth hormone most characterized role is stimulating longitudinal bone growth. In addition, GH impacts numerous physiological systems that include prominent roles in cell growth, differentiation, and metabolism in different tissues, including liver, adipose tissue, skeletal muscle, and bone (Bartke, 2021a; Chia, 2014; K. Kim & Choe, 2019). GH exerts its effects by influencing gene expression profiles. IGF-1 is a critical transcriptional target of GH signaling in the liver and other tissues, as it is the primary mediator of GH's effects (Chia, 2014).

High levels of GH/IGF-1 are crucial for cell growth in the early stages of development and adolescence. Blood levels of GH/ IGF-1 remain low throughout childhood and reach the highest during adolescence, and then it gradually declines to become almost negligible in the elderly. The age-associated decline of GH/IGF-1 can cause a reduction in protein synthesis, a decrease in lean body mass, and a decline in immune function (Chahal & Drake, 2007). In addition, based on GH metabolism-enhancing effects, GH/IGF-1 decline might contribute to sarcopenic obesity in aged individuals. GH treatment of older adults with low plasma IGF-1 levels increases lean body mass and bone density while decreasing adipose tissue mass (K. Kim & Choe, 2019)

Paradoxically, GH/IGF-1 age-related decline is associated with increased lifespan (López-otín et al., 2013). Transgenic mice overexpressing GH have consistently exhibited a shorter lifespan than control wild-types (K. Kim & Choe, 2019). According to López-Otín et al. (2013), the GH/IGF-1 and subsequently IIS pathway decline represents a defensive response aimed at

minimizing cell growth and metabolism to reduce rates of cellular damage and ultimately extend longevity (López-otín et al., 2013). These contradictory observations illustrate the critical role of the hypothalamus in aging by timely controlling the GH release throughout life.

The age-associated decline in GH/IGF-1 levels remains unclear. However, studies in aged rates revealed that the alterations of expression of GHRH/SST and sensitivity of somatotrophs are possible clues for the age-dependent decrease in plasma GH levels (K. Kim & Choe, 2019).

2.2.3 - Shifts in the circadian rhythm

The circadian rhythm plays a vital role in health by providing an adaptive mechanism for organisms to coordinate cellular processes, physiological functions, and behaviors with the predictable daily 24-hour cycle of light and dark prevenient from Earth's rotation (Acosta-Rodríguez et al., 2021; Hood & Amir, 2017; K. Kim & Choe, 2019; Kondratova & Kondratov, 2012). The circadian rhythm machinery regulates several physiological processes, including the prominent sleep and wake cycle and the daily oscillations of metabolism, hormone secretion, and cardiac function, to name a few (K. Kim & Choe, 2019; Kondratova & Kondratov, 2012).

The central circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. In the SCN, two major neuronal populations, the arginine vasopressin (AVP) positive neurons, and the vasoactive intestinal peptide (VIP) neurons, form a synchronized neuronal network responsible for maintaining the 24h oscillation by synchronizing internal processes with environmental timing cues (Acosta-Rodríguez et al., 2021; K. Kim & Choe, 2019; Kondratova & Kondratov, 2012). The internal coordination of multiple oscillators within and between various organ systems is critical for organismal fitness while providing the most efficient response to environmental cues, ensuring optimal organismal adaptation (Acosta-Rodríguez et al., 2021; Kondratova & Kondratov, 2012).

With increasing age, the circadian system undergoes significant changes that detrimentally affect rhythms of behaviors, sleep quality, cognitive function, temperature regulation, hormone release, metabolism, and inflammation. Dysfunction of the circadian rhythm is associated with significant increases in the risk of developing metabolic syndromes, cardiovascular diseases, chronic inflammation, neurodegenerative diseases, and cancer (Hood & Amir, 2017; Kondratova & Kondratov, 2012).

Among several age-associated changes contributing to the disruption of circadian rhythm, age-related changes in SCN have a considerable impact. Evidence suggests that the number of AVP-positive decreases in aged rats. Additionally, the synchronized electric activity generated from

the SCN, mediated by the coordinated activity of AVP and VIP neurons, decreases with aging (K. Kim & Choe, 2019). Thus, the age-dependent changes in AVP and VIP neurons that underlie the desynchronization of the master clock could be crucial in systematic aging.

2.2.4 - Reproductive decline

The hypothalamus-pituitary–gonad axis (HPG axis) plays a crucial role in regulating mammalian reproduction (K. Kim & Choe, 2019). At the center of HPG axis is the machinery driving the pulsatile Gonadotropin-releasing hormone (GnRH) secretion, the so-called GnRH pulse generator; consisting of the intrinsic property of the GnRH neurons located in the pre-optic area of the hypothalamus to generate coordinated bursts of GnRH release (Krsmanovic et al., 2009). The secreted GnRH is released into the portal vessel to stimulate the synthesis and pulsatile secretion of gonadotropic hormones, luteinizing hormone (LH), and follicle stimulation hormone (FSH) from the anterior pituitary gland (K. Kim & Choe, 2019). These gonadotropic hormones have multiple functions within the reproductive system, such as stimulating the secretion of gonadal steroids and promoting the maturation of gonads (R. Ke et al., 2019).

The natural decline in reproductive functions with age is associated with the dysfunction of the HPG axis. In particular, hypothalamic defects involving an altered release of GnRH constitute a significant contributor to HPG axis dysfunction (Brann & Mahesh, 2005). In humans, female reproductive aging is characterized by menopause, which involves a dramatic reproductive and endocrinological shift, leading to the cessation of the menstrual cycle and, subsequently, the end of fertility. As for males, both humans and rodents experience an aging-associated decrease in reproductive activity, testosterone, gonadotropins, and GnRH, while preserving their fertility, to some extent. The activities of the GnRH pulse generator, as reflected in the pulsatility of LH, are significantly reduced in aged animals in both females and males, supporting the idea that the GnRH pulse generator is a crucial mediator of reproductive aging. In addition, decreases in sex hormone level and their pulsatility contributes to other aging phenotypes, such as metabolic disturbance and cognitive impairment (K. Kim & Choe, 2019).

Although the number of GnRH neurons was found not to change with age, KNDy (Kisspeptin/neurokinin B/dynorphin) neurons that are part of regulatory feedback loop upstream GnRH pulse generator decrease with age in both female and male rats. Therefore, age-related changes in hypothalamic neurons constituting the GnRH pulse generator and its regulators contribute to reproductive aging (K. Kim & Choe, 2019).

2.2.5 - Underlying cellular mechanisms

There are several underlying mechanisms for the hypothalamus-mediated aging progression (**Erro! A origem da referência não foi encontrada.**), and these can be correlated with the following López-otín et al.(2013) hallmarks of aging: deregulated nutrient sensing (mTOR), altered intercellular communication (NF-κB), stem cell exhaustion (htNSC - hypothalamic neural stem cells), proteostasis loss (Autophagy), and epigenetic alterations (SIRT1)(K. Kim & Choe, 2019; López-otín et al., 2013).

Table 1 – A summary of the critical factor underlying hypothalamus-mediated aging. Adapted from Kim & Choe, 2019

Cellular substrate	Hypothalamic region/neuron	Levels in old	Related physiological effect
mTOR	POMC neurons	Increased activity	Obesity, food intake, glucose tolerance, leptin level
NF-κB	MBH	Increased activity	Longevity, cognition, and muscle endurance
htNSC	MBH	Decreased number	Longevity, cognition, and muscle endurance
Autophagy	POMC neurons	Decreased activity	Obesity, glucose tolerance, insulin tolerance
SIRT1	SCN	Decrease	Circadian rhythm
	DMH, LHA	Transgenic overexpression	Longevity, circadian rhythm

Abbreviations: POMC – proopiomelanocortin; **MBH** - Mediobasal Hypothalamus ; **SNC** - Suprachiasmatic nucleus; **DMH** - Dorsomedial hypothalamic nucleus; **LHA** – lateral hypothalamic area.

2.2.6 - mTOR (Nutrient Sensing)

The mammalian target of rapamycin (mTOR), as the catalytic subunit of two protein complexes, mTORC1, and mTORC2, is a central regulator of metabolic processes (K. Kim & Choe, 2019; López-otín et al., 2013; Sabatini, 2017). Both of its protein complexes are crucial for energy homeostasis. mTORC1 acts as a sensor of energy status in the hypothalamus and regulates feeding behavior and body weight via leptin and ghrelin signaling, adipogenesis and gluconeogenesis in various peripheral tissues. On the other hand, mTORC2 controls neurons' size, number and morphology, and energy balance in the hypothalamus (K. Kim & Choe, 2019; Pena-Leon et al., 2020).

The mTOR activity in POMC neurons increases with aging, as shown by studies in aged mice (Yang et al., 2012). The increased mTOR signaling contributes to age-dependent obesity by enlarging POMC neuronal soma and decreasing its neurite projection to the paraventricular nucleus (PVN), impairing POMC's anorexigenic effects (Mori et al., 2009). In addition, mTOR inhibition via intracerebral injection of rapamycin reduces food intake and body weight by improving the excitability and neurite projection of POMC neurons (Harrison et al., 2009).

Thus, mTOR inhibition could be a valid therapeutic strategy to ameliorate age-related hypothalamic disruption of energy homeostasis and subsequent age-dependent obesity.

2.2.7 - NF-κB (Intercellular communication/ Inflammation)

Emerging data reveals that microglial activation plays a pivotal role in developing hypothalamic inflammation. Activation of microglial cells is highly susceptible to perturbations in homeostatic balances in the brain, triggering an inflammatory response through pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), Interleukin 6 (IL-6), and interleukin-1 beta (IL-1β). Excessive and prolonged microglial activation in the hypothalamus affects its functionality and structural levels, leading to a loss of hypothalamic homeostatic regulation (K. Kim & Choe, 2019).

Studies in aged mice reveal that age-associated hypothalamic inflammation is mediated by increased activity of IκB kinase-β (IKK-β) and nuclear factor κB (NF-κB) in the microglia and, subsequently, in nearby neurons through the microglia–neuron interaction in the mediobasal hypothalamus. Inhibiting the activity of IKK-β or NF-κB in the hypothalamus or brain can delay aging phenotype and improve lifespan. In addition, the age-associated decline in GnRH might partly be due to the increased IKK-β and NF-κB activity, which significantly downregulates GnRH transcription. Age-associated decrease in GnRH contributes to reproductive dysfunction and is also associated with metabolic syndromes, obesity, and type 2 diabetes (K. Kim & Choe, 2019). Hence, an age-dependent increase in hypothalamic inflammation mediated by increased IKK-β and NF-κB activity could play a significant role in systematic aging.

2.2.8 - Hypothalamic neural stem cells (stem cell exhaustion)

The decrease in neurogenesis is one of the most apparent physiological changes in the aging brain, resulting in a diminished generation of neurons in adulthood. Studies on aged mice revealed that hypothalamus neural stem cells (htNSC) decrease significantly with aging. The study reported on the role of htNSC in systematic aging by generating two separate mouse models by injecting into the hypothalamus with two different lentivirus vectors for the depletion of two specific htNSC, Bmi1- and Sox2-positive stem cells. The Bmi1+ cell-depleted mice presented a significant decline in physiological functions, including decreased muscle endurance, cognition, coordination, sociality, and spatial memory, while Sox2+ cell-depleted mice exhibited a reduced lifespan (Y. Zhang et al., 2017). Additionally, evidence suggests that htNCS is directly associated with energy homeostasis regulation (Kokoeva et al., 2005). Thus,

the loss of htNCS with aging might also contribute to the disruption of energy homeostasis in the hypothalamus.

Furthermore, age-related loss of neural stem cells could also contribute to neuroinflammation and systemic physiological changes accompanying aging. Studies with exosomes from the adipose-derived stem (ADSC) revealed that ADSC exosomes could inhibit the activation of microglia cells by suppressing NF-kB and MAPK pathways(Feng et al., 2019). Although htNCS and ADSCs are different stem cells, they might share similar exosome-mediated immunosuppressive effects. In addition, microRNAs secreted from htNCS exosomes might restore GnRH secretion contributing to the anti-aging effects of these cells (Y. Zhang et al., 2017). Therefore, the loss of htNCs plays a crucial role in aging by contributing to hypothalamic inflammation and systemic physiological changes that accompany aging. Furthermore, exosomal miRNAs could be essential for the role of htNSCs in the control of aging.

2.2.9 - Autophagy (Loss of proteostasis)

Autophagy is a critical process in maintaining cellular homeostasis, and thus it is vital for the hypothalamus as the master regulator of homeostasis. Autophagy maintains cellular homeostasis by regulating the turnover of cytoplasmatic components (Aveleira et al., 2015). Notably, basal autophagy is essential for maintaining the quality of proteins and organelles and, therefore, fundamental for preserving cell function and growth (Coupé et al., 2012). The basal autophagy activity decreases with aging, and its deficiency contributes to different aspects of the aging phenotype and aggravation of age-related diseases (Aveleira et al., 2015; K. Kim & Choe, 2019). In particular, decreased hypothalamic autophagy might contribute to age-related metabolic dysregulation (Kaushik et al., 2012).

Studies in aged mice suggest that age-related decline in hypothalamic autophagy is associated with age-dependent reduction of neuropeptide Y (NPY) levels, one of the major neuropeptides present in the hypothalamus (Aveleira et al., 2015). Transgenic rats overexpressing NPY exhibited improved resistance to stress and increased lifespan (Michalkiewicz et al., 2003). Furthermore, NPY overexpression can stimulate autophagy in hypothalamic neuronal cells and mice hypothalamus. Aveleira and colleagues observed that NPY increased autophagic activity in hypothalamic neurons through activation of NPY Y1 or Y5 receptors. The activation of these receptors is mediated by the PI3K (Phosphoinositide 3-kinases), MEK/ERK (Mitogen-activated protein kinase/ extracellular-signal-regulated kinase), and PKA (protein kinase A) signaling pathways (Aveleira et al., 2015). This result illustrates that NPY plays a relevant role in aging and lifespan and that the stimulation of hypothalamic autophagy could be the underlying mechanism.

Additionally, the decreased hypothalamic autophagy might contribute to the metabolic dysregulation observed with age through autophagosome-dependent mechanisms in the nutrient-sensing hypothalamic neurons, affecting nutrient-driven peptide processing and secretion (Kaushik et al., 2012). A study with typically aged mice reported a reduction in hypothalamic ATG7 (Autophagy Related 7) and LC3-II (microtubule-associated protein light chain 3 – II) levels and decreased autophagy flux rates, suggesting reduced autophagosome content and autophagolysosomal fusion. These mice also presented a significant accumulation of p62/SQSTM1 (sequestosome 1) predominantly within POMC neurons, indicating increased sensitivity of POMC neurons to reduced autophagy with age. In the ATG7 knockout mice, it was shown that reduced autophagy during aging increased hypothalamic POMC protein levels and reduced those of α-MSH. Furthermore, the ATG7 knockout mice presented similar defects in adiposis and lipolysis as aged mice, as evidenced by increased body weight, fat mass, and reduced fasting-induced in serum-free fatty acids and glycerol compared with young mice. These results highlight the importance of POMC neuronal autophagy for maintaining energy balance mediated by α-MSH and that the decreased autophagy in POMC neurons might contribute, in part, to the metabolic disturbances observed during aging (Kaushik et al., 2012; K. Kim & Choe, 2019).

2.2.10 - Sirtuin 1 (epigeneic alterations)

SIRT1 is a NAD-dependent deacetylase that controls several physiological pathways and is particularly important for healthy aging (K. Kim & Choe, 2019; López-otín et al., 2013). Studies with Brain-specific SIRT1-overexpressing (BRASTO) transgenic mice reported a significant increase in median and maximal lifespan in both males and females. Compared to the wild-type control, these mice also exhibited enhanced physiological phenotypes in physical activity, body temperature, oxygen consumption, and sleep quality (K. Kim & Choe, 2019). The underlying mechanism of these phenotypes might be attributed to the enhancement of the neural activity in the dorsomedial and lateral hypothalamic nuclei (DMH and LHA, respectively), through the augmented expression of orexin type 2 receptor (Ox2r) by SIRT1 and its partner NKX2-1 (NK2 homeobox 1) (K. Kim & Choe, 2019).

Furthermore, SIRT1 regulates the circadian rhythm in the SCN by activating the transcription of two essential circadian clock components, BMAL1 (brain and muscle ARNT-Like 1), and CLOCK, thus amplifying the magnitude of the molecular clock machinery. Evidence suggests that the expression level of SIRT1 in the SCN decreases during aging. Additionally, the silencing of the Sirt1 gene in the brain of young mice phenocopies age-related variations of the circadian rhythm, which the brain-specific overexpression of SIRT1 can reverse. Hence, SIRT1

decrease with aging might be strongly implicated in disrupting the circadian rhythm as an underlying mechanism (K. Kim & Choe, 2019).

3 - Protein synthesis and Lifespan control

Proteins are the main executor biomolecules in the cellular arsenal of numerous organisms, ranging from the simple unicellular prokaryotes to complex multicellular eukaryotes (Charmpilas et al., 2015). Almost all physiological processes are dependent on proteins, and therefore the preservation of proteome integrity is imperative for the organism life. Several specialized factors are dedicated to preserving the cellular proteome integrity, starting from the pre-translation process to the end of the protein functional lifecycle. The maintenance of cellular homeostasis directly correlates with maintaining a precise balance of synthesis, degradation, and function of every protein. When this balance is perturbed, damaged proteins accumulate progressively, leading to a detrimental state and even cell death. The shift in this balance naturally occurs with aging as the integrity and effectiveness of protein synthesis machinery and proteostasis network systems gradually diminish due to the inevitable accumulation of damage with age (Anisimova et al., 2018; Charmpilas et al., 2015). Thus, understanding how protein synthesis, maintenance, and degradation systems change with age is fundamental for understanding aging and lifespan control mechanisms.

3.1 - mRNA translation

To understand how age affects protein synthesis, it is necessary to elucidate the mechanisms by which translational components function and their interplay with the different signaling pathways governing translation control (Figure 7). mRNA translation is a crucial and ubiquitous process in almost all organisms. In the translation process, the genetic codons are translated from mRNA to protein by ribosome translocation after the genetic information contained in DNA is transcribed to the mRNA. The translation process involves three leading players: the mRNA (genetic template), the ribosome (assembly machinery), and the aminoacyl transfer RNAs (aa-tRNAs), and is conceptually divided into three phases: initiation, elongation, and termination (Zhao & Krishnan, 2014).

Different signaling pathways tightly regulate each phase of the translation process. However, the central signaling pathway regulating the whole process is the TOR pathway. mTOR

integrates four significant input signals, nutrient and energy supply, growth factors, and stress, to modulate translation control according to the cell's metabolic needs (Tavernarakis, 2008).

Upon activation, mTOR primarily regulates translation initiation, the rate-limiting step of protein synthesis (Figure 7). mTOR stimulates translation initiation by phosphorylating 4E-BP (the eukaryotic initiation factor eIF4E binding protein), leading to its dissociation from eIF4E. Unbound eIF4E recognizes and binds to the 5′ cap structure of mRNAs and, together with eIF4G and eIF4A, forms the eIF4F complex (Gonskikh & Polacek, 2017).

After that eIF4G in the eIF4F complex interacts with the poly(A)-binding protein (PABP) at the mRNA 3'end. The interaction between eIF4G, eIF4E, and PABP is crucial for the circularization of the mRNA by bringing together the 5'- and 3'-ends. In addition, eIF4G also recruits eIF4A to the cap for the local unwinding of mRNA secondary structure elements. The unwinding of mRNA and interactions of eIF4G with eIF3, eIF5, or eIF1 facilitates the recruitment of the 43S pre-initiation complex. Hence, the interaction between eIF4E and eIF4G is considered critical for initiating mRNA translation (Gonskikh & Polacek, 2017).

The pre-initiation complex then incorporates the initiator methionyl-tRNA (Met-tRNA) bound on eIF2 and assembles with the 60S ribosomal subunit at the ATG start codon to form the 80S initiation complex, releasing the translation initiation factors. The 80S ribosome then initiates elongation of the polypeptide chain with the assistance of two eukaryotic translation elongation factors (eEFs). eEF1 supplies the ribosome with the appropriate amino acid-loaded tRNA, while eEF2 promotes the GTP-dependent translocation of the ribosome along the mRNA (Troulinaki & Tavernarakis, 2009). However, eEF2 is inactivated by the highly specific eEF2 protein kinase through the phosphorylation of its Threonine 56. It is by activating eIF2 that mTOR also controls translation elongation. The activation of mTOR promotes phosphorylation of the eEF2 kinase at least at three serine residues, leading to its inactivation. As a result, mTOR activates eEF2 and enhances translation elongation (Gonskikh & Polacek, 2017). mRNA translation is terminated upon encountering a stop codon. The eukaryotic release factor (eRF) mediates the ribosome dissociation from the mRNA and releases the 40S and 60S ribosomal subunits (Tavernarakis, 2008).

Furthermore, mTOR regulates translation through two additional targets, the S6 kinases, S6K1, and S6K2. Activating these kinases by mTOR leads to subsequent phosphorylation of several translation machinery components, such as the 40S ribosomal protein S6, eIF4B, and eEF2 kinase. S6 kinases are also activated by the Insulin/IGF-1 signaling (IIS), and thus it is another signaling pathway involved in the translational control. IIS pathway through the insulin receptor activates a downstream cascade of kinases. One of the activated kinases, Akt,

activates the mTOR signaling pathway, while the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) stimulate the S6 kinase (S6K), which phosphorylates the small ribosomal subunit protein S6 (Anisimova et al., 2018; Gonskikh & Polacek, 2017).

ERK1 and ERK2, together with the p38 mitogen-activated protein kinases (p38 MAPK), also activate another component of the translation machinery: mitogen-activated protein kinase-interacting kinase (Mnk1). Mnk1 is a member of the eIF4F complex, and it binds directly to eIF4G. Mnk1 phosphorylates eIF4E stabilize its interaction with eIF4G and 5′ cap of mRNA, enhancing in such a way translation initiation. Thus, the mitogen-activated protein kinases (MAPK) pathway through the p38 protein kinase can also regulate mRNA translation (Anisimova et al., 2018; Gonskikh & Polacek, 2017).

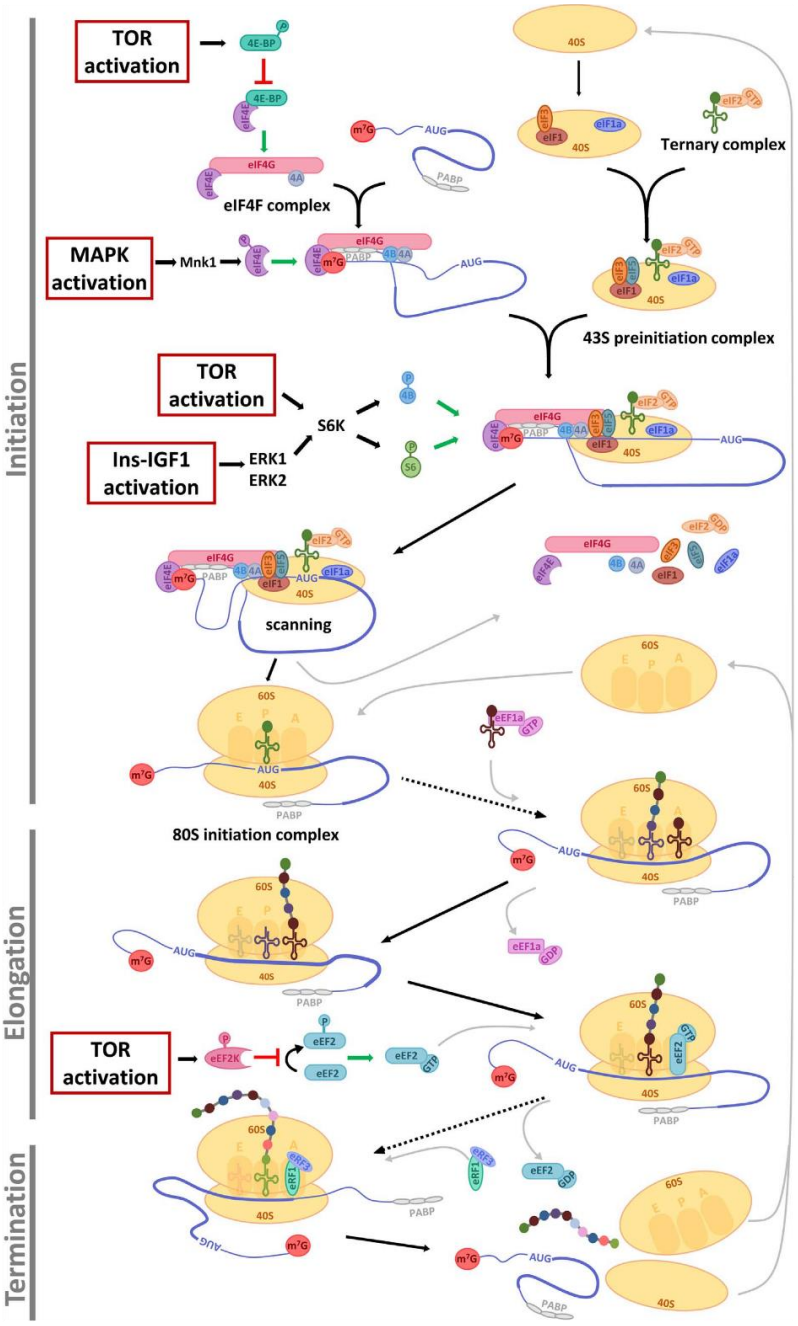

Figure 7 – Schematic overview of the eukaryotic mRNA translation process, highlighting the aging signaling pathways that regulate the initiation elongation and termination phases of mRNA translation.

Red bar lines represent the inhibitory effect, while green arrows represent stimulatory effects. Activation of TOR signaling stimulates translation initiation via phosphorylation of 4E-BP, dissociating from eIF4E. In turn, eIF4E binds to eIF4G forming the eIF4F complex. TOR phosphorylates and inactivates eEF2 kinase, enhancing translation elongation. Activation of ERK1 and ERK2 through IIS pathway, and together with TOR activates S6K, which enhances translation initiation by phosphorylating the small subunit protein S6 and eIF4B. MAPK activation leads to activation of MnK1, which in turn phosphorylates eIF4E and thus stimulates translation inhibition. (adapted from Gonskikh & Polacek, 2017)

3.2 - Changes in protein synthesis rate and translation machinery with age

Several studies have established that overall protein synthesis rates decline with aging in various cells, tissues, organs, and organisms. These studies monitored protein synthesis by the *in vitro* incorporation of radioactively labeled amino acids using cell-free extracts derived from different organisms. Most of these studies reported a significant decrease in protein synthesis (from 20 to 75%) with increasing age (Gonskikh & Polacek, 2017). In addition, these studies observed an attenuated activity and levels of crucial initiation (eIF) and elongation (eEF) factors and a decreased ribosome abundance (Figure 8). They also observed a reduction in mitochondrial protein synthesis activity with aging (Anisimova et al., 2018; Tavernarakis, 2008).

In particular, it was observed that the amount of eukaryotic initiation factor 2 (eIF2) relative to total protein diminishes with increasing age in several rat tissues. eIF2 plays a crucial role in the first step in peptide-chain initiation, the binding Met-tRNAiMet to the 40S ribosomal subunit, to assemble the 43S pre-initiation complex, and thus a decrease in its levels leads to a decline of protein synthesis. In fact, the eIF2 content was directly proportional to the rate of protein synthesis in the tissues examined (Kimball et al., 1992).

As for elongation factors, it was reported that the abundance and activity of eukaryotic elongation factor 1 alpha (eEF1α) declined in aging adult *Drosophila melanogaster*, and this decline was the leading cause of the decreased synthesis of total protein. In another study, a two-fold decrease in eukaryotic elongation factor 2 (eEF2) protein abundance was observed in the pineal gland of old rats. The same factors were assayed in a cell-free translation system, and eEF1α was shown to be more active in preparations taken from young rats, but contradictory results for eIF2 preparation (Anisimova et al., 2018; Witkowski et al., 2021).

Regarding the age-related changes in ribosomes, the data is somewhat contradictory. Reportedly, there is a reduction in mRNA levels encoding the four large subunits of ribosomes, resulting in fewer ribosomes in old cells. However, replicative aged budding yeast exhibited an increase in the number of ribosome proteins, while the output of ribosomal protein mRNA translation and overall translation efficiency decreased. Such difference was also observed in the brains of old rats. The proposed explanation for this discrepancy is that dysfunctional ribosomes were not correctly recognized and recycled in old cells (Anisimova et al., 2018).

Furthermore, evidence suggests that the distribution of rRNA gene copy numbers is narrowed in the genomes of older adults, indicating that there might be an optimal amount of rRNA (and

hence, ribosomes) for the cell to live and thrive long. A low copy number might not be sufficient for maintaining the function of an aging organism, whereas a high copy number may also represent a disadvantage during aging (Anisimova et al., 2018; Witkowski et al., 2021). This hypothesis was illustrated in the budding yeast, where older cells with a reduced amount of ribosomal proteins exhibited a longer replicative lifespan, while in younger cells, the ribosomal protein abundance correlated positively with the lifespan. Thus, the downregulation of ribosome biogenesis exhibited in some aged tissues may represent a protective mechanism to increase lifespan (Witkowski et al., 2021).

3.3 - Changes in translation fidelity with age

Aside from the effects of aging on the translation machinery itself, age-related changes in translation fidelity are another aspect of protein synthesis that attracts the attention of researchers in the aging field. Loss of translation fidelity with age refers to translation errors, namely decoding errors (insertion of the wrong amino acid) and stop codon readthrough events (not terminating translation in the right place), which occur with increasing age (Figure 8). Early studies (in the 1980s) in translation fidelity are contradictory, with many authors unable to detect age-related changes in translation fidelity. These studies used cell-free conditions, and the methodology available at that time did not allow for the detection of subtle changes in translation fidelity (Anisimova et al., 2018; Witkowski et al., 2021).

Nevertheless, studies in cell systems were also unable to provide a straightforward elucidation. On the one hand, the number of translational errors found in cultured human fibroblasts that completed 55 population doublings (PD) was 7-fold higher than in the fibroblasts that completed only 28 PDs. On the other hand, no change in translational fidelity was observed in a study investigating human skin fibroblasts obtained from healthy subjects and progeria patients, and compared the fidelity of translation in cells from early and late passages. Until now, most of the data on the subject is controversial, and it seems that dramatic decreases in translational fidelity do not accompany aging. Nevertheless, further studies are needed to establish if changed translational fidelity is an essential factor in the aging of human cells (Anisimova et al., 2018; Witkowski et al., 2021).

Despite the contradictory results on translation fidelity, the number of translational errors correlates with lifespan in several animal species. The naked mole-rat, with a lifespan exceeding two decades at body mass like the rat, demonstrates translational fidelity about 10 times higher than the mouse (Anisimova et al., 2018; Azpurua et al., 2013; Witkowski et al.,

2021). This study used fibroblasts isolated from the skin of mice and naked mole rats transfected with various luciferase-based reporter constructs that could only detect translation errors. The results suggest that high translational fidelity (decreasing translational errors) might have co-evolved with increasing lifespan. However, the rate of stop codon readthrough did not correlate with the lifespan as reported by a follow-up study, suggesting a higher selective pressure for translation termination fidelity due to the high energetic cost of failing to recognize a stop codon (Anisimova et al., 2018; Z. Ke et al., 2017). Thus, translation fidelity does not dramatically reduce with age and positively correlates with maximal lifespan. Still, more studies are necessary to elucidate the role of translation fidelity in determining the lifespan of different individuals of the same species (Witkowski et al., 2021).

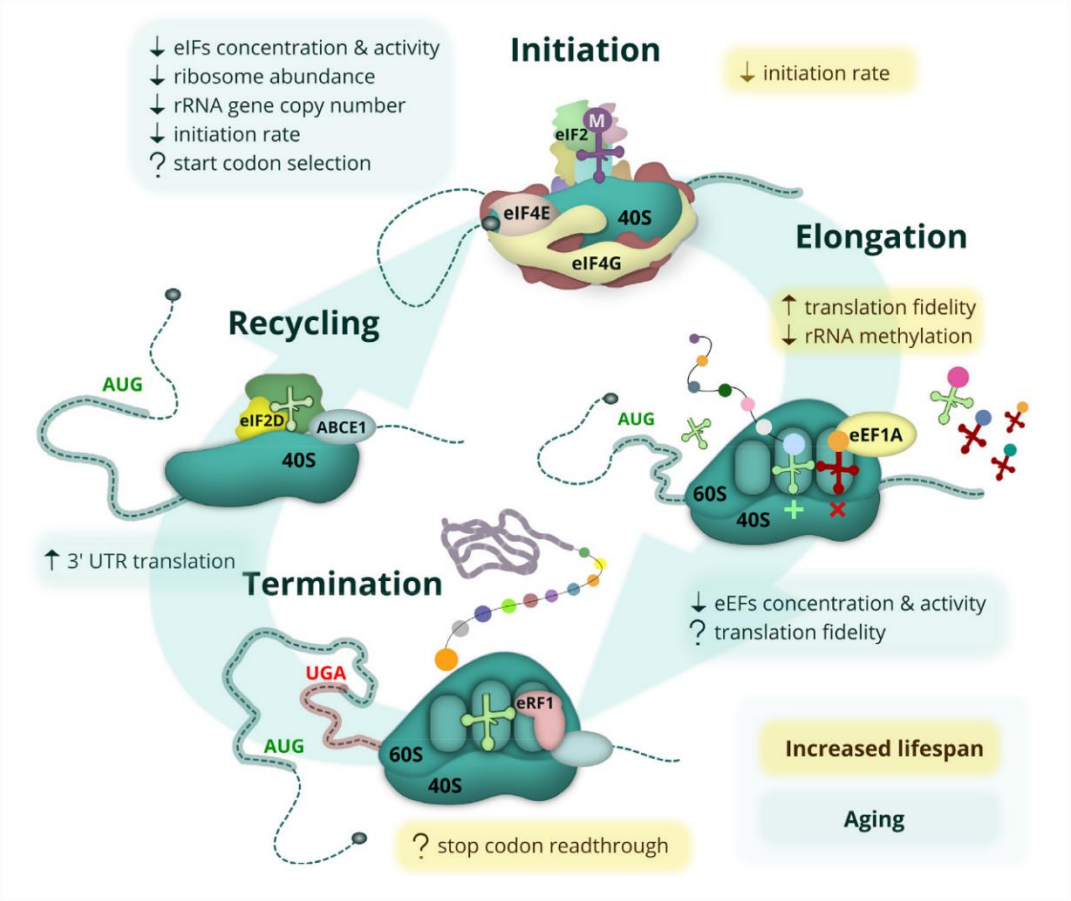

Figure 8 - Age-related changes and lifespan modulating aspects of protein synthesis
Schematic overview of the eukaryotic mRNA translation cycle featuring the age-related changes (light-green boxes) or linked to lifespan control (yellow boxes). Known positive and negative effects are shown by up and down arrows, respectively, while a question mark indicates controversial or potential regulation. (Adapted from Anisimova et al., 2018)

3.4 - Extending lifespan through modulation of the protein synthesis and translation-related signaling

3.4.1 – Modulation of the lifespan via the manipulation of the translation machinery

An aging organism experiences an overall reduction of protein synthesis; however, the observed correlation between translation control and aging brings into question whether the attenuation of protein synthesis is a causative agent of aging or the consequence of physiological changes accompanying aging. To answer this question, several studies in different model organisms, including yeast, nematodes, and the fruit fly, have demonstrated that specific deletion of different components of the translational machinery and thus reduction of protein synthesis significantly increase their lifespans (Gonskikh & Polacek, 2017).

Affecting translation initiation is perhaps the best illustration of this correlation. A screening of 2700 genes for the search for pro-longevity ones using siRNA mediated knockdown in *C. elegans* revealed that inhibition of translation initiation factors increased lifespan up to 50%. Depletion or downregulation of the IFE-2 somatic isoform of eIF4E reduces global protein synthesis and extends the lifespan. In addition, inhibition of the translation initiation factors eIF2 and eIF4G significantly extended lifespans in nematodes (Anisimova et al., 2018; Gonskikh & Polacek, 2017). In particular, eIF4G increases the average lifespan of nematodes by more than 30%, while deletion of two subunits of eIF3 results in a 40% extension. Downregulation of one of the subunits of the eIF2B also improves lifespan in nematodes (Anisimova et al., 2018). In *D. melanogaster*, overexpression of the translation repressor 4E-BP (the eukaryotic initiation factor eIF4E binding protein) significantly increased lifespan by enhancing mitochondrial activity (Zid et al., 2009). Conversely, the overexpression of eIF4E, the target of 4E-BP, induces cellular senescence in mouse embryonic fibroblasts and B cells (Gonskikh & Polacek, 2017).

Similar effects were made by affecting ribosomal proteins and numerous components of the ribosome biosynthesis apparatus. Reducing the levels of several ribosomal proteins (rpl3, rpl6b, rpl10, rps6, and rps18) in yeast strains significantly increases their replicative life span. Similarly, in *C. elegans,* depletion of six ribosomal proteins of the 40S subunit and five of the 60S subunit by siRNA-mediated knockdown reduced the translation level and concomitantly extended the lifespan (Gonskikh & Polacek, 2017). Additionally, mutations in some ribosomal

proteins also extended the lifespan of the filamentous fungi, *Podospora anserina* (Anisimova et al., 2018).

Moreover, depletion of factors involved in ribosomal biogenesis and tRNA synthesis, such as rsks-1 (the worm homolog of S6 kinase), leads to lifespan extension in *C. elegans* (Gonskikh & Polacek, 2017). Likewise, in *S. cerevisiae*, inhibition of 60S subunit biogenesis by deletion of its encoding genes, processing factors, or diazaborine treatment (inhibitor of 60S subunit biogenesis) is sufficient to increase replicative lifespan (Gonskikh & Polacek, 2017; Steffen et al., 2008).

In regard to translation elongation factors, the results are somewhat controversial. In early studies, fruit flies with increased expression of eEF1A exhibited a longer lifespan, while future experiments revealed no effects on lifespan. However, the inhibition of eEF2 by EFK-1 kinase increases the longevity of *C. elegans* both during starvation and under nutrient-rich conditions illustrating that modulation of translation elongation factors could also affect lifespan (Anisimova et al., 2018).

3.4.2 - Signaling pathways controlling longevity via modulation of protein synthesis

Several cellular signaling mechanisms converge to influence the rate of mRNA translation, in response to various stimuli, by modulating the activity or the availability of critical translational regulators (Tavernarakis, 2008). The signaling pathways, such as TOR, IIS, and MAPK, tightly regulate aging onset in various organisms by controlling different components of the translation machinery affecting the global rate of protein synthesis. Multiple genetic manipulations or pharmacological treatments affecting different levels of these pathways have been reported to extend lifespan in multiple model organisms (Charmpilas et al., 2015; Gonskikh & Polacek, 2017).

mTORC1 and mTORC2 multiprotein kinase complexes with a shared catalytic subunit mTOR, lie at the center of the metabolic state-sensing pathways of eukaryotic cells (Charmpilas et al., 2015). It has been demonstrated that the inhibition of the TOR signaling pathway by rapamycin treatment, gene deletion or by depleting different upstream and downstream effectors significantly extends the lifespan in yeast, nematodes, fruit flies and mice (Gonskikh & Polacek, 2017). The main mTORC1 targets that affect translation are ribosomal S6 kinase (S6K), eIF4E binding protein (4E-BP), and eukaryotic elongation factor 2 kinase (eEF2 kinase). mTORC1 directly phosphorylates these components to stimulate translation, and thus

mTOR is believed to increase the general protein synthesis rate and subsequently negatively impact lifespan(Anisimova et al., 2018; Charmpilas et al., 2015; Tavernarakis, 2008). mTORC1 phosphorylates the translational repressor 4E-BP to promote its dissociation from eIF4E, allowing for the recruitment of eIF4G and eIF4A translational factors to the mRNA cap structure. One way that direct mTOR kinase inhibitors, such as Torin1 or PP242, can extend lifespan is by promoting the dephosphorylation of 4E-BPs and subsequently decreasing translation efficiency, since the hypo phosphorylated forms of 4E-BPs bind the cap-binding protein eIF4E, preventing its interaction with its partner eIF4G and other components of the translation apparatus. Indeed, translation downregulation mediated by hyperactive 4E-BP improves proteostasis and extends lifespan in flies, similarly to mTORC1 inhibition (Anisimova et al., 2018).

In addition, mTORC1 also upregulates translation via S6 kinase activation, which, among others, phosphorylates the S6 protein of the 40S ribosomal subunit and the eIF4B initiation factor (Anisimova et al., 2018; Tavernarakis, 2008). S6K was shown to be elevated in old mice and animals such as *S. cerevisiae*, *C. elegans*, and *D. melanogaster* lacking S6k1 exhibited extended lifespan (Anisimova et al., 2018; Lamming et al., 2013). mTORC1 inhibition by its specific inhibitor, rapamycin, and subsequent S6k decreased activity is a path by which rapamycin mediates cell-type-specific repression of mRNA translation (Choo et al., 2008; Lamming et al., 2013). However, overexpression of a constitutively active form of S6K completely overrides the beneficial longevity effects of rapamycin, proving that reducing protein synthesis rates is pivotal for the manifestation of the enhanced survival phenotype (Charmpilas et al., 2015).

Another crucial signaling pathway regulating translation is the insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway, the first nutrient-sensing pathway discovered to affect the aging process (Mathew et al., 2017). Reduced insulin/IGF-1 signaling by mutation of different components consistently extended the lifespan of worms, flies, and mice (Gonskikh & Polacek, 2017). While the underlying molecular mechanisms mediating these effects on lifespan are not clear, a consistent decrease in the rate of protein synthesis was found in several of these model organisms. In this context, attenuation of mRNA translation is the result of reduced insulin–IGF-1 signaling through PI3K/Akt, ERK1/2 and p38 MAPK, which in turn reduce key translation regulators such as mTORC1, ribosomal S6K, Mnk1 kinase (mitogen-activated protein kinase-interacting kinase) and subsequently and the translation initiation factors eIF4E and 4E-binding protein 1 (4E-BP1) (Anisimova et al., 2018; Tavernarakis, 2008). Indeed, phosphorylation levels of most of the signaling molecules of this axis (ERK 1/2, S6K, Mnk1 and p38 MAPK) were higher in older men's muscles (Anisimova et al., 2018).

In addition, AMP-activated protein kinase (AMPK) signaling pathways can also regulate the translation process. AMPK signaling is crucial for energy metabolism and stress responses and, as indicated by its name, is triggered when the intracellular ATP/AMP ratio is low (Sen, 2018). This pathway exerts its regulation during translation elongation by phosphorylating eEF2K, a specific kinase for translation elongation factor eEF2. This phosphorylation leads to a reduction of eEF2 translocase activity, which subsequently slows down the elongation process. *C. elegans* deficient in EFK-1, an ortholog of eEF2K, exhibited a shorter lifespan not only in response to starvation but also under nutrient-rich conditions, while the increase in AMPK subunit levels, in turn, prolonged the worm's life (Anisimova et al., 2018).

3.5 – Possible reasons for reduced protein synthesis promoting longevity

A rapid rate of protein synthesis is accompanied by the production of damaged proteins due to transcriptional or translational error and co-translational misfolding. As a result, these proteins cannot effectively maintain their native function and tend to accumulate, forming potentially toxic aggregates. Attenuation of global protein synthesis induced by the depletion of components of the translation machinery decreases the production of normal as well as damaged proteins, which as a result might reduce the risk of accumulating toxic proteins. Furthermore, aging is associated with a decline on the protein quality control systems, which under normal circumstances, recognize damaged or misfolded proteins, to either assist in protein folding or target for degradation. Aged cells proteostasis network become overloaded with all the damaged proteins, resulting in the accumulation of cytotoxic aggregates. Slowing down translation prevents producing the excessive amounts of misfolded proteins, which reduces the load on the proteostasis network (Gonskikh & Polacek, 2017).

Another aspect that possibly mediates the lifespan extension effects of reducing overall protein synthesis is in the context of cellular energy. Protein synthesis is one of the most energy-consuming cellular processes, requiring approximately 75% of total cell's energy. Consequently, the reduction of mRNA translation increases overall energy availability, allowing energy diversion towards cellular maintenance and repair processes, thus promoting longevity. This hypothesis is supported by the depletion of ribosomal proteins and translational factors in several model organisms which decreases protein synthesis and increases stress resistance, extending lifespan (Gonskikh & Polacek, 2017; Tavernarakis, 2008).

4 - The proteostasis network

Intracellular proteins are highly dynamic molecules that can adopt many possible conformations to fulfill their intended function, and thus the folding process is inherently error-prone. Proteins undergo frequent conformational changes with partial or complete unfolding/refolding to translocate across membranes, assemble into functional structures, or regulate self and interacting proteins function, which often expose hydrophobic regions hidden in their core that could lead to undesired interactions within or non-specific binding with other proteins (Figure 9) (Klaips et al., 2018; Koga et al., 2011)

Moreover, stress conditions or stochastic errors of protein synthesis occurring at the level of transcription and mRNA maturation and translation further increase the production of misfolded proteins, which should be recognized and degraded to avoid aberrant interactions. This challenge is exacerbated in the case of aging and, if left unresolved, can lead to the formation of toxic aggregate species. Therefore, to maintain proteostasis, cells have evolved a wide variety of molecular chaperones and protein quality-control factors that are functionally linked with protein degradation machineries. This system is referred to as the proteostasis network (PN), and operationally, it can be divided into three branches composed of factors belonging to major processes: (1) protein synthesis, (2) folding and conformational maintenance (often coupled to transport and/or assembly), and (3) protein degradation (the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP). Molecular chaperones and their regulatory cofactors serve as the liaisons connecting all these processes (Klaips et al., 2018).

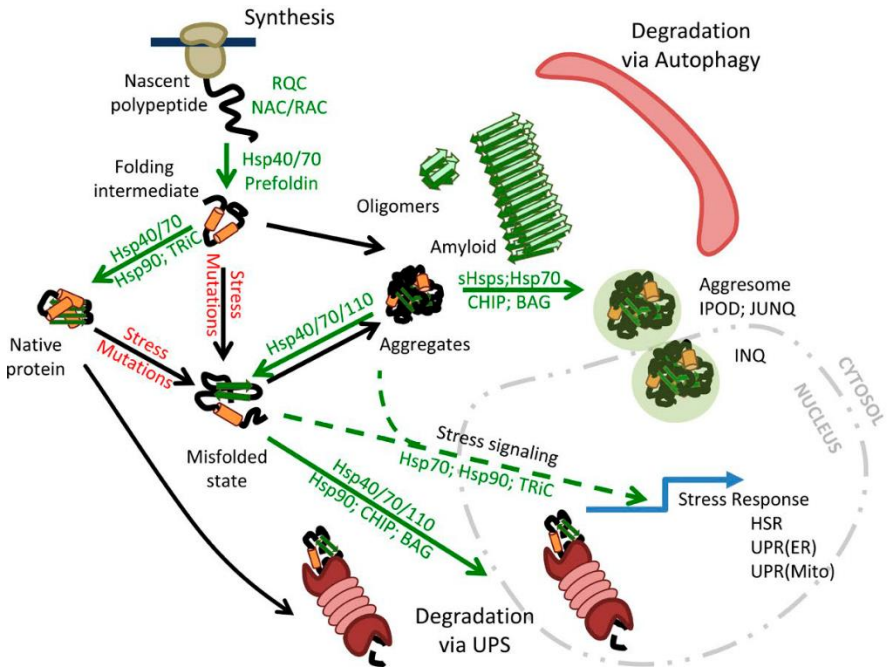

Figure 9 – The proteostasis network (PN)
Proteins rely on the proteostasis network during many stages of their lifecycle. Most newly synthesized polypeptides require the assistance of chaperones to fold as they exit the ribosome. At the end of a protein functional lifecycle, proteins that are unable to be folded are efficiently targeted for degradation via the ubiquitin-proteasome system (UPS) or chaperone-mediated autophagy (CMA) (not shown). Conditions of stress or mutations cause misfolding and aggregation of proteins. Cells activate the stress response to increase the PN capacity and restore homeostasis. With aging, the accumulation of mutations and the overall decrease in PN capacity cause an accumulation of misfolded proteins that may aggregate and/or be sequestered into large structures (aggresomes). A subset of misfolded species may form amyloid fibers that, if not degraded by autophagy, can further interfere with cellular processes. Chaperones' interventions are shown in green. (adapted from Klaips et al., 2018)

4.1 - Molecular chaperones

Most newly-synthesized proteins must fold into well-defined three-dimensional structures to obtain biological function, except for proteins with intrinsically disordered regions that may acquire structure only when interacting with partner molecules (Hipp et al., 2019). Polypeptide chains fold by sequestering hydrophobic residues and forming stabilizing intramolecular interactions to achieve a low-free energy state (Klaips et al., 2018). Generally, proteins' folded (or native) states are thermodynamically favorable and easily obtainable in an unrestricted *in vitro* environment (Hipp et al., 2019; Klaips et al., 2018). However, the highly crowded cellular environment imposes significant challenges for the folding process due to partially folded states with exposed hydrophobic amino acid residues having a high propensity for misfolding and aggregating. Aberrant folding is a consequence of the complex energy landscape that proteins must navigate during the folding process, in which partially folded intermediates can adopt kinetically stable (trapped in localized energy minima) nonnative structures that also

tend to engage in aberrant intermolecular interactions, forming aggregates that may be thermodynamically more stable than the native state (Hipp et al., 2019; Klaips et al., 2018).

Aberrant folding may occur during de novo synthesis or in conditions of conformational stress, where preexisting proteins fail to maintain their folded states (Klaips et al., 2018). Indeed, the bulk of protein synthesis must be adjusted to the protein folding capacity of the cell to avoid the accumulation of misfolded proteins, especially in conditions of conformational stress (Anisimova et al., 2018). Furthermore, destabilizing mutations or the presence of intrinsically unstructured regions can also predispose proteins to misfold (Klaips et al., 2018).

In order to overcome the challenges of protein folding and solubility, cells have evolved molecular chaperones, defined as proteins that assist in the folding, assembly, conformational maintenance, or regulation of another protein without becoming part of its final structure. Chaperones participate extensively in de novo folding by recognizing generic structural features of nonnative conformation, such as exposed hydrophobic amino acid residues and the substrate protein's unstructured backbone. They promote folding by kinetic partitioning of nonnative states (Hipp et al., 2019; Klaips et al., 2018). Molecular chaperones are also known as stress proteins or heat shock proteins (HSPs) due to being upregulated under stress conditions in which the concentrations of aggregation-prone folding intermediates increase. Thus, in addition to their function in de novo folding, chaperones are also involved in other proteome-maintenance functions refolding of stress-denatured proteins, oligomeric assembly, protein trafficking and assistance in proteolytic degradation (Hartl et al., 2011; Klaips et al., 2018; Koga et al., 2011).

Several classes of structurally unrelated chaperones exist in cells, forming cooperative pathways and networks (Hartl et al., 2011). They are highly conserved molecules from bacteria to mammals and are classified according to their molecular weight into five major families: HSP100, HSP90, HSP70, HSP60 (chaperonins), and the small heat shock proteins (sHSP) with molecular weights between 12 to 43 kDa, (Table 2) (Hartl et al., 2011; Hipp et al., 2019; Koga et al., 2011). Members of each class can be found in different cellular subcompartments and contribute to maintaining a different subset of proteins. Some chaperones, such as HSP70 or HSP60, act over a large pool of proteins in the cells by promiscuously recognizing ubiquitous motifs in proteins, such as hydrophobic patches or frequent post-translational modifications. While specific chaperones are solely dedicated to the surveillance of a very limited subset of proteins in which they recognize a defined binding region (i.e. those acting over collagen or modulating the dynamics of actin or intermediate filaments) (Koga et al., 2011).

Table 2 – Main chaperone families and their functions in eukaryotes (adapted from Hipp et al., 2019)

Molecular chaperone family	Function
HSP70 ~70 kDa ATP-dependent	HSP70 is one of the major chaperone families, consisting of at least eight homologous chaperone proteins in the cytoplasm, mitochondria, and the ER. HSP70 chaperones are required for folding newly synthesized proteins, conformational maintenance, and aggregation prevention. Moreover, HSP70 cooperates with HSP40 and HSP110 in protein disaggregation.
HSP40 (also known as J proteins) ~40 kDa	HSP40 chaperone family is A diverse group of proteins containing the HSP70-interacting J-domain, with homologs in the cytoplasm, mitochondria, and the ER. They function as co-chaperones of HSP70 and regulators of the HSP70 ATPase cycle of protein substrate binding and release. Additionally, they recruit HSP70 to different substrates and cellular locations.
HSP110 ~100 kDa	Function as a nucleotide exchange factor for HSP70. HSP110 cooperates with HSP70 in protein folding, degradation of misfolded proteins, and disaggregation.
HSP90 ~90 kDa ATP-dependent	HSP90 functions as a homodimer in the folding and conformational maintenance of functionally and structurally diverse client proteins involved in several cellular pathways. Its major substrate classes are kinases, steroid receptors, and other signaling proteins. HSP90 cooperates with multiple cochaperones containing TPR (tetratricopeptide repeat) domains.
HSP60 ~60 kDa subunit ATP-dependent	HSP60 is the mitochondria chaperonin. It consists of two heptameric rings with ~60 kDa subunits stacked together. HSP60 cooperates with the HSP10 cofactor and functions in folding a subset of mitochondrial proteins after translocation from the cytosol.
TRiC/CCT ~1 MDa ATP-dependent	TRiC/CCT is the eukaryotic cytosol chaperonin that consists of two octameric rings with ~60 kDa subunits stacked together. TRiC is required to fold a subset of cytosolic proteins, including actin and tubulins. It can also interfere with the aggregation of Huntingtin protein.
HSP100 ~100 kDa subunit ATP-dependent	HSP100 is a family of chaperones in fungi, bacteria, and chloroplasts in plants (Hsp104, Hsp78, ClpA, ClpB, ClpC, ClpX, and HsIU) that belongs to a large superfamily of AAA+ ATPases. These chaperones are typically composed of hexameric rings. Hsp104 in yeast and other fungi mediates protein disaggregation in cooperation with Hsp70 and Hsp40.
Small HSP ~12-45 kDa subunit	sHSP is a group of ATP-independent chaperones that forms large heterogeneous oligomers. sHSP subunits contain a conserved α-crystallin domain, packed with β-sheets and involved in oligomerization. They have ten different forms in humans (HspB1-10). They are involved in preventing aggregation by binding to non-native states ('holdase'-function). In addition, sHSP also mediate the sequestration of misfolded proteins into less toxic aggregates.

4.2 - Chaperones in protein synthesis

The folding of newly-synthesized proteins requires the assistance of the chaperone network, either on the cytosol or intracellular compartments such as the reticulum endoplasmatic (ER). Moreover, folding may also be required in the case of cytosolic native proteins that translocate across membranes to reach organelle lumens, where they will be functional (Koga et al., 2011). The chaperones that participate broadly in de novo protein folding and refolding, such as the HSP70s, HSP90s, and the chaperonins (TRiC/CCT, HSP60s), are multicomponent molecular machines that promote folding through ATP- and cofactor-regulated binding and release cycles (Table 2). They typically recognize hydrophobic amino-acid side chains exposed by nonnative proteins and may functionally cooperate with ATP-independent chaperones, such as the small HSPs (Hartl et al., 2011).

The nascent polypeptide chain's first interaction with chaperones occurs near the exit site of the ribosome (Figure 10). Here, the first tier of ribosome-associated chaperones stabilizes nascent polypeptides on ribosomes and initiates their folding (Klaips et al., 2018; Vabulas et al., 2010). These are specialized chaperone complexes such as nascent chain-associated and ribosome-associated complexes (trigger factor in bacteria) that interact with exposed hydrophobic sequences of the emerging nascent chain and act to prevent premature (mis)folding, maintaining the polypeptide in a nonaggregated, folding competent state until sufficient structural elements for productive folding are available (Klaips et al., 2018).

The Second tier of chaperone components acts downstream of ribosome-associated chaperones, cotranslationally or post-translationally, to ensure the folding process's completion in the case of longer polypeptide chains or incomplete folding (Vabulas et al., 2010). The ubiquitous HSP70 family cooperates with regulatory chaperone cofactors of the HSP40 class, which typically bind first to exposed hydrophobic patches on nonnative proteins and recruit HSP70. The C-terminal of HSP70 then binds to the short hydrophobic peptide sequences on the nascent and nonnative protein substrates allowing their folding in one or several cycles of ATP-dependent binding and release by HSP40-mediated hydrolysis (Klaips et al., 2018; Vabulas et al., 2010). There are ~50 different HSP40 proteins in human cells, conferring broad functionality and versatility to the HSP70 system, allowing these chaperones to participate in the de novo folding, conformational maintenance, disaggregation, and targeting terminally misfolded proteins for degradation (Klaips et al., 2018).

Proteins that fail to fold through HSP70 cycles go through another folding round by the chaperonin class of chaperones (GroEL/GroES in bacteria, HSP60 in mitochondria, and TRiC/CCT in the eukaryotic cytosol). These chaperonin proteins form multimeric, cylindrical complexes that function by transiently encapsulating individual nonnative proteins so they can fold, unimpaired by aggregation. Only ~10% of the proteome requires a chaperonin to fold, even though substrates include essential and very abundant proteins, such as tubulin and actin (Klaips et al., 2018).

Alternatively, HSP90 is another important chaperone system that functions downstream of HSP70 in the final structural maturation and conformational regulation of several signaling molecules and transcription factors (Vabulas et al., 2010). HSP90 is active as a homodimer and mediates protein folding via ATP-dependent structural changes in cooperation with several regulators and cochaperones, many of which contain tetratricopeptide repeat (TPR) domains (Klaips et al., 2018; Vabulas et al., 2010). HSP90 can bind near-native substrates, thereby stabilizing metastable proteins, such as kinases and steroid receptor molecules, in a conformation poised for activation by ligand binding (Klaips et al., 2018).

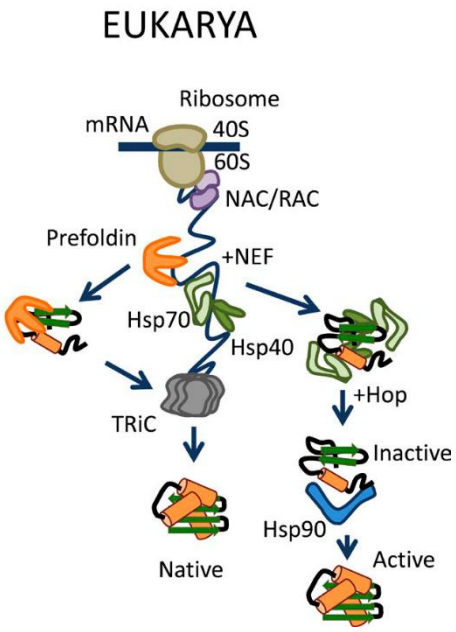

Figure 10 – Newly synthesized protein folding in eukaryotic cytosol
Most newly synthesized proteins require folding assistance on ribosome exit. Nascent chain-associated complex (NAC) and ribosome-associated complex (RAC) are chaperone complexes that assist in folding these proteins. Downstream HSP70s cooperate with HSP40 and nucleotide exchange factors (NEFs) in protein folding. Some proteins must be transferred to the chaperonin class of chaperones for further folding (TRiC). Pre-foldin can transfer some substrates directly to TRiC. Cofactors such as Hop (HSP70-HSP90 organizing protein) can mediate interactions with HSP90, which also acts downstream of HSP70 in folding a subset of proteins mainly engaged in cell signaling. (adapted from Klaips et al., 2018)

4.2.1 - Chaperones in the maintenance of conformational stability

After initial folding, many proteins require continual chaperone surveillance to maintain their conformational integrity. Some proteins are biologically functional under conditions just at the cusp of stability, and their functional conformational states may be challenged by additional destabilizing factors, such as mutations or external stressors (e.g. increased temperature or exposure to oxidative agents) (Hipp et al., 2019; Klaips et al., 2018). Proteins comprising the metastable proteome are particularly vulnerable to stress-induced misfolding and aggregation, and they include up to 75% of signaling molecules in mammalian cells. These proteins contain intrinsically unstructured regions or sequences of low amino acid complexity essential for their function, subsequently compromising stability. They only acquire a stable structure upon binding to a ligand or other macromolecular surface. In order to adapt and maintain a functional proteome in conditions of acute stress, cells activate the stress-response pathway to

increase the transcription of chaperones and other proteostasis components, decrease the substrate load and resolve misfolded proteins and aggregation (Klaips et al., 2018).

The cytosolic stress response is regulated mainly by the Heat shock factor 1 (HSF1). In non-stressed cells, HSF1 is maintained in an inactive state by association with HSP90 and HSP70. Under stress conditions, these chaperones are tittered away from HSF1, allowing HSF1 to trimerize and induce the transcription of a wide range of proteostasis components while attenuating general protein synthesis to reduce the production of new substrate clients (Hipp et al., 2019; Klaips et al., 2018). Among the chaperones induced under the stress response, the most prominent are sHSP and members of the HSP70 family. The expression of these chaperones is essential to counter the accumulation of misfolded proteins and aggregates under stress conditions such as heat shock. In fact, overexpression of these chaperones alone is enough to confer cell resistance to heat shock in several organisms (Koga et al., 2011). Finally, once the stressor is removed, a negative-feedback loop on Hsf1 activity allows rebinding of HSP90/HSP70 to ensure a return to stasis (Hipp et al., 2019; Klaips et al., 2018).

Similar stress-response pathways include the unfolded protein response (UPR) in the ER and mitochondria (Hipp et al., 2019; Klaips et al., 2018). The importance of the ER chaperones is such that a very complex network of proteins and factors is dedicated to upregulating ER chaperone synthesis when the amount of unfolded products reaches critical levels that could compromise ER homeostasis (Koga et al., 2011). ER transmembrane signaling proteins such as IRE-1 (inositol-requiring transmembrane kinase/endonuclease 1 alpha), PERK (protein kinase RNA-like ER kinase), and ATF6 (Activating transcription factor 6) constitute distinct arms of the UPR and function to activate transcription factors to produce a multiplicity of proteostasis components. PERK activation also leads to phosphorylation and subsequent inhibition of translation initiation factor 2α (eIF2α) and thus attenuates overall translation to alleviate ER clogging. Even though the exact mode of activation of the UPR is different from that of the cytosolic heat shock response, the overall goals are similar: an up-regulation of quality-control components and a decrease in potentially misfolded substrates through transient attenuation of translation (Klaips et al., 2018).

4.2.2 - Chaperones in protein degradation

Proteins that cannot fold or refold, despite intervention by chaperones, must be degraded to prevent the accumulation of potentially toxic aggregate species. Therefore, terminally misfolded proteins undergo proteolytic degradation mainly by the ubiquitin-proteasome system (UPS) or chaperone-mediated autophagy (CMA). These proteolytic systems are intimately linked to major cytosolic chaperones, the HSP70 and HSP90 systems, and

chaperone cofactors. In particular, the UPS cooperates with these chaperone systems through specific E3 ubiquitin ligases, such as the carboxyl-terminus of HSP70-interacting protein (CHIP). CHIP interacts via its tetratricopeptide repeat (TPR) domain with the carboxy-terminus of HSP70 or HSP90 to ubiquitylate misfolded chaperone-bound proteins, which targets them to the proteasome. As for CMA, HSC70 (the constitutively expressed member of the HSP70 family) participates in the degradation of soluble cytosolic proteins containing a pentapeptide motif in lysosomes.

4.3 - Age-related changes in the chaperone system

The age-dependent decline in the ability of cells to maintain a functional proteome is regarded as a significant driver of age-related cellular dysfunction and degenerative diseases (Hipp et al., 2019). The accumulation of misfolded proteins in cells during aging challenges the proteostasis network and eventually results in the deposition of aggregates, as shown in model organisms such as *C.elegans* and *Drosophila*. The inability of cells to restore normal proteostasis may result in disease and even cell death. Indeed, several diseases are associated with aberrant protein folding. Toxic gain-of-function diseases such as type 2 diabetes and major neurodegenerative conditions (Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Alzheimer's disease) are typically age-related and caused by the accumulation of amyloid or amyloid-like aggregates of the disease protein. Hence, the age-related decline in proteostasis and specifically in the incapacity to upregulate chaperones in response to conformational stresses would trigger disease manifestation and, in turn, accelerate proteostasis collapse (Brehme et al., 2019).

Indeed, many age-related diseases show primary or secondary deficits in chaperone function (Koga et al., 2011). During human brain aging, the expression of approximately 30% of ATP-dependent chaperones such as HSP90s, HSP70s, or HSP60s is repressed. However, the expression of approximately 20% of ATP-independent chaperones and cochaperones, such as sHSPs, is induced. The repression and induction of these chaperone families are aggravated in Alzheimer's, Huntington's, and Parkinson's brains (Brehme et al., 2019). Changes in chaperone sHSP content represent the cellular attempt to accommodate the pathogenic condition and preserve cellular homeostasis, at least during the first stages of the disease. For example, HSP27, a heat-inducible sHSP ubiquitously expressed in the human brain, has been detected at elevated levels in degenerating areas of Alzheimer's disease brains (Koga et al., 2011). On the one hand, the formation of larger amyloid aggregates may exert a relative protective effect by sequestering the more toxic oligomers. On the other hand, these amyloid

aggregates and oligomers may interact aberrantly with key proteostasis network components such as chaperones and sequester them into insoluble deposits contributing to cellular disfunction (Klaips et al., 2018). Another possible explanation for the age-related shift in chaperone content is the availability of ATP content in the cell. Deterioration of cellular energetics with age and disruption of fatty acid and glucose mechanism reduces the amount of available ATP, causing the age-related change in chaperone induction and activity, ultimately accumulating damaged proteins. The aging cell's preference to downregulate ATP-dependent chaperones in favor of an increased expression of ATP-independent chaperones and cochaperones represents an adaptation to the energetic deficit (Anisimova et al., 2018).

Stress response pathways are also affected since the stress-induced synthesis of cytosolic and organelle-specific chaperones is significantly impaired in aging (Koga et al., 2011; López-otín et al., 2013). In cultures of senescent fibroblast and tissues from old organisms of different species, a decreased transcriptional upregulation of HSP70 was observed in response to different stressors. Cells from human centenarians exhibit preserved chaperone upregulation during stress. Likewise, several studies support that increasing chaperone induction increases longevity in uni- and multicellular organisms. Flies and worms carrying extra copies of an *hsp*-70 family member or sHSPs are long-lived (Koga et al., 2011). Mutant mice deficient in a cochaperone of the heat-shock family exhibit accelerated aging phenotypes, whereas long-lived mouse strains exhibit a marked upregulation of some heat-shock proteins (López-otín et al., 2013). Furthermore, many long-lived mutants (such as those with lower insulin signaling or with mutations that mimic caloric restriction) have upregulated *hsp*-16 genes, and higher levels of the protein product have also been detected in long-lived mutant worms during and after heat stress, with a high rate of accumulation in the aggregate fraction (Hipp et al., 2019; Koga et al., 2011). The recruitment of HSP16 and other sHSP chaperones into insoluble aggregates deposits may reflect the organism's controlled attempt to sequester protein aggregates (as inclusion bodies), consistent with the age-dependent formation of aggregates being a regulated, protective response to lower the pressure on the proteostasis network (Hipp et al., 2019).

Overexpression of the gene encoding the *hsp* gene transcriptional activator, heat shock factor (HSF), increases longevity and thermotolerance in nematodes. Moreover, one of the reasons for the cell's failure to upregulate chaperone transcription with age, at least in the case of HSP70, has been narrowed to the inability of HSF to bind to the heat shock element on the chaperone gene promoter. Indeed, specific polymorphisms in the promoter region of the HSP70 gene affect the probability of attaining longevity. In mammals, different factors connected to longevity modulates HSF, such as the histone deacetylase 6 (HDAC6), which is required to activate HSF1 during proteasome inhibition, or SIRT1, another histone deacetylase

that activates HSF-1, whereas SIRT1 downregulation attenuates heat shock response (Koga et al., 2011; López-otín et al., 2013).

The CHIP ubiquitin ligase/cochaperone also impacts lifespan. CHIP-defective mice have reduced longevity and display an accelerated aging phenotype. Unsurprisingly, these animals also present a higher level of damaged proteins and declined proteasome activity since this chaperone is known to deliver cytosolic proteins for degradation through this proteolytic complex (Koga et al., 2011).

Organelle-specific chaperones also contribute to longevity. The levels and activity of essential ER chaperones, such as BiP (Binding immunoglobulin protein), calnexin, and protein disulfide isomerase (PDI), decline with age in many tissues of old rodents. Age-induced unbalanced expression of pro-apoptotic versus pro-survival markers of the UPR has also been proposed to be responsible for the lower resistance to stress of old organisms. As for mitochondrial chaperones, overexpression of mortalin, a mitochondrial HSP70 increases the lifespan of worms and human fibroblasts in culture. Additionally, the overexpression of another mitochondrial chaperone, the sHSP HSP22, also increases flies' lifespan and health span (Koga et al., 2011).

Another critical aspect in which age-related chaperone dysfunction contributes to aging proteotoxicity is a markedly reduced ability to maintain metastable proteins in their soluble states. The pool of metastable proteins in all cells is highly dependent on chaperones to maintain their functional conformational state. Due to their instability, metastable proteins are particularly vulnerable to misfolding under conditions of conformational stress and have a high propensity to aggregate (Klaips et al., 2018; Koga et al., 2011). Therefore, a healthy chaperone network is required to maintain the metastable proteome and prevent the accumulation of toxic aggregate species. These examples support the hypothesis that an improved cell capacity to adapt to various stresses, in which chaperones play a leading role contributes to lifespan extension (Klaips et al., 2018).

4.4 - Protein degradation mechanisms

4.4.1 - The ubiquitin/proteasome system

The ubiquitin/proteasome system (UPS) is a central component of the proteostasis network, serving as the primary selective proteolytic system in eukaryotic cells. It is a precise mechanism responsible for ~80% of protein turnover, and thus it is crucial for the maintenance of proper

concentration of many regulatory proteins involved in multiple biological processes (Figure 11) (Jayaraj et al., 2020; Vilchez et al., 2014).

Functionally, the UPS pathway relies on the coordinated action of its two main components: the ubiquitination machinery, i.e., the tagging system responsible for substrate targeting, and the proteasome core, responsible for substrate degradation (Koga et al., 2011). The sequential ubiquitin conjugation on the substrate protein allows for precise recognition and selective degradation by the proteasome core (Vilchez et al., 2014). Ubiquitin is a small (8 kDa) heat-stable protein that targets proteasome substrates for degradation through covalent linkage and self-conjugation, resulting in polyubiquitin chains. The linkage of ubiquitin to the substrate is mediated by a group of distinct classes of enzymes, including ubiquitin-activated enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which act sequentially to activate ubiquitin, present it to the substrate, and catalyze the conjugation, respectively (Koga et al., 2011). There are only two E1's, while there are several E2 enzymes and many E3 ubiquitin ligases, each of which recognizes one or several protein motifs; thus, E3 ubiquitin ligases for the selectivity of the process targeting specific proteins to degradation (Vilchez et al., 2014).

The proteasome is a multi-catalytic complex with a proteolytic core, known as a 20S core particle or 20S proteosome, which exhibits a Cylindric-like structure resulting from the association of 28 subunits assembled in four heptameric rings (Figure 11). The two outer rings are formed of seven α-subunits (α1 to α7), while the inner rings are composed of seven β-subunits (β1 to β7). The α-rings regulate the entrance of substrate into the catalytic cavity, while three β-rings contain proteolytic active sites in three of its subunits: β1, β2, and β5 have caspase-like, trypsin-like, and chymotrypsin-like activities, respectively. These β-subunits are exchangeable, allowing proteasomes with different catalytic activities to coexist. For example, β5t is a homolog of β5 uniquely found in the thymus, and its integration into the proteasome reduces the chymotrypsin-like activity of the proteolytic machine. Another example is the immunoproteasome, a specialized form of the 20S proteosome prevalent in antigen-presenting cells, generated by replacing the catalytic subunits with β1i, β2i, and β5i (Vilchez et al., 2014).

The 20S proteasome is considered inactive and unable to degrade polyubiquitinated proteins until binding to proteasome activators. Active proteasome exists in several configurations depending on the assembly of different regulatory subunits that dock on one or both ends of the 20S proteasome. The primary configuration is the 26S proteasome (or 30S, if double capped), formed by assembling the 20S proteasome to the 19S regulatory subunit. The 19S regulates the activity of the proteolytic chamber by recognizing, unfolding, and translocating polyubiquitinated proteins into the 20S catalytic core for degradation in an ATP-dependent process. The components of the regulatory subunit are primarily chaperones, ATPases, and

enzymes, distributed within two subcomplexes: a base adjacent to the 20S core particle and a lid on top of the base. The base contains six distinct AAA-ATPases (Rpt1–6) and three non-ATPases subunits (Rpn1–2 and Rpn10). AAA-ATPases are generators of mechanical force to unfold substrates, open the proteasome barrel, and push the substrate into the catalytic core. The lid contains eight subunits (Rpn3, Rpn5–9, and Rpn11–12) crucial for substrate recognition and deubiquitination (Vilchez et al., 2014).

Alternatively, the 20S proteasome can also be activated by other proteasome regulatory particles such as the Blm10/PA200 family or PA28 (11S). These regulatory subunits open the 20S core but lack deubiquitinating enzymes (DUBs) and ATPase activity and thus promote proteolytic degradation in an ATP- and ubiquitin-independent manner. PA200 binds to one end of the 20S proteasome with 19S on the opposite end to form a hybrid complex (Aladdin et al., 2020; Vilchez et al., 2014). While PA200 substrates are less characterized than those of 19S, emerging data suggests PA200 facilitates the degradation of acetylated core histones during DNA repair and replication stress, and unstructured proteins such as Tau and N-terminal Huntingtin fragments (N-Htt) involved in the pathogenesis of age-related neurodegenerative such as Alzheimer's and Parkinson's disease, and Huntington's disease, respectively (Aladdin et al., 2020).

The PA28 binds to both ends of the 20S proteasome and considerabily enhances its capacity to degrade short peptide substrates. Like PA200, PA28 can also associate with the free end of asymmetric 26S proteasomes (19S-20S) to form hybrid proteasomes, which allows the hydrolyze tri- and tetra-peptides at higher rates than canonical 26S particles. PA28 is composed of two homologous subunits, namely; PA28α and PA28β, both of which are induced by -interferon-γ. Another member of the PA28 family is PA28γ, which is mainly located in the nucleus and is not INF-γ-inducible (Cascio, 2014). These proteins assemble into hetero-heptamer rings (PA28α and PA28β) and homo-heptamer rings (PA28γ) (Kors, 2019). Studies suggest that PA28αβ plays a role in MHC class I antigen presentation and generation of specific viral antigens, while PA28γ is involved in cell cycle regulation by promoting the degradation of p21. In addition, PA28 has a vital role in the degradation of oxidized proteins. PA28 can provide a backup to support the degradation of oxidized proteins since oxidative stress causes the disassembly of the 26S/30S proteasome towards 20S. Thus, proteasome activity is tightly modulated by a vast spectrum of proteasome regulators and conditions (Cascio, 2014; Vilchez et al., 2014).

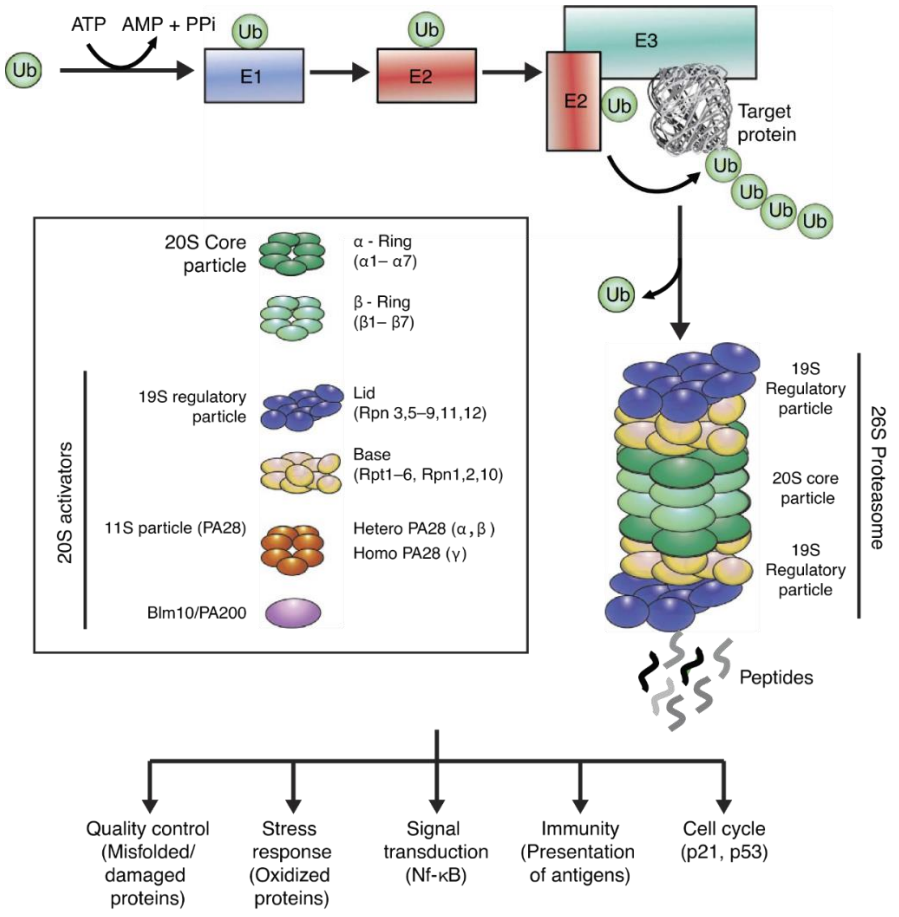

Figure 11 - The ubiquitin–proteasome system (UPS)
The first step in the degradation process of the UPS is the conjugation of ubiquitin to the substrate protein, which is done with the assistance of three distinct classes of enzymes: E1 (ubiquitin-acting enzyme) activates the c-terminal glycine residue of ubiquitin in an ATP-dependent manner; activated ubiquitin is then transferred to a cysteine site of E2 (ubiquitin-conjugating enzyme). E3 (ubiquitin ligase) links ubiquitin from the E2 enzyme to a lysine residue of the target protein. E3 ubiquitin ligases are responsible for the selectivity of the targeting process. The same cascade links additional ubiquitin molecules to the first one via ubiquitin lysines, forming a ubiquitin chain. After ubiquitination, the substrate protein is recognized and degraded by the proteasome. Free intracellular 20S proteasome must first be activated by binding to 20S activators to degrade proteins. The major assembly of the 20S proteasome is with the 19S regulatory protein, which recognizes polyubiquitylated substrates, removes ubiquitin molecules, and unfolds the substrate to translocate it into the 20S proteolytic chamber. The 20S can also be activated by the PA28 complex or the Blm10/PA200 protein. The UPS is involved in various cellular functions, such as quality control of the proteome, stress response, or cell cycle regulation. (adapted from Vilchez et al., 2014)

4.4.2 - Age-related changes in the ubiquitin–proteasome system

The activities of the two central proteolytic systems decline with aging and, in conjunction with the widespread, aberrant changes in translation and generalized downregulation of chaperones, contribute to the collapsing proteostasis characteristic of old age (López-otín et al., 2013; Vilchez et al., 2014).

Multiple studies have reported a decline in proteasome function during aging in different mammalian tissues and cells, although contrarily to the lysosomal system, the decline in activity with age appears not to be universal. The age-related proteasome dysfunction occurs

at different levels, such as down-regulated expression of proteasome subunits, unbalanced levels of α and β catalytic subunits, defective expression of regulatory subunits, and damaging post-translational modification in critical proteasome subunits (Koga et al., 2011; Vilchez et al., 2014). In addition, factors extrinsic to the proteasome could also interfere with their function in aging cells. For example, reduced ATP content, oxidation and aggregation of proteasome substrates have been shown to inhibit the assembly or activity of the 26S proteasome. Inactivation of the proteasome could induce catastrophic proteostasis feedback since the loss of proteostasis can cause the accumulation of protein aggregates, which obstruct and further inhibit proteasome activity (Vilchez et al., 2014).

Age-related changes in specific components of the ubiquitin system also contribute to the dysfunction of the proteasome. Studies have reported an age-dependent decrease in the levels of free ubiquitin and transcriptional downregulation of two ubiquitin-conjugating enzymes and an E3 ubiquitin ligase in some aged tissues. However, in other tissues, higher constitutive levels of these enzymes are present in old animals but instead fail to upregulate their activity in response to stressors. In addition, with age, there is an increased content of mutant forms of ubiquitin due to molecular misreading that could also contribute to the inefficient clearance of polyubiquitinated proteins in aging (Koga et al., 2011).

Several model systems support connections between longevity and the UPS. Compared to human fibroblasts with fewer passages, senescent fibroblasts have significantly reduced proteasome activity and lower proteasome subunit levels (Vilchez et al., 2014). Additionally, Fibroblasts treated with proteasome inhibitors have a shortened lifespan and present a senescent-like phenotype. Unsurprisingly, fibroblasts from healthy centenarians have a more active proteasome activity than fibroblasts of regular old donors, which could contribute to their successful aging (Koga et al., 2011). In *C.elegans*, the expression of RLE-1, an E3 ubiquitin ligase, regulates aging by catalyzing DAF-16 polyubiquitination. Disruption of RLE-1 expression increases *C.elegans* lifespan (Li et al., 2007)

Moreover, defects or missing regulatory components of the proteasome impact the lifespan of worms. For example, the knockdown of 19S and 20S subunits during *C.elegans* adulthood shortens their lifespan (Vilchez et al., 2014). Worms lacking AIP-1 (homolog of the mammalian regulatory subunit AIRAP) exhibit reduced lifespan and hypersensitivity to misfolding proteins. Mutations in CUL-1 E3 ligase complex genes diminish the extended lifespan of DAF-2 *C. Elegans* mutants (Koga et al., 2011).

In addition, CHIP deficient mice have a shortened lifespan and aggravation of aging phenotypes. CHIP is an E3 ubiquitin ligase that interacts with HSP70 and promotes the

proteolysis of its unfolded substrates, and its deficiency provides a link between chaperones, proteasomes, and aging (Koga et al., 2011; Vilchez et al., 2014). Transgenic mice with decreased chymotrypsin-like activity via β5 subunit replacement with β5t also exhibit a reduced lifespan, accelerated age-related phenotypes, and aggravation of age-related metabolic disorders. Also PA28γ deficiency promotes premature ageing in mice (Vilchez et al., 2014).

All these reports suggest that UPS activity indeed declines with aging. A proper proteasome function is required to attain maximal lifespan extension in long-lived mutants, underlying an important role for this proteolytic system in lifespan (Koga et al., 2011).

4.4.3 - The Autophagy/lysosomal pathway

The Autophagy/lysosomal pathway (ALP) is another component of the proteostasis network with an essential role as a quality control mechanism of proteins. Autophagy is an intracellular catabolic process in which cytosol fractions, organelles, and macromolecules are degraded within the lysosome, an organelle devoted to degradation (Koga et al., 2011). ALP is a vital pro-survival pathway, acting as a bulk intracellular degradation system in response to metabolic stress (nutrient deprivation, DNA damage, hypoxia) or oxidative and ER stress to maintain energy balance and cellular homeostasis (Vilchez et al., 2014). In addition to its role as a stress response pathway, autophagy performs a crucial function in quality control by promoting basal turnover of long-lived proteins and organelles as well as selectively degrading damaged cellular components (Murrow & Debnath, 2013). Emerging evidence suggests that autophagy is a selective mechanism to degrade misfolded and aggregated proteins, including neurodegenerative-associated proteins such as tau, α-synuclein, and polyglutamine-expanded proteins. It is a role that has arisen in light of a newly found interaction between Autophagy and UPS, in which specific receptors and adaptors such as p62 (SQSTM1) recognize ubiquitinated proteins and target these substrates to the autophagy machinery (Vilchez et al., 2014).

Furthermore, due to the capacity to engulf entire cellular regions, autophagy is crucial in every process that requires extensive cellular remodeling, such as embryogenesis, cellular differentiation, or cellular death. Autophagy is also involved in acquired and innate immunity through antigen presentation and degradation from external pathogens (Vilchez et al., 2014).

4.4.3.1 – The autophagy machinery and the major autophagic pathways

The central catalytic component of the autophagy machinery is the lysosome, a single-membrane vesicle containing a large variety of cellular hydrolases in its lumen, including proteases, lipases, nucleotidases, and glycosidases, that assist in the degradation of

intracellular components. Protein degradation in the lysosome is highly processive since it results from the collective action of endo- and exoproteases leading to the conversion of proteins into small di- and tri-peptides and free amino acids, released into the cytosol through permeases at the lysosomal membrane. These peptides and free amino acids serve to obtain energy or are recycled to synthesize new proteins (Figure 12) (Vilchez et al., 2014; Wong & Cuervo, 2010).

Depending on the type of cargo and delivery to lysosomes, mammalian autophagy can be characterized in three different modalities: macroautophagy, microautophagy, and chaperone-mediate autophagy (CMA). In macro- and microautophagy, both proteins and organelles are degraded, whereas CMA only participates in the degradation of proteins (Koga et al., 2011; Vilchez et al., 2014; Wong & Cuervo, 2010).

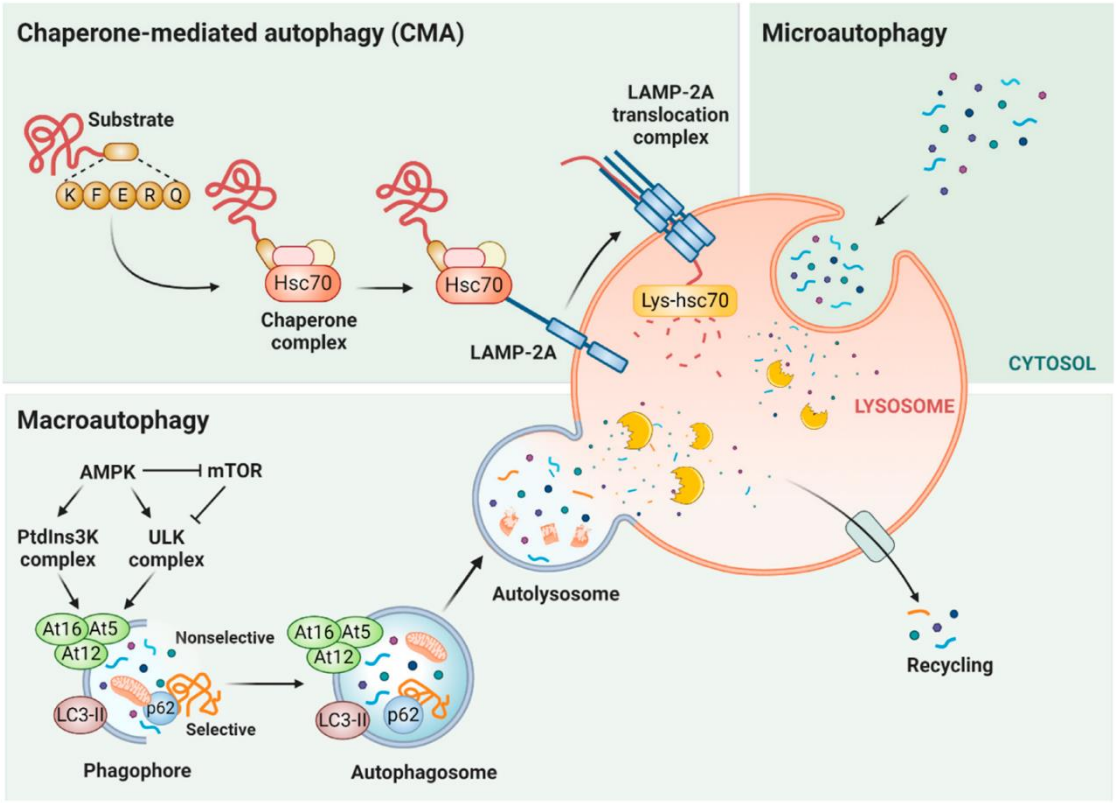

Figure 12 – Schematic representation of the three forms of autophagy: Chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy.
CMA is a selective form of autophagy in which target proteins with the pentapeptide motif (KFERQ sequence) are recognized by cytosolic chaperone HSC70 and cochaperones (chaperone complex). Next, the CMA substrate is taken to the lysosome-associated membrane protein type 2A (LAMP-2A) and translocated across the lysosomal membrane to the lysosomal lumen with the assistance LAMP-2A translocation complex and lys-HSC70. In microautophagy, the substrate is directly engulfed via lysosomal membrane invagination and rearrangement. Macroautophagy is controlled by mTOR and AMPK. mTOR is inhibited by AMPK, which stimulates the unc-51-like autophagy-activating kinase (ULK) and class III phosphatidylinositol 3-kinase (PtdIn3K) complexes to catalyze phagophore formation. LC3 and Atg12 conclude phagophore maturation. Then the autophagosome fuses with the lysosome for substrate degradation and later recycling of metabolic precursors. Macroautophagy can be both selective and non-selective. In selective macroautophagy, specific receptors like p62 recognize the substrate protein and target them to the autophagosome. In non-selective macroautophagy, the substrate is carried to degradation through autophagosomes. (image created with Biorender, adapted from Andrade-Tomaz et al., 2020)

In macroautophagy, an entire region of the cytosol cargo (including organelles and proteins) is sequestered inside a double membrane vesicle (autophagosome), fusing with lysosomes for cargo degradation (Koga et al., 2011). The blending of the luminal content of autophagosomes and lysosomes gives the lysosomal hydrolases access to the sequestered cytosolic cargo, initiating its degradation. Approximately 35 genes, known as autophagy-related genes or ATG, participate in macroautophagy. Their protein products, or Atg proteins (Table 3), are arranged into functional complexes that regulate each of the steps of macroautophagy (Vilchez et al., 2014; Wong & Cuervo, 2010). Recruitment of these autophagic complexes to specific regions of intracellular membranes initiates the formation of a limiting membrane or phagophore, which elongates and fuses to form an autophagosome (Koga et al., 2011; Wong & Cuervo, 2010). The mitochondria, endoplasmic reticulum, and the plasma membrane are confirmed sites of autophagosome formation. In these sites of autophagosome formation, specific Atgs (Autophagy-related genes) act as a platform for assembling other Atgs to the membrane (Wong & Cuervo, 2010). Complexes recruited to the membrane consist of: **(1)** Atgs proteins involved in two conjugation cascades, a protein-protein conjugation and a protein-lipid conjugation system, together with the enzymes that catalyze these conjugation events; **(2)** a beclin-1 containing kinase complex that brings along Class III PI3K Vps34 responsible for the enrichment of the lipid phosphatidylinositol 3-phosphate (PtdIns3P), expanding the phagophore that will originate the autophagosome; and **(3)** Atg1/ULK1 kinase complex, that on activation of autophagy dissociates from the negative regulator of autophagy, mTOR, and mobilizes to the phagophore assembly site to initiate autophagosome formation (Koga et al., 2011; Murrow & Debnath, 2013; Wong & Cuervo, 2010; Yin et al., 2016). The fusion of the autophagosome with lysosomes involves microtubules and proteins in both membranes, to modulate the fusion process and mix the content between both compartments (Wong & Cuervo, 2010).

Induction of macroautophagy is subjected to the negative regulatory effect of mTOR and the components associated with this kinase as part of the mTORC1 complex. Under normal nutrient conditions, mTORC1 is activated by the insulin-like growth factor receptor (IGF1R) pathway in a PI3K/AKT-dependent manner. Activated mTORC1 phosphorylates and inactivates the Atg1/ULK1 complex. Conversely, mTOR is inhibited under starving conditions, and the Atg1/ULK1 complex consequently triggers autophagosomal vesicle formation. Hence, mTOR inhibitors, such as rapamycin, are widely used as macroautophagy activators. In addition, macroautophagy is controlled by another protein kinase complex, the AMP-activated protein kinase (AMPK). Under conditions of low intracellular energy, activated AMPK induces autophagy both by phosphorylating ULK1 and activating it and by inhibiting mTORC1 via phosphorylation of Raptor (Murrow & Debnath, 2013; Vilchez et al., 2014). Furthermore,

SIRT1, a deacetylase that regulates cellular metabolism and survival, can also activate autophagy by deacetylation of several Atg proteins and transcription factors, such as FOXO1,-3 and -4(Vilchez et al., 2014).

Microautophagy, a pathway characterized to a lesser extent also involves sequestration of entire cytosolic regions but, unlike macroautophagy, the engulfment happens directly by the lysosomal membrane. It is a process that is better characterized in the yeast, in which studies have shown a subset of gene products that contribute to the emergence of membrane projections from the surface of the vacuole (equivalent of the lysosome in yeast). These membrane projections sequester soluble proteins and organelles and internalize them inside small vesicles in the lumen of the vacuole. The ausence of mammalian homologs for the microautophagy yeast genes has made it difficult to understand the pathophysiology of this process (Koga et al., 2011; Wong & Cuervo, 2010).

In CMA, substrate proteins are directly translocated across the lysosomal membrane with the assistance of a chaperone. This process requires the recognition of a pentapeptide motif in the substrate protein by the cytosolic heat shock cognate chaperone of 70kDa (Hsc70). The substrate is targeted to the lysosomal surface, where it interacts with the lysosomal-associated membrane protein type 2A (LAMP-2A) receptor (Koga et al., 2011). On substrate binding, the LAMP-2A multimerizes to form a complex that facilitates substrate translocation (Wong & Cuervo, 2010). Substrate internalization into the lysosomal lumen is mediated by a lumenal chaperone (lys-hsc70), and it requires complete unfolding of the substrate protein before translocation. CMA is maximally activated in response to stress (starvation, oxidative stress and conditions that cause protein damage). Under these conditions, the selectivity of CMA allows the removal of altered proteins without affecting functional ones. CMA has only been described in mammalian species, and LAMP2A variant is not conserved in other species, supporting that CMA was acquired late in evolution (Koga et al., 2011).

Table 3 – Summary of autophagy-related proteins (adapted from Vilchez et al., 2014)

Autophagy step	Yeast	Mamals
Formation of phagophore	Atg1	ULK1/2
	Atg13	Atg13
	Atg17	FIP200
	Atg14	Atg14
	Atg6	Beclin-1
Elongation of phagophore/ Maturation of phagophore	Atg12	Atg12
	Atg5	Atg15
	Atg16	Atg16L
	Atg7	Atg17
	Atg10	Atg10
Maturation of phagophore	Atg8	LC3/MAP1LC3A
	Atg3	Atg3
	Atg4	Atg4

Abbreviations: Atg - Autophagy-related genes; ULK1/2 - Unc-51 Like Autophagy Activating Kinase 1 and 2; LC3/MAP1LC3A - microtubule-associated protein light chain 3

4.4.4 - Age-related changes in the autophagic system

Similar to the UPS activity, autophagic-lysosomal potential also decreases with aging, and its decline has been described in different mammalian tissues. Indeed, macroautophagy dysfunction was described in the livers of old rodents even before a complete molecular characterization of this pathway. This early study showed that even though cargo sequestration by autophagosomes still takes place in old tissues, the clearance of these vesicles by fusion with the lysosomal compartment was severely impaired. Incomplete degradation of cargo inside the lysosomal compartment leads to the accumulation of undigested products, which in the case of protein aggregates and other cross-linked products, exert a potent inhibitory effect on lysosomal proteolysis by directly inducing undesired post-translation modifications in the enzymatic machinery and also by altering the proprieties of the intralysosomal environment which is optimized for the functionality of the lysosomal enzymes (Koga et al., 2011). The molecular mechanisms mediating the age-related demise of macroautophagy are still not fully elucidated; however, several studies show that Atg proteins, autophagy inductors, and the cellular response to hormones are the primary triggers for the autophagic degradation decline with age. Atg5, Atg7 and Beclin-1 are downregulated in the aged human brain. Additionally, the levels of Beclin-1, ULK1 and LC3 are reduced in age-related pathologies such as osteoarthritis (Vilchez et al., 2014). Nevertheless, as in the proteasome case, the macroautophagy defect seems to be tissue-dependent (Koga et al., 2011).

Further studies in livers of old rodents revealed alterations in the hormonal regulation of macroautophagy. Insulin and glucagon induce opposite inhibitory and stimulatory effects on macroautophagy. However, the stimulatory effect of glucagon is compromised primarily due to increased basal signaling activity from insulin receptor, which is upregulated due to the oxidative stress characteristic of old organisms. Dietary restriction and lipogenic treatment can prevent the dysregulation of macroautophagy in old rodents, even though the mechanism by which they employ their action is still under investigation (Koga et al., 2011).

As for CMA, its activity decreases in almost all tissues of old rodents and senescent human fibroblasts in culture. A comparative analysis of CMA in livers of young and old rodents revealed a decrease with age in binding and lysosomal translocation of substrate proteins due to progressively lower levels of LAMP-2A receptor. The mechanisms of LAMP-2A downregulation seem to be at the post-translational level since RNA levels of this receptor are unchanged with age. One of the possible mechanisms behind the instability of LAMP-2A in old organisms is the change in lipid composition of the lysosomal membrane (Koga et al., 2011). Another one is the decline in the availability of chaperones during aging, particularly the HSP90, which is critical for the assembly of LAMP-2A receptor. Thus, the decreased HSP90 in

aged liver could explain the impairment in trafficking and stability of LAMP-2A (Vilchez et al., 2014).

Several genetic and functional studies have supported a causal effect of decreased autophagy and aging. Reduced expression of several autophagic genes (atg1, atg7, atg12, bec1 and atg18) in *C. elegans* shortens its lifespan, supporting the role of autophagy in longevity. Furthermore, in *Drosophila melanogaster*, a reduced expression of autophagy genes was found in the neurons of aged flies, and loss of function of Atg1, Atg8 and *Drosophila* sestrin (dSesn, a protein required for autophagy) leads to a reduction of lifespan (Vilchez et al., 2014). In mice, tissue-specific knockout of several autophagy genes precipitates aging and aging-associated phenotypes:

- Atg5, Atg7 or Atg17/FIP200 knockout in the central nervous system-specific of leads to the accumulation of ubiquitin-positive inclusion bodies with subsequent neuronal loss and premature death (Vilchez et al., 2014);
- Purkinje cell-specific knockout of Atg7 causes axonal dystrophy and degeneration of axon terminals with subsequent Purkinje cell death and cerebellar ataxia (Vilchez et al., 2014);
- knockout of Atg7 in the skeletal muscle leads to muscle atrophy, age-dependent decrease in force, accumulation of abnormal mitochondria, disorganization of sarcomeres and exacerbates muscle loss during denervation and fasting (Vilchez et al., 2014);
- knockout of Atg7 on hepatocytes promotes the accumulation of lipid droplets containing triglycerides and cholesterol, induces ER stress and causes insulin stress resistance (Vilchez et al., 2014);
- β cell-specific deletion of Atg7 induces degeneration of pancreatic islets, impaired glucose tolerance, decreased serum insulin level, accumulation of ubiquitinylated proteins colocalized with p62, mitochondrial swelling, and ER distension (Vilchez et al., 2014);
- Podocytes-specific knockout of Atg5 induces spontaneous age-dependent late onset of glomerulosclerosis with an accumulation of oxidized and ubiquitinylated proteins, damaged mitochondria, ER stress, and podocyte loss (Vilchez et al., 2014).

It is essential to highlight that these findings provide evidence of cross-talk between the UPS and the autophagy system. Accumulation of ubiquitinylated products colocalized with p62, even with a functional intact proteasome, indicates the existence of an autophagic ubiquitinated-mediated proteolysis mechanism (Vilchez et al., 2014). It also suggests that aging seems to disrupt the balanced cross-talk between these two proteolytic systems since the

accumulation of ubiquitinylated products represents an unbalanced compensatory attempt of the UPS to mitigate the defective autophagic (Anisimova et al., 2018).

5 - Poly(A) Binding Proteins (PABPs)

All facets of life, including physiological aging, depend on tight regulation of gene expression, highlighting the importance of post-transcriptional control mechanisms (Smith et al., 2014). Translation control is an essential means by which cells regulate gene expression, and therefore the role of RNA-binding proteins (RBPs) cannot be understated. RBPs play a crucial role in translational control, and among them are the Poly(A) Binding Proteins (PABPs) family, comprising proteins that regulate numerous aspects of eukaryotic mRNA fate. PABPs constitute a significant class of regulatory proteins that interact with the 3' poly(A) tail of mRNA and are highly conserved in eukaryotes ranging from yeast to humans (Goss & Kleiman, 2013; Smith et al., 2014).

Based on their intracellular location and phylogeny, PABPs have been divided into two broad categories: nuclear PABPs (PABPNs) and cytoplasmatic PABPs (PABPCs). Both types bind poly(a) tail but consist of different domains (Figure 13) and have distinct steady-state intracellular functions and distributions (Gorgoni & Gray, 2004; Goss & Kleiman, 2013). The number of genes encoding both types also varies across species. Usually, only one gene encoding PABPC is found in single-cell eukaryotes such as yeasts, whereas multiple PAPBC genes are present in metazoans and plants. As for PABPN, a single gene has been found in some species (cow, frog, human, mouse, fly, worm, and yeasts) (Mangus et al., 2003; Smith et al., 2014).

In humans, three lineages of PABP proteins have been identified: cytoplasmic PABPs (PABPC1, PABPC3, and iPABP); nuclear PABP (PABPN1), and X-linked PABP (PABPC5) (Mangus et al., 2003). Among these, PABPC1 (also referred as PABP1) appears to be ubiquitously expressed and is the only PABP whose function in mRNA translation and stability has been extensively addressed and therefore is the focus of this study.

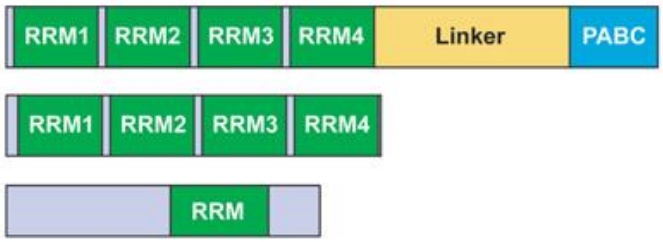

Figure 13 – Domain organization of PABPs
Domain organization of PABPs. Top: vertebrate PABPC1, PABPC4, ePABP, tPABP (mammalian-specific), *D. melanogaster* dPABP and *C. elegans/C. briggsae* PAB-1 and PAB-2 (all predominately cytoplasmic). Middle: mammalian-specific PABPC5 (cytoplasmic). Bottom: PABPN1 (nuclear) and ePABP2 (cytoplasmic). Linker, proline/glutamine-rich variable linker region. **Abbreviations:** RRM - RNA-recognition motif; PABPC1 - Poly(A) Binding Protein Cytoplasmic 1 , also called PABP1; PABPCn - Poly(A) Binding Protein Cytoplasmic number, eg.: PABPC1, PABPC4, PABPC5; dPABP - drosophila PABP; ePABP - Embryonic PABP; tPABP – testis PABP; PABPN - nuclear PABPs; PAB - Poly(A)-Binding Protein in C. elegans/C. briggsae, mainly PAB1 and PAB2. (Adapted from Smith et al., 2014a)

5.1 - Structure of PABP1

The structure of PABP1 is highly conserved, and it has a molecular weight of approximately 70 kDa. It contains, in its N-terminal portion, four repeats of a standard RNA binding domain, the so-called RNP (ribonucleoprotein) domain or RRM (RNA recognition motif), and a highly conserved PABC (PABP C-terminal) domain connected to the four RNP domains by a proline- and glutamine-rich 'linker' (Kühn & Wahle, 2004). The RRM domain, typically 90-100 amino acids long, is the most prevalent domain used in RNA recognition, as illustrated by its presence in more than 200 putative RBPs. Its broad distribution among proteins suggests that this is an ancestral motif with important functions in RNA biology. Solution nuclear magnetic resonance (NMR) and X-ray crystallographic studies have demonstrated that the RRM is a globular domain consisting of four antiparallel β sheets flanked by two α helices (Mangus et al., 2003). Two conserved sequence motifs, RNP1 and RNP2, are located in the central two ß strands, which mediate mRNA poly(A) tail recognition via Van der Waals contacts, hydrogen bonds, and stacking interactions with conserved residues within them (Goss & Kleiman, 2013; Mangus et al., 2003).

The highly conserved carboxy-terminal domain, also known as PABC, is an approximately 75 amino acid domain that folds independently into four or five alpha-helices. The carboxy-terminal domain is not required for RNA recognition and is dispensable for cell viability in yeast. However, this domain is the site of interaction with factors regulating polyadenylation, deadenylation, translation initiation, and translation termination (Goss & Kleiman, 2013; Mangus et al., 2003). Such factors are, for instance, eukaryotic translation termination factor 3 (eRF3), PABP interacting proteins 1 and 2 (PAIP1 and PAIP2), and PAN3 (poly(A) nuclease

3) deadenylase, that have a PABP interaction motif (PAM-2) thus regulating PABP1 functions (Goss & Kleiman, 2013).

5.2 - PABP1 localization and functions

PABP1 exhibits a predominantly diffused cytoplasmatic distribution, but it can also be found in high concentration at sites of localized translation such as neuronal dendrites or leading edges of migrating fibroblasts. Some family members can shuttle to and from the nucleus, and in situations of cell stress, they accumulate in the nucleus or the cytoplasmic foci, such as stress granules (Smith et al., 2014).

The PABP1 has a crucial role in gene expression pathways (Mangus et al., 2003). The primary function binds to nascent pre-mRNAs poly(A) tails and enhances translation initiation by interacting with the translation initiation factors at the 5' end (Figure 14 A). The interplay between PABP1 and the initiation factors allows them to stabilize their interaction with mRNA and therefore enhance the recruitment of ribosomal subunits. However, it is essential to highlight that the extent to which translation of individual mRNAs is stimulated can also be influenced by the regulation of their poly(A) tails lengths (Smith et al., 2014). Thus, stabilization of the interaction between mRNA and initiation factors process also depends on factors regulating the polyadenylation process and ultimately determining the size of poly(A) tail (Mangus et al., 2003).

Interestingly, PABP1 also binds (directly or indirectly) to sites other than the poly(A) tail, and by doing so, PABP1 can activate or repress translation in an 'mRNA-specific' manner (Figure 14 F and G). mRNA-specific translation activation or repression depends on the location of its alternative binding sites and the protein which PABP1 interacts with (Smith et al., 2014).

Additionally, PABP1 has other less-characterized functions in mRNA turnover. Among these is the PABP1 role in protecting poly(A) tail from deadenylation, which is the first rate-limiting step in RNA turnover (Figure 14 B). Conversely, PABP1 is also involved in deadenylation, specifically by recruiting deadenylases to mRNA and coordinating translation termination, therefore, regulating mRNA lifespan. PABP1 can also regulate the stability in an mRNA-specific manner either as part of regulatory complexes bound to sites within mRNAs or by interacting with stabilizing or destabilizing complexes when bound to the poly(A) tail (Figure 14 C). Moreover, PABP1 is also implicated in miRNA-mediated translational repression and/or deadenylation (Figure 14 E). Finally, PABP1 is associated with discerning mRNAs that should

undergo nonsense-mediated decay (NMD) due to the presence of premature stop codons (Figure 14 D) (Smith et al., 2014).

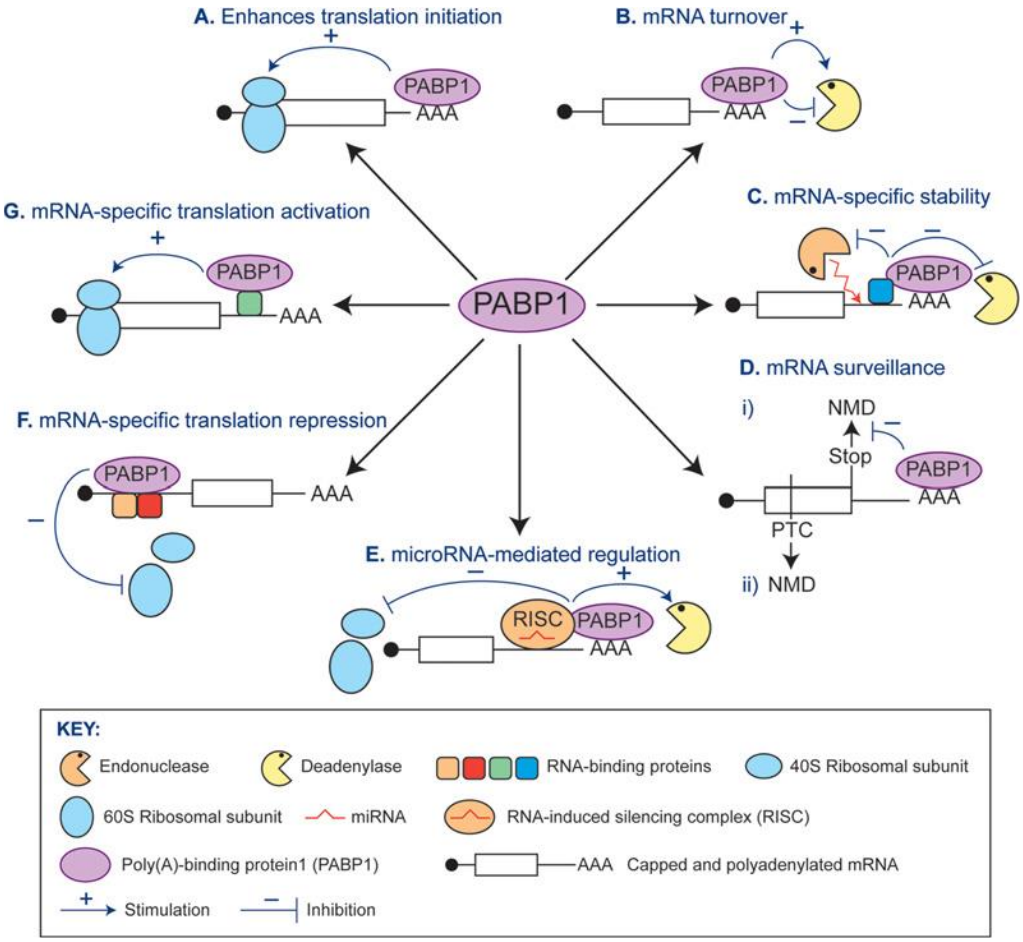

Figure 14 – Molecular functions associated with PABP1

(**A**) PABP1 enhances global translation initiation by binding to mRNA's poly(A) tail and interacting with initiation factors at the 5'cap to recruit ribosomal subunits. (**B**) PABP1 regulates mRNA turnover by blocking access of deadenylases to the poly(a) tail and recruits deadenylases to the mRNA (in translation termination). (**C**) PABP1 has an mRNA-specific role in stability by interacting with 3'-UTR-binding proteins to block deadenylation and endonucleolytic cleavage within 3'-UTR. (**D** panel i) PABP1 ensures correct termination at stop codons by inhibiting the nonsense-mediated decay pathway (NMD); (**D** panel ii) PABP1 only allows NMD in the cases of aberrant termination at premature termination codons (PTCs). (**E**) PABP1 enhances miRNA-mediated translation repression and deadenylation by interacting with the RISC complex. (**F**) PABP1 can participate in translation repression in an mRNA-specific manner by binding to the 5'-UTR repression complex, which blocks ribosome assembly. (**G**) PABP1 can also act as an mRNA-specific activator when recruited to 3'-UTR by other RNA binding proteins (RBPs) or regulatory elements. (Adapted from Smith et al., 2014)

5.3 - PABP1 role in mRNA translation

PABP1 is considered to be a central regulator of mRNA translation and stability (Gray et al., 2015). Its role in translation mRNA circularization is imperative for the process of translation. After mRNA enters the cytoplasm, the association of PABP1 with its poly(A) tail promotes 5' - 3' interactions that stimulate the initiation of its translation. The resulting mRNA circularization ('end-to-end complex' or 'closed loop') promotes translation by recruiting 40S ribosomal subunits (figure 15). This recruitment is dependent on interactions between initiation factor eIF4G and PABP1 and simultaneous interactions between eIF4G and the cap-binding protein eIF4E (Kühn & Wahle, 2004).

This interaction is also crucial to enhancing the affinity of eIF4E for the 5' cap of the mRNA by lowering its dissociation rate and to stimulating the RNA-binding activity of PABP1 for poly(A), effectively locking them onto both ends of the mRNA (Gorgoni & Gray, 2004; Kühn & Wahle, 2004). Moreover, it also increases the ATPase and RNA helicase activities of eIF4A, eIF4B, and eIF4F. The synergy of these effects also provides an effective means for the protein synthesis apparatus to ensure preferential translation of mRNAs containing both a cap and a poly(A)tail and may create an opportunity for ribosomes to recycle from the 3' to the 5' end of the same mRNA (Kühn & Wahle, 2004). PABP1 can also influence later steps in translation, including 60S subunit joining and termination, which PABP1 regulates via interaction with eRF3 (Goss & Kleiman, 2013).

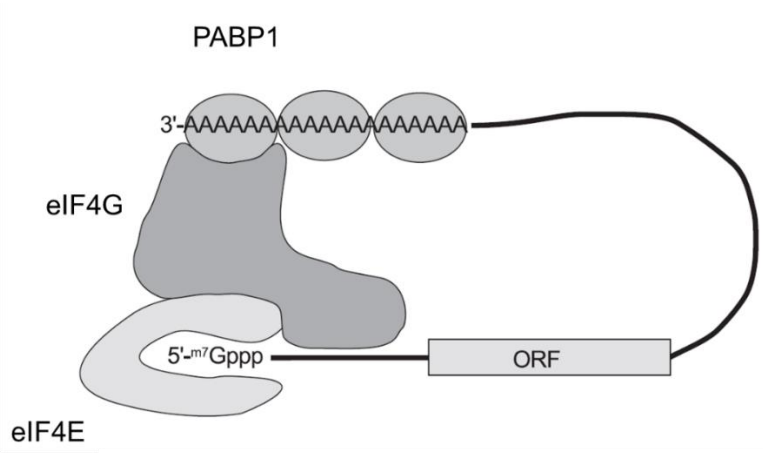

Figure 15 – The closed-loop model of translation
The initiation factor eIF4E binds the cap, whereas PABP1 binds the poly(A) tail. Both proteins interact with eIF4G, which functions in the recruitment of the 40S subunit. (Adapted from Kühn & Wahle, 2004)

5.4 – Regulation of PABP1

Two independent mechanisms tightly regulate the expression of PABP1. Firtstly, PABP1 is able to autoregulate its own mRNA by binding to an adenine-rich sequence within its 5' UTR, resulting in the repression when the levels of PABP1 are too elevated. Alternatively, a 5' terminal oligopyrimidine tract (5' TOP) can also regulate the expression levels of PABP1 in response to cell growth. Moreover, TOPs are also present in other translation machinery components, allowing for coordinated growth regulation (Gorgoni & Gray, 2004).

PABP can also be regulated via phosphorylation and methylation at a protein level. Phosphorylation is a well-established means of controlling global translation, as illustrated by the translation factors eIF4E and eIF2. There is evidence of multiple phosphorylation forms of PABPs in plants, yeast, and sea urchins. For instance, phosphorylation of wheat germ PABP enhances its cooperative binding to poly (A) tail, suggesting that its ability to homodimerize is increased. Furthermore, the ability of eIF4G and eIF4B to interact with the PABP1/Poly (A) tail seems to be differentially influenced by the PABP1 phosphorylation state. PABP1 is also the substrate for the coactivator-associated arginine methyltransferase (CARM1), which methylates arginines in the proline-rich linker region, which may affect homodimerization, RNA or protein interactions, or even intracellular localization (Gorgoni & Gray, 2004).

Other proteins also regulate PABP1 protein. Rotavirus nonstructural protein 3 (NSP3) was the first protein shown to modulate PABP1 activity by competing for the binding to eIF4G, disrupting PABP1-eIF4G interaction. PAIP1 and 2 (Poly(A) Binding Protein Interacting Protein 1 and 2) can also regulate PABP1. PAIP2 acts as a negative regulator of PABP1 by binding within RRM 2-3 and PABC, inhibiting its interaction with poly (A) tail and PAIP1, a translational stimulator. PABPs may also be subject to proteolytic cleavage during viral infection and apoptosis. Enterovirus protease 3Cpro cleaves PABP1 at three sites within the proline-rich linker region. Cleavage releases the PABC domain from RRM, leading to inhibition of poly (A)-dependent translation. During apoptosis, caspase mediates the cleavage of several translation factors such as eIF4G and eIF4B, disrupting their interaction with PABP1 and the formation of closed loop complex (Gorgoni & Gray, 2004).

6 - Objectives

Aging is associated with an accumulation of damage that affects all physiological functions, including protein synthesis. Indeed, recent studies have unraveled an intricate connection between overall protein synthesis and aging. Accumulating evidence suggests that protein synthesis and translation control could significantly influence lifespan. This notion is reinforced by the fact that signaling pathways known for regulating the aging process tightly regulate protein synthesis. However, the mechanisms by which translation control and protein synthesis mediate lifespan control are still elusive.

Therefore, this study aims to elucidate the role of protein synthesis and translation control in aging and lifespan and understand their functional connection with several hallmarks of aging. To this end, we focused on PABP1, a central protein in translation control, to establish a link between aging and protein synthesis.

Chapter 2 - Materials and Methods

1 - Animals

Sixteen adult C57BL/6J mice, bred in the animal house facility of the Algarve Biomedical Center Research Institute of the University of Algarve, were used in this experiment. The animals, average weight of 20-25g, were housed in a temperature-controlled room (22±2ºC) on a 12h light–12 h dark cycle. Food and water were provided *ad libitum*. After *in vivo* stereotaxic injection of lentiviral vectors (LV), the animals were separated into three experimental groups: six animals injected with PABP1 (one died), four animals injected with GFP, and six animals not injected. The experiments were carried out in accordance with the European Community directive (86/609/EEC) for the care and use of laboratory animals. The researchers received certified training (FELASA-certified course) and approval to perform the experiments from the Portuguese authorities (Direcção Geral de Alimentação e Veterinária - DGAV) in the project Neuropath (421/2019).

2 - Lentiviral vectors

Lentiviral particles (LVs) were produced from cDNA encoding for human PABP1 and GFP under the control of mouse phosphoglycerate kinase 1 (PGK). The viral vectors encoding the two different constructs were produced and quantified in HEK 293T cells using a four-plasmid system described previously (Pereira De Almeida et al., 2002). Viral samples were stored at -80°C until used.

3 - *In vivo* injection of lentiviral vectors

For the stereotaxic injection of lentiviral vectors, concentrated viral stocks were thawed on ice and resuspended by vortexing. Adult (12 months-old) C57BL/6J mice (both males and females) were anesthetized through intraperitoneal injection (IP) of a mixture of ketamine (75 mg/kg, Dechra) and medetomidine (0,75 mg/kg, Esteve). LVs were stereotaxic injected bilaterally into the arcuate nucleus of the hypothalamus following brain coordinates relative to bregma: anteroposterior, -1,65mm; lateral, +/- 0,5 mm; ventral, -5,8 mm (Paxinos & Franklin, 2019). Particle content of lentiviral vectors was matched to 400 ng of p24 antigen. For the injections, animals received a single injection in each hemisphere at a rate of 0.2 µl/min by

means of an automatic injector (Stoelting Co., Wood Dale, IL, USA) into mice striatum using a 34-gauge blunt-tip needle linked to a Hamilton syringe (Hamilton, Reno, NV, USA). Animals were kept for 8 weeks post-injection before being sacrificed for subsequent analyses, with weight monitoring once a week.

4 - Tissue preparation

Eight weeks after stereotaxic injection, animals were sacrificed by sodium pentobarbital overdose, and the brains were directly removed without transcardial perfusion. Brains were dissected fresh, then punches with a Harris Uni-Core pen (with 2.0 mm diameter) were performed to collect the hypothalamus; both were preserved at -80°C, and the hypothalamus was used later for Western Blot and RT-PCR analysis. Subsequently, the liver, spleen, white adipose tissue (WAT), and brown adipose tissue (BAT) were collected and weighed. They were all preserved in 10% neutral buffered formalin for posterior analysis.

5 - Protein and RNA extraction

Frozen punches were slightly thawed at room temperature, and the protein and RNA extraction was performed with 1100 µl of QIAzol™ lysis reagent and then homogenized for 5 minutes. Next, 200 µL of d-chloroform was added and left to rest for 2-3 minutes at room temperature. The samples were centrifugated at 12.000 rpm for 15 min at 4°C, the aqueous phase (~500 µL) was transferred to an RNAse-free Eppendorf for RNA extraction, and the other phases were used for protein extraction.

The Nucleospin RNA kit (NZYtech) protocol was used for RNA extraction. The aqueous phase was cleaned up using DNase digestion at the NZYtech spin column according to the manufacturer's recommendations. The RNA concentration and purity were determined with NanoDrop 2000c (Thermo Scientific).

For the protein extraction, 300 µL of 100% ethanol was added to the interphase and phenol phase, then mixed by inversion and incubated at room temperature for 2-3 min. After this period, the samples were centrifuged at 2000 g for 2 min at 4°C to sediment DNA. The phenol/ethanol supernatant containing the protein fraction was transferred to a 15 mL Falcon, and 1,5 mL of isopropanol was added to precipitate the protein and mix by inversion for 15 sec. The volume was separated into two 1,5 mL Eppendorf and incubated at room temperature for 10 min. In order to remove the supernatant, the samples were centrifugated again at 12.000 g

for 10 min at 4ºC. Next, 1 mL of guanidine-ethanol solution (to each Eppendorf) was added to the protein pellet; the samples were vortexed, incubated at room temperature for 20 min, and centrifugated at 7500 g for 5 min at room temperature. After this process, the supernatant was removed, and then the previous steps after 12000g centrifugation were repeated two more times. 1 mL of 100% ethanol was added to each pellet containing the proteins, and afterward, it was vortexed, incubated at room temperature for 20 min, centrifugated at 7500 g for 5 min at room temperature, the supernatant was removed, and the pellet was air-dried for 10min. Afterward, a 50 µL solution of Urea/DTT was added, and the samples were incubated at room temperature for 1 hour. Again, the samples were incubated for another 3min at 95ºC and subsequently placed on ice. Finally, the proteins sample were quantified by the Bradford method and subsequently preserved at -80ºC for later use.

6 - Western Blot

Cells were first lysed in RIPA-buffer solution (50 mm Tris HCl pH = 8; 150 nm NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) containing protease inhibitors (Roche diagnostics, Risch-Rotkreuz, Switzerland), followed by sonication of 5s ultrasound chase (1 pulse/s). Total protein lysates were stored at −80◦C, and protein concentration was determined through the PierceTM BCA Protein Assay Kit (Thermo Scientific, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Thirty or fifty micrograms of protein extract were resolved in SDS-polyacrylamide gels (10%). Then proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare) according to standard protocols. Immunoblotting was performed using the antibodies shown in Table 4. Immunoreactive bands were visualized by chemiluminescence with the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, USA) in a ChemiDoc™ XRS+ System (Bio-Rad, USA).

Table 4 – Antibodies used in Western blot procedures

	Antibody name	Species	Dilution	Incubation	Manufacturer
Primary antibody	anti β-actin	mouse monoclonal	1:5000	ON, 4 ºC	Sigma
	anti-β-tubulin		1:5000	ON, 4 ºC	Sigma
	anti-PABP1		1:1000	ON, 4 ºC	Millipore
	anti-puromycin		1:20000	ON, 4 ºC	Millipore
	anit-eIF2α	rabbit monoclonal	1:1000	ON, 4 ºC	Cell Signalling
Secondary antibody	anti-mouse IgG	sheep polyclonal	1:10000	2 h, RT	GE Healthcare
	anti-rabbit IgG	sheep polyclonal	1:10000	2 h, RT	GE Healthcare

Abbreviations: **IgG** – immunoglobulin **ON** – overnight, **RT** – room temperature

7 - Reverse-transcription polymerase chain reaction (RT-PCR)

cDNA synthesis was performed with iScriptTM cDNA Synthesis Kit (Bio-Rad) from 1 mg of total RNA according to the manufacturer's instructions. PCR was performed in real-time quantitative PCR with the SsoAdvancedTM SYBR® Green Supermix Kit (Bio- Rad). The primers used for target and housekeeping genes are shown in Table 5. Briefly, 2.5 ml of the cDNA obtained in the reverse transcription reaction diluted 10-fold with DNase free deionized water were used. The quantitative PCR was performed as follows: one single cycle at 95°C for 30 s, followed by 45 cycles of two steps, first step of 5 s at 95°C, second step of 15 s at 55°C. The melting curve protocol started immediately after the quantitative PCR and consisted of 5 s heating at 65°C with a 0.5°C temperature increase in each step until 95°C is reached. The cycle threshold (Ct) values were generated automatically by the QuantStudio Software (Applied Biosystems - Termofisher). To each gene, and in each experiment, a standard curve was performed, and quantitative PCR efficiency was determined by the software.

Table 5 – Primers used in PCR procedures

Gene	Primer sequence	Dilution	Manufacturer
PABP1	**Fwd:** 5'-GAATATGCCCGGTGCTATCCG-3' **Rev:** 5'-ACTGTGGAACCTGTGAGGAA-3'	1:10	Invitrogen
mTOR	**Fwd:** 5'-TCCTGCGCAAGATGCTCATC-3' **Rev:** 5'-TGTGCTCCAGCTCTGTCAGGA-3'	1:5	Invitrogen
p62	**Fwd:** 5'-ATGCTGCCATGGGTTTCTC-3' **Rev:** 5'-GGTGGAGGGTGCTTTGAATA-3'	1:10	Invitrogen
PGC-1α	**Fwd:** 5'-AAACTTGCTAGGGGTCCTCA-3' **Rev:** 5'-TGGCTGGTGCCAGTAAGAG-3'	1:5	Invitrogen
GapDH	**N.d.**	1:10/1:5	Invitrogen
LC3B	**Fwd:** 5'-GACGCTTCCTGTACATGGTTT-3' **Rev:** 5'-TGGAGTCTTACACAGCCATTGC-3'	1:10	Invitrogen
NFκB	**Fwd:** 5'-GAAATTTCCTGATCCAGACAAAAC-3' **Rev:** 5'-ATCACTTCAATGGCCTCTGTGTAG-3'	1:5	Invitrogen
IL-6	**Fwd:** 5'-ACGGCCTTCCCTACTTCACA-3' **Rev:** 5'-CATTTCCACGATTTCCCAGA-3'	1:10	Invitrogen
HPRT	**N.d.**	1:10	Invitrogen
ATXN2	**N.d.**	1:5	Invitrogen

Abbreviations: **N.d.** – not disclosed, **Fwd** – forward primer, **Rev** – reverse primer

8 - Cell culture, transfection and H_2O_2 treatment

Human embryonic kidney 293 cells containing SV40 T-antigen (HEK293T) and human fibroblast were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% and 15% fetal bovine serum (FBS), respectively, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) at 37◦C in 5% CO2/air atmosphere. Every 2/3 days, the culture medium was changed as cells were split upon reaching 80-90% average confluence.

For the HEK293T transfection, the cells were seeded in 12-well plates and were transfected with 500 ng of plasmid DNA and 3 µL of polyethylenimine (PEI, 1 mg/ml, Polysciences Inc.,

USA) per well, and then cultured for 24h. Transfection reagents were left in cells for a 48h-period incubation, following translation rate assay and cells harvest for western blot assays. These cells were transfected with progerin or lamina A. For the HEK293T treatment with H_2O_2 (peroxide), the cells were seeded in 6-well or 12-well plates and were treated with medium containing 0,1 mM H_2O_2 for 30min, following translation rate assay and cells harvest for western blot assays. The human fibroblast cells (HGPS and wild-type fibroblast) were harvested directly from the 75cm² or 175 cm² for translation rate and western blot assays.

9 - Translation rate assay

A previously described method was used that allows the monitoring and quantification of global protein synthesis based on the incorporation of puromycin during translation (Schmidt et al., 2009). Briefly, cells were incubated with 10 mg/ml of puromycin (Sigma) for 15 min, and later lysed for western blot posterior processing. As positive control for the translation inhibition, some cells were incubated first with 10 mM of cycloheximide (CHX, Sigma) for 15 min, and then incubated with 10 mg/ml of puromycin (Sigma) for 15 min. Some cells were not treated with puromycin as the control for the experiment; wild-type HEK293T cells (untreated and untransfected without progerin or lamina A) and wild-type human fibroblast were also used in these experiments as additional controls.

10 - Statistical analysis

All statistical analysis was performed using Student's t-test o using GraphPad software (La Jolla, USA). Results are normally expressed as mean ± SEM. Significant thresholds were set at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, as defined in the text.

Chapter 3 – Results

1 – Overall protein synthesis is significantly lower in HGPS fibroblasts than in control fibroblasts

In several organisms, aging is associated with extensive alterations in general and specific protein synthesis (Tavernarakis, 2008). While several studies have established that protein synthesis declines during aging, a complete consensus on this topic has not been achieved yet (Anisimova et al., 2018; Tavernarakis, 2008). To contribute to the clarification on how the level of protein synthesis rate varies with age, we measured the overall protein synthesis in fibroblasts, using Western blot SUnSET (SUrface SEnsing of Translation). We used fibroblasts from a HGPS patient, as a model for aging, which we compared fibroblasts from a healthy control. We observed a significant reduction of overall protein synthesis on HGPS fibroblasts compared to control fibroblasts (Figure 16).

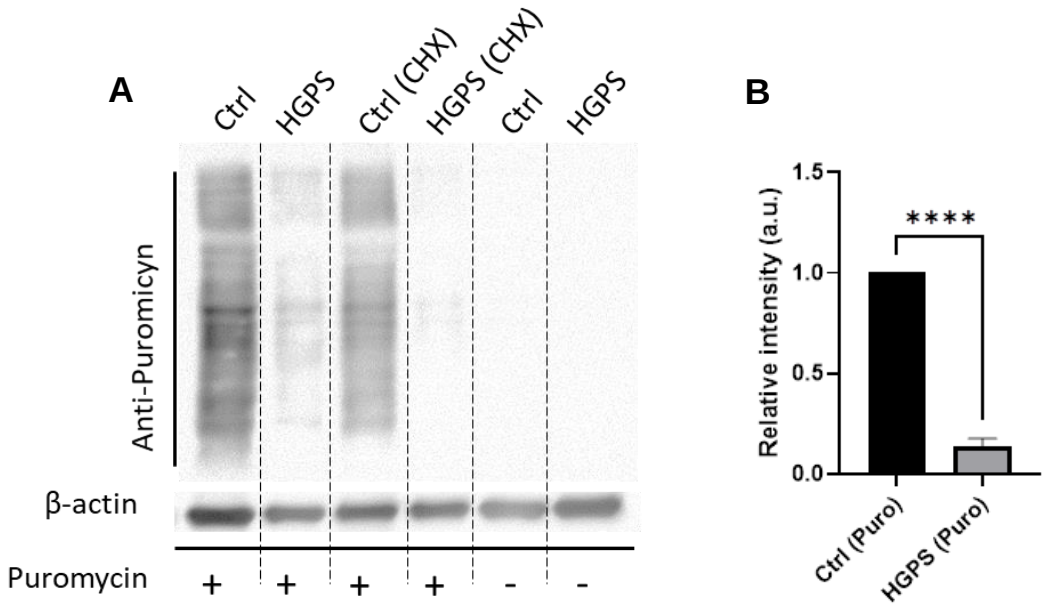

Figure 16 - Translation rate comparison between HGPS fibroblast and control fibroblast
(**A**) Western blot for protein lysates from HGPS fibroblast and wild-type fibroblast as control, for the puromycin as a measure of translation rate. CHX was used as a negative control (CHX=cycloheximide) (n=3). (**B**) HGPS fibroblast (n=3) have a decreased translation rate compared to the controls (n=3).
Values are expressed as mean ± SEM . ****P <0.0001 (unpaired Student's t-test)

2 – Level of endogenous PABP1 and EIF2α did not correlate with the observed decrease of protein synthesis rate in HGPS fibroblasts

After observing a significant reduction in overall protein synthesis in HGPS fibroblast, we next investigated whether important players of translation were also altered and thus could explain the observed results. PABP1 is a multifunctional protein with roles in global and mRNA-specific translation and stability and thus is a crucial regulator of mRNA fate (Smith et al., 2014). The interacting eukaryotic translation initiation factors 2 alpha (eIF2α) is a regulatory node that controls protein synthesis initiation by its phosphorylation or dephosphorylation (Liu et al., 2020). PABP1 enhances translation initiation by interacting eukaryotic translation initiation factors, and together they have a central role in cytoplasmatic mRNA metabolism (Smith et al., 2014). Through Western blot, we measured PABP1 and eIF2α in HGPS and control fibroblasts. No significant differences were found between both experimental groups, although there is trend for a reduction of both markers in HGPS fibroblasts (Figure 17).

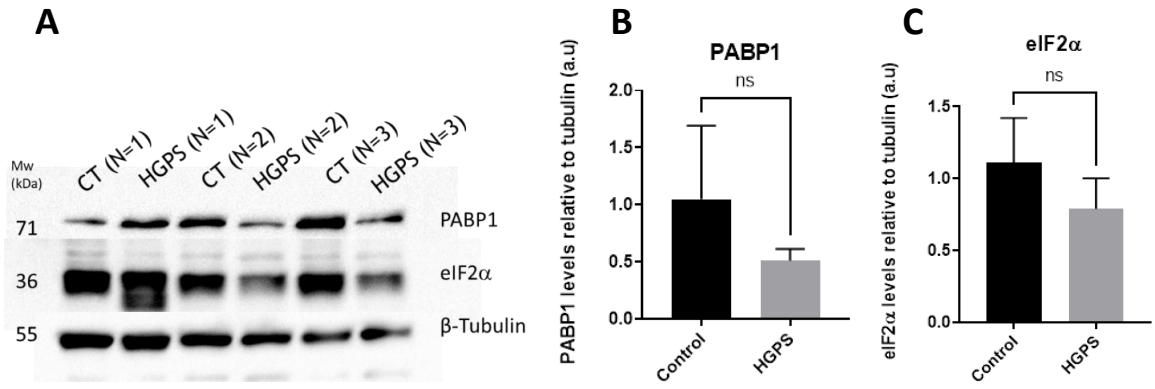

Figure 17 - Levels of PABP1 and eIF2α in HGPS and control fibroblast
(**A**) Western blot for PBAP1 and eIF2α protein lysates from HGPS fibroblast and wild-type fibroblast as control. (**B** e **C**) No significant differences between HGPS fibroblast (n=3) and the controls (n=3), in both the PABP1 [CT (n = 3) vs HGPS (n = 3) P-value = 0.4356 unpaired Student's t-test] and eIF2α [CT (n = 3) vs HGPS (n = 3) P-value = 0.4563 unpaired Student's t-test].

3 – Inducing genetic instability and oxidative stress in HEK293T cells increases overall protein synthesis

Next, we investigate how protein synthesis rate varies, while inducing oxidative stress and genomic instability to promote cellular aging using two models: hydrogen peroxide (H_2O_2) treatment and progerin transfection, respectively. Exposure to H_2O_2 is a widely used procedure to cause oxidative damage/stress in cellular models (Ransy et al., 2020). Progerin, a mutant form of lamina A, alters nuclear structure and heterochromatin, affecting cell cycle progression, gene expression, and genomic stability (Niedernhofer et al., 2011).

For both of these models, we used HEK293T and then measured global protein synthesis using WB SUnSET. In the first experiment, we treated HEK293T cells with H2O2 and then compared the overall protein synthesis to untreated HEK293T. We observed a significant increase in protein synthesis in the H2O2 treated group, as reflected by the intense labeling of puromycin compared to the untreated group (Figure 18). In the second model, we transfected HEK293T cells with progerin and Lamina A, as the positive control of the transfection and also used non-transfect HEK293T as an additional control. We observed a non-significant trend of higher overall protein synthesis rate in the HEK293T cells transfected with progerin compared with Lamin A transfected HEK293T group (Figure 19 A and B), but a non-significant decrease compared to the control group (Figure 19 A and C).

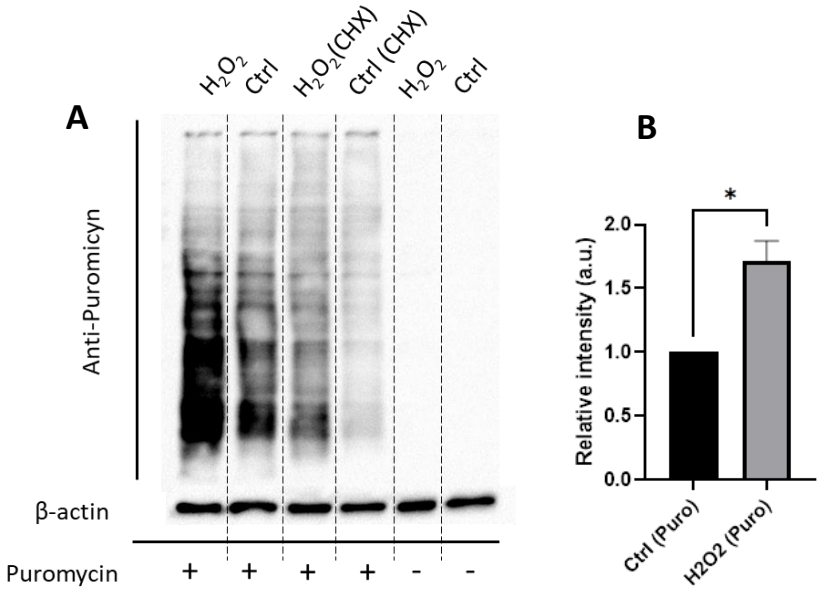

Figure 18 - Measurement of translation rate on HEK293T under oxidative stress
(**A**) Western blot for protein lysates from HEK293T cells treated with H_2O_2 and untreated HEK293T cells as control, for the puromycin as a measure of translation rate. (**B**) HEK293T treated with H_2O_2 have an increased translation rate compared to the controls [CT (n = 3) vs H_2O_2 (n = 3) *P-value* = 0. 0105 *unpaired Student's t-test*]. Values are expressed as mean ± SEM. *P <0.01 (unpaired Student's t-test)

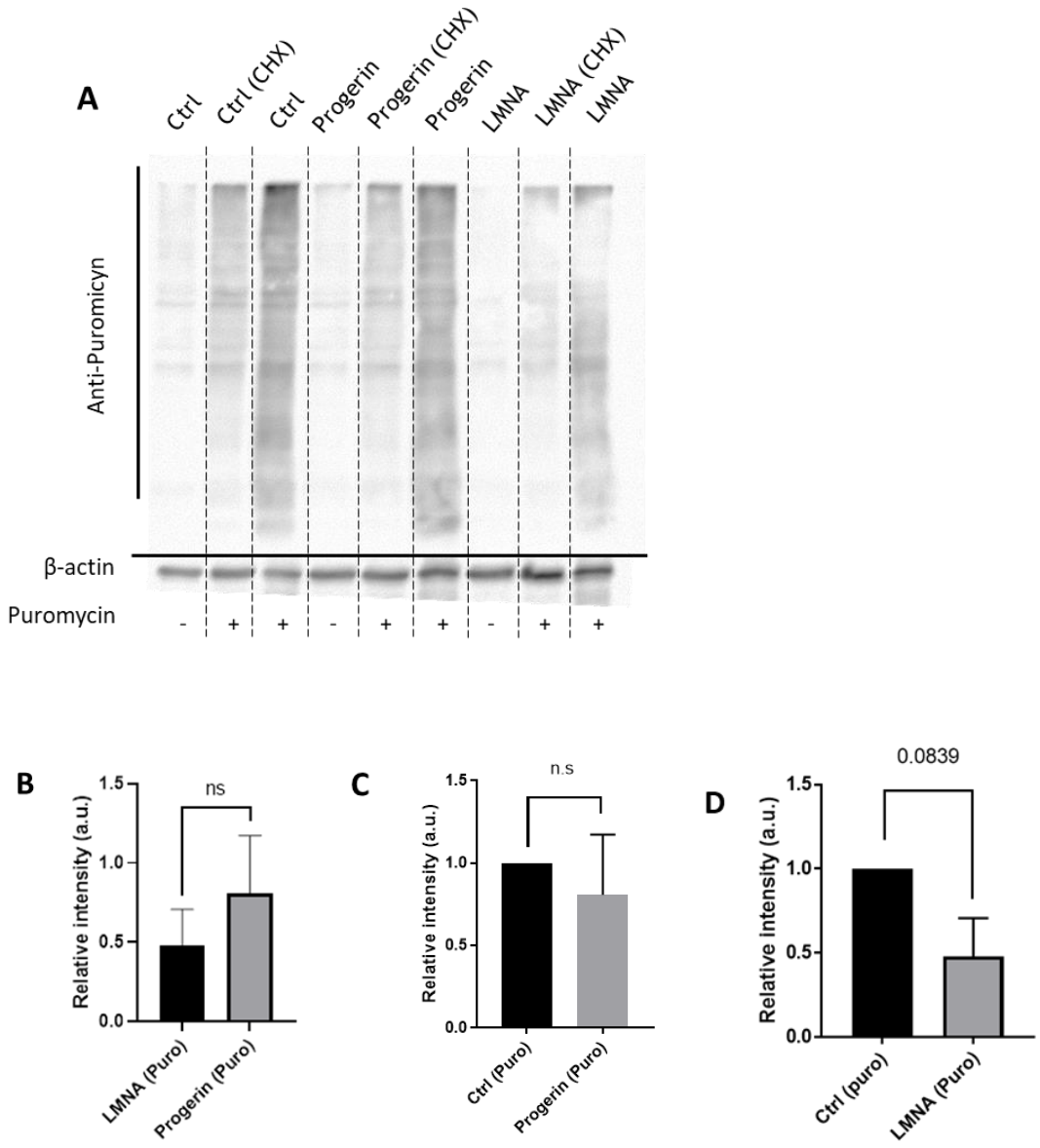

Figure 19 - Measurement of translation rate on HEK293T under genomic instability
(**A**) Western blot for protein lysates from HEK293T cells and HEK293T cells expressing Progerin or Lamina A (LMNA) for the puromycin as a measure of translation rate. (**B**) No significant differences between Lamina A and Progerin [LMNA Puro (n = 3) vs Progerin puro (n = 3) *P-value* = 0.4880 *unpaired Student's t-test*]. (**C**) No significant differences between Progerin and control [CT puro (n = 3) vs Progerin puro (n = 3) *P-value* = 0.6272 *unpaired Student's t-test*]. (**D**) No significant differences between LMNA and control [CT puro (n = 3) vs LMNA Puro (n = 3) *P-value* = 0.0839 *unpaired Student's t-test*].

4 – Endogenous PABP1 expression levels are higher in the hypothalamus of old mice, as compared to young mice

Hypothalamus governs several physiological functions that decline with age, such as metabolism, reproduction, circadian rhythm, and homeostasis, and thus it is a central regulator of aging (K. Kim & Choe, 2019).

To further elucidate the role of protein synthesis in systematic aging, we analyzed its endogenous PABP1 mRNA levels in the hypothalamus of wild-type old mice (18 months) compared to wild-type young mice (2 months). We found that the mRNA expression levels of endogenous PABP1 was higher in the hypothalamus of old mice than those of young mice, even though the result was not statistically significant (Figure 20).

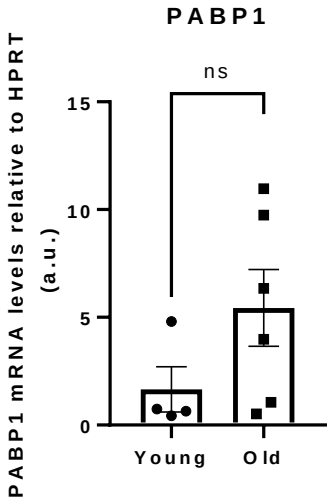

Figure 20 - Comparison of the endogenous PABP1 expression levels between old and young mice hypothalamus
No significant differences in PABP1 between young and old mice [Young (n = 3) vs Old (n = 5) *P-value* = 0.1505 *unpaired Student's t-test*]

5 – Overexpression of PABP1 in the mice hypothalamus increases the levels of endogenous PABP1, while not affecting the levels of Ataxin-2

To further investigate the role of PABP1 in systematic aging, we overexpressed PABP1 in mice's hypothalamus and analyzed the expression levels of several key target mRNAs involved in aging. First, we intended to understand if the overexpression of PABP1 could be affected by endogenous PABP1. It is reported that PABP1 autoregulates its mRNA by binding an A-rich sequence within its 5'UTR, to repress it when its levels are high (Gorgoni & Gray, 2004). We observed a significant increase in the endogenous mRNA levels of PABP1 in the hypothalamus of mice overexpressing PABP1 compared to the non-injected group (Figure 21 A). In addition, we analyzed the mRNA levels of Ataxin-2, which is known to interact with PABP1 to regulate RNA translation (Carmo-Silva et al., 2017; Nóbrega et al., 2015). However, we did not find a significant difference between the two groups (Figure 21 B)

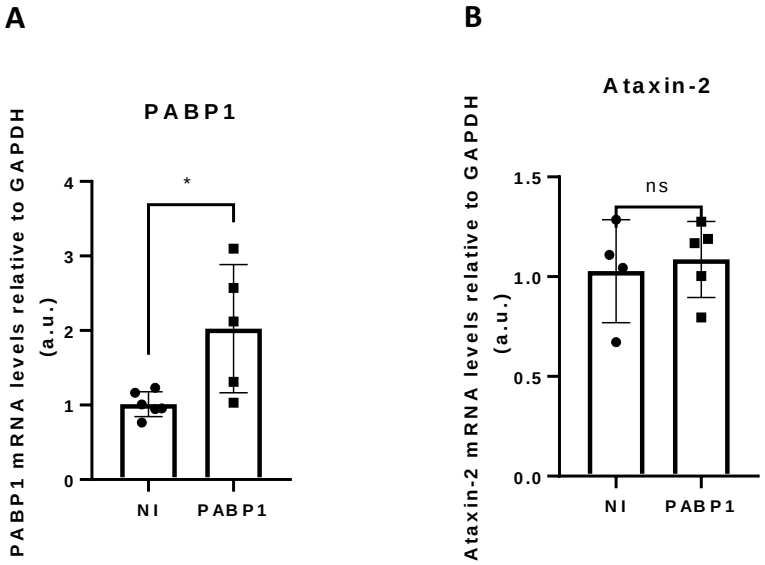

Figure 21 - Endogenous PABP1 and Ataxin-2 levels in the hypothalamus of mice overexpressing PABP1
(**A**) A significant increase in the endogenous mRNA levels of PABP1 in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n= 6) vs PABP1 (n = 5) *P-value* = 0.018 *unpaired Student's t-test*] (**B**) No significant differences in the mRNA levels of Ataxin-2 in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n= 4) vs PABP1 (n = 5) *P-value* = 0.7049 *unpaired Student's t-test*]. Values are expressed as mean ± SEM. *P <0.01 (unpaired Student's t-test)

6 – Overexpression of PABP1 in the mice hypothalamus did not affect the levels of mTOR

The mTOR kinase is a central component of cellular metabolism that integrates nutrient sensing with cellular processes that fuel cell growth and proliferation (López-otín et al., 2013; Papadopoli et al., 2019). In addition to nutrient-sensing, mTOR is implicated in many processes associated with aging, including cellular senescence, immune responses, cell stem regulation, autophagy, mitochondrial function, and protein homeostasis. Thus, it is regarded as a central regulator of lifespan and aging (Papadopoli et al., 2019). Since both mTOR and PABP1 are vital components of protein synthesis, we investigated whether the overexpression of PABP1 could affect mTOR expression levels. However, we did not find significant differences between the hypothalamus of mice overexpressing PABP1 and the non-injected group (Figure 22).

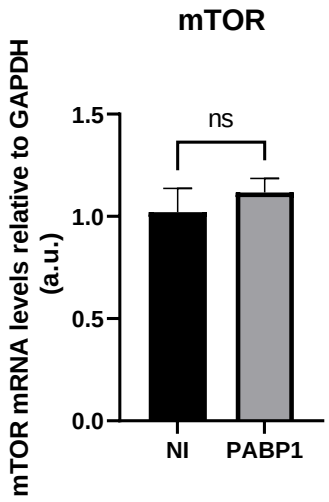

Figure 22 - mTOR levels in the hypothalamus of mice overexpressing PABP1
No significant differences on mTOR levels in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n= 4) vs PABP1 (n = 5) *P-value* = 0.4799 *unpaired Student's t-test*].

7 – Overexpression of PABP1 in the mice hypothalamus affects autophagy

Next, we analyzed whether the overexpression of PABP1 could affect autophagy. As previously mentioned, dysregulation of autophagy contributes to the loss of proteostasis, an essential hallmark of aging (López-otín et al., 2013). We observed an almost significant increased in mRNA levels of p62, which is an autophagy substrate, in the hypothalamus of mice overexpressing PABP compared to the control group. This could indicate a defective clearance of protein aggregates and an inhibition or downregulation of autophagy (Figure 23 A). However, we did not observe significant differences in mRNA levels of LC3B between the two groups (Figure 23 B).

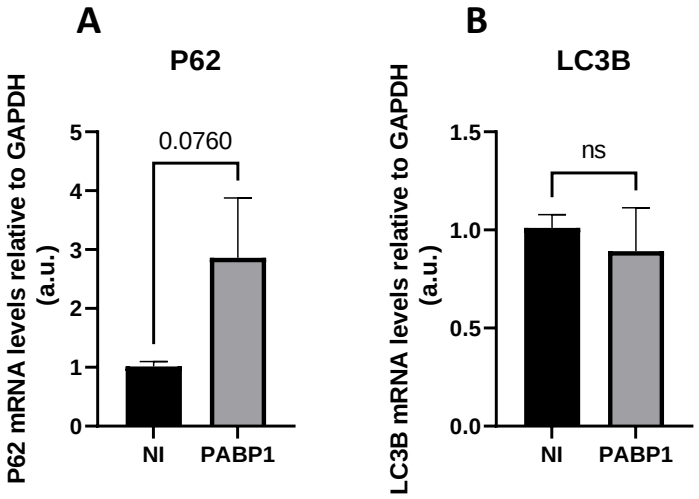

Figure 23 - p62 and LC3B levels in the hypothalamus of mice overexpressing PABP1
(**A**) No significant trend to increase in the mRNA levels of p62 in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n= 6) vs PABP1 (n = 5) *P-value* = 0.0760 *unpaired Student's t-test*] (**B**) No significant differences on LC3B levels in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n= 6) vs PABP1 (n = 5) *P-value* = 0.5913 *unpaired Student's t-test*].

8 – Overexpression of PABP1 in the mice hypothalamus decreases the level of a critical inflammatory mediator

Inflammaging is a prominent aging-associated alteration in intercellular communication, a hallmark of aging (López-otín et al., 2013). To investigate a possible impact of PABP1 in inflammation, we analyzed the expression levels of two crucial mediators of inflammation, IL-6 and NF-κB. We observed a significant decline in mRNA levels of IL-6 in the hypothalamus of mice overexpressing PABP1, compared to the control group (Figure 24A). As for the NF-kB, we observed a non-significant trend of an increased expression of NF-κB in the hypothalamus of mice overexpressing PABP1 (Figure 24B).

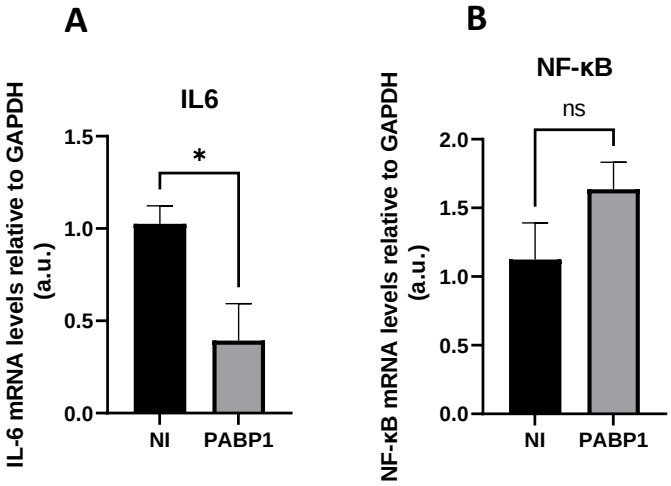

Figure 24 - IL-6 and NF-κB levels in the hypothalamus of mice overexpressing PABP1
(**A**) A significant decrease in the mRNA levels of IL-6 in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n = 6) vs PABP1 (n = 5) P-value = 0.0149 *unpaired Student's t-test*] (**B**) No significant differences in the mRNA levels of NF-κB in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n = 4) vs PABP1 (n = 5) P-value = 0.1578 *unpaired Student's t-test*]. Values are expressed as mean ± SEM. *P <0.01 (unpaired Student's t-test)

9 – Overexpression of PABP1 in the mice hypothalamus did not affect the levels of PGC-1α

To investigate a possible impact of PABP1 in mitochondrial dysfunction, another hallmark of aging, we analyzed the levels of PGC-1α, a transcriptional coactivator that is crucial for mitochondrial biogenesis in cells (López-otín et al., 2013)(Austin & St-Pierre, 2012). We did not observe significant differences in PGC-1α mRNA levels between the group overexpressing PABP1 and the control group (Figure 25).

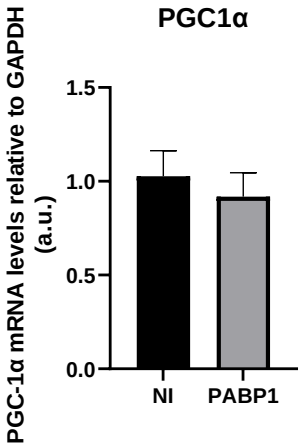

Figure 25 – PGC-1α levels in the hypothalamus of mice overexpressing PABP1
No significant differences on PGC-1α mRNA levels in the hypothalamus of mice overexpressing PABP1, compared to the non-injected group [NI (n = 4) vs PABP1 (n = 5) P-value = 0.5817 *unpaired Student's t-test*].

Chapter 4 – Conclusion

1 – Discussion

Aging is related with a gradual decline of organismal function and fitness, correlating with changes in the proteome. The accumulation of molecular damage and other deleterious changes with aging affects the crucial balance between protein synthesis, quality control, and degradation required to maintain cellular homeostasis (Anisimova et al., 2020). Indeed, disruption of proteostasis is a well-established hallmark of aging, associated with several age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson´s disease, and Huntington's disease (López-otín et al., 2013; Tavernarakis, 2008). Proteome maintenance includes distinct but tightly connected biological processes conditionally divided into phases of protein synthesis, folding, activity, post-translational modifications, and degradation. Among these, only protein folding and degradation is relatively well understood in the context of aging, while protein synthesis changes with age remain notably less explored (Anisimova et al., 2020).

Several studies in the second half of the 20th century reported that the overall rate of protein synthesis decreases with age. Underlying this decline is the deregulated stoichiometry of translation components such as eukaryotic initiation (eIF) and elongation (eEF) factors, ribosome abundance, and components ribosomal biogenesis. Moreover, the role of protein synthesis in aging is supported by indirect evidence that its attenuation generally leads to an increased lifespan in several animal models. Overexpression of translation initiation repressors such as 4E-BP1, suppression of metabolism by caloric restriction or inhibition of nutrient signaling (Rapamycin), are valid interventions that increase the lifespan through the regulation of protein synthesis (Anisimova et al., 2020). A possible explanation behind the lifespan extension mediated attenuation of protein synthesis is the reduced load on the proteostasis network and decreased energy use (Anisimova et al., 2018; Gonskikh & Polacek, 2017; Tavernarakis, 2008). Despite these findings and the growing number of studies, several aspects of protein synthesis in the context of aging remain elusive, especially at a mechanistic level.

In this work, we attempted further to elucidate the role of overall protein synthesis in aging. First, we measured the translation rate in human HGPS (Hutchinson-Gilford progeria syndrome) fibroblasts compared to human wild-type (WT) fibroblasts as the control. While HGPS is not a complete representation of physiological aging, it can be referenced as a model of human aging characterized by clinical features mimicking physiological aging at an early

age, providing insights into the mechanisms of natural aging (Ding & Shen, 2008). We observed a significant reduction in overall protein translation in HGPS fibroblasts compared to WT fibroblasts, which is consistent with the already established idea that the overall rate of protein synthesis decreases with aging(Anisimova et al., 2018; Tavernarakis, 2008). However, a recent study by Buchwalter and colleagues investigating the effect of progerin on nuclear protein homeostasis reported a different outcome in which they observed a significant increase in the overall rate of protein synthesis in HGPS fibroblasts compared to WT fibroblasts. According to this study, progerin acts as a dominant-negative mutant to prevent a previously unappreciated role of lamin A in organizing nucleoli and limiting ribosome biogenesis. Progerin induces the disruption of the nuclear organization, depleting the heterochromatin repressive marks on rDNA loci, subsequently increasing the abundance of mature 28S and 18S rRNAs, ultimately enhancing ribosome biogenesis and increasing protein synthesis. The discrepancy between our results could be due to the unsustainable nature of the increased protein production rate and cellular proliferation of HGPS fibroblasts since these cells in culture become senescence at earlier passages than WT cells (Buchwalter & Hetzer, 2017). Therefore, the increased protein synthesis observed by Buchwalter and colleagues could be in the early passages of HGPS cell culture, while we used HGPS fibroblast with a relatively high passage, in which senescence was a considered factor.

Interestingly, these data offer important insights into protein synthesis in the context of aging. Even though protein synthesis declines with aging, the rate of this decline varies in different phases of the organismal's life. The variation in the overall rate of protein synthesis could be caused by genomic instability, stress conditions, or other forms of damage, subsequently increasing protein translation rate and thus affecting longevity. To test this hypothesis, we induced mild oxidative stress via H_2O_2 exposure and genomic instability via progerin disruption of the nuclear organization in HEK293T cells and analyzed translation rate profiles. We observed a significant increase in overall protein synthesis in HEK293T treated with H_2O_2 compared to untreated cells, while HEK293T transfected with progerin presents a non-significant trend of higher overall protein synthesis than the group transfected with lamina A. Furthermore, the cells transfected with progerin present no significant difference in overall protein synthesis rate compared to the non-transfected cells. In contrast, the lamina A group presents an almost significant reduction in overall protein synthesis compared to the non-transfected group, which is consistent with the role of nucleoplasmic lamin meshwork in suppressing ribosome biogenesis (Buchwalter & Hetzer, 2017).

Paradoxically, global attenuation of protein synthesis is a typical response to oxidative stress since ROS induces a marked increase in the eIF2 phosphorylation via the unfolded protein response (UPR) (Shenton et al., 2006). Moreover, H_2O_2 at low concentrations acts as a

secondary messenger regulating protein synthesis at different levels to promote beneficial aspects supporting health and longevity, consistent with the mitohormesis phenomenon (López-otín et al., 2013; Picazo & Molin, 2021). The contrasting result in our study suggests that at least during the initial exposure to H_2O_2 or the low concentration used (0.1 mM) could induce protein synthesis of antioxidants to detoxify and remove H_2O_2 and its metabolic products, which transiently increases overall protein synthesis (Shenton et al., 2006). In support of this hypothesis, low doses of H_2O_2 can also induce phosphorylation of 4E-binding protein 1 (4E-BP1), enhancing translation initiation (Sanchez et al., 2019). Altogether these data support that genomic instability and oxidative stress induces variations in the overall protein synthesis rate, which could negatively impact lifespan. In the case of oxidative stress, ROS could increase the translation of proteins involved in adaptive cell response, resulting in a transient increase in the overall rate of protein synthesis.

Furthermore, we sought to investigate whether key proteins in protein synthesis, such as PABP1, are altered with aging. PABP1 is a central regulator of mRNA translation and stability (Gorgoni & Gray, 2004). Therefore PABP1 could play an essential role in mediating changes in protein synthesis with age. PABP1 binds to the poly (A) tail, enhancing translation initiation by interacting with eIF4G and eIF4E at the mRNA 5' end, subsequently resulting in the circularization of the mRNA, stabilizing and enhancing translation initiation (Smith et al., 2014). In addition, we also analyzed the levels of eIF2, a crucial initiation factor involved in the delivery of Met-tRNAi to ribosomes and ensuring accurate recognition of the start codon. Phosphorylation of the eIF2α subunit is sufficient to attenuate protein synthesis(Sanchez et al., 2019). We found no differences in the levels of both of these proteins in HGPS fibroblasts compared to WT fibroblasts, although there is a non-significant trend of lower expression of both proteins in HGPS fibroblasts. We also analyzed the expression levels of PABP1 in the hypothalamus of old mice compared to those of young mice and observed an increase in the levels of PABP1 with age, suggesting that PABP1 could play a role in aging, although the result was not significant. The discrepancy between these results could be due to the variability of PABP1 expression between species, organs, and tissues or the different methodology used.

To further elucidate the role of PABP1 in mediating the age-related alteration of protein synthesis, we overexpressed PABP1 in the hypothalamus of old mice and analyzed the expression levels of crucial target proteins representing different hallmarks of aging (López-otín et al., 2013). The hypothalamus controls several physiological functions that decline with aging, such as metabolism, homeostasis, development, and cycadean rhythm, and therefore is considered the master regulator of systematic aging (K. Kim & Choe, 2019). The hypothalamus could be a potential gateway mediating the age-related changes in overall protein synthesis at a systematic level. First, we investigated whether the overexpression of PABP1 in the

hypothalamus could be repressed by endogenous PABP1 protein. The levels of PABP1 mRNA are tightly regulated by its own protein or by 5' terminal tract oligopyrimidine (5' TOP) (Gorgoni & Gray, 2004). However, we found a significant increase in the expression levels of PABP1 in the injected group compared to the non-injected group, validating its overexpression. Next, we analyzed the levels of Ataxin-2, known for interacting with PABP1 via the PABP interacting motif (PAM) to regulate mRNA translation (Nóbrega et al., 2015). We found no significant differences between the inject and non-inject groups, indicating that the PABP1 overexpression did not affect the translation by altering the levels of Ataxin-2. PABP1 overexpression also did not affect the expression levels of mTOR kinase, which plays a pivotal role in the regulation of diverse aspects of cellular physiology implicated with aging, such as body metabolism, cell growth, protein synthesis, cell size, autophagy, and cell differentiation(Papadopoli et al., 2019). We also found no significant differences in the expression levels of PGC-1α, a crucial transcriptional coactivator crucial in mitochondrial biogenesis (López-otín et al., 2013). Therefore, our data indicate that PABP1 overexpression does not affect the expression levels of mTOR or PGC-1α, two critical proteins tightly linked to the aging process.

We also investigated if PABP1 overexpression could affect components of the proteostasis network. We found an almost significant increase in the expression levels of p62/SQSTM1 in the hypothalamus of the injected group compared to the non-injected group. p62 is a selective autophagy receptor that also has roles in the ubiquitin-proteasome system, cellular metabolism, signaling, and apoptosis. p62 is characterized by a decline in its expression with age in several animal models, including in human and mouse brains with Alzheimer's disease. Moreover, p62 expression has both positive and negative roles in lifespan. On the one hand, Increased p62 leads to hyperactivation of nuclear factor erythroid 2–related factor 2 (Nrf2) target genes, which protects against oxidative damage and inflammation. On the other hand, p62 accumulation represents a loss of autophagy or UPS. p62 is a crucial mediator of the crosstalk between autophagy and UPS. Proteasome inhibition and ubiquitin overexpression cause the accumulation of ubiquitinated p62 that activates autophagy. Alternatively, autophagy inhibition also causes the accumulation of p62, which delays proteasomal substrate delivery compromising the UPS (Kumar et al., 2022). We also examined the expression levels of LC3B, another autophagy marker, with a crucial role in autophagosome biogenesis(Lee & Lee, 2016). We found no significant differences between the two groups. How PABP1 induce the expression of p62 is unclear; however, this result indicates that PABP1 expression could indirectly impact positively or negatively lifespan by inducing p62 and thus affect several biological processes, including proteostasis. More studies are necessary to elucidate the implications of PABP1-induced expression of p62 in the context of aging.

Finally, we investigated the impact of PABP1 on inflammation, since inflammaging is a prominent aging-associated alteration in the dynamics of intercellular communication(López-otín et al., 2013). We found a significant decline in the expression levels of the cytokine IL-6 in the injected group compared to the non-injected group; however, we found no such differences for NF-κB, a key mediator of inflammation. IL-6 reduction could be the consequence of the p62 overexpression. Studies show that p62 overexpression inhibits the expression of multiple cytokines, including IL-6, whereas p62 repression can markedly elevate cytokines expression levels(J. Y. Kim & Ozato, 2009).

In summary, our data point to a key role of PABP1 in aging by inducing the expression of p62. PABP1-induced expression of p62 could potentially have a role in inflammaging, as it could reduce the levels of crucial cytokines such as IL-6. However, the current data is insufficient to discern if the PABP1-induced expression of p62 is associated with its role in protein synthesis. We found that the overall rate of protein synthesis is indeed altered with aging. Nevertheless, we were unable to demonstrate if alterations of PABP1 mediate the age-dependent in global protein synthesis. Moreover, we found that cellular damage generating genomic instability or stress conditions could impact the overall rate of protein synthesis, possibly affecting lifespan. ROS could potentially induce an transient increase in overall protein synthesis in an effort to promote an adaptive cellular response to oxidative stress. Altogether our data revealed a complex relationship between protein synthesis and aging, and therefore more studies are necessary to elucidate the role of protein synthesis in aging.

2 – Conclusion and future perspectives

Aging is correlated with extensive changes in the overall rate of protein synthesis, but the causal relevance between aging and protein synthesis is complex. The overall rate of protein synthesis decreases with aging, but how it varies in different stages of an organismal's life remains to be elucidated. Protein synthesis could be altered by cellular damage affecting translation machinery components, such as mutations causing genomic instability or oxidative stress, subsequently impacting longevity. PABP1, a central component in the translation machinery, could have a role in the context of aging by inducing the expression of p62/SQSTM1, which is involved in several cellular processes that are implicated with aging. PABP1 could also potentially decrease inflammation in the hypothalamus by inducing p62, mitigating several pathologies associated with inflammaging.

However, important questions remain to be addressed better to understand the intricate relationship between aging and protein synthesis and validate the role of PABP1 in aging:

- To better understand how the overall rate of protein synthesis varies with aging, we should include a broader range of ages than just both extremes, long lived animal models and cells from healthy centenarians;
- More tissues should be analyzed to evaluate the effects of PABP1 and overall protein synthesis in systemic aging, such as liver, kidney, and muscle tissues, applying a range of different molecular biology techniques as well as histological analysis;
- To better understand the role of PABP1 in aging and to validate if PABP1 is mediating the age-depend changes in overall protein synthesis is necessary to expand the analysis to more components of the translation machinery and other proteins with prominent roles in aging;
- We should investigate how proteins regulating PABP1, such as PAIP1 and PAIP2 are altered with aging.

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